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R. W. SCHRAMM, TERESA MOSSOR and CZ. GIERCZAK

INFLUENCE OF LIGHT ON THE CONTENT OF CITRATE AND MALATE IN GERMINATING SEEDS AND YOUNG SEEDLINGS OF HORSEBEAN (*VICIA FABA* L. *MINOR*)

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1. The light affects in a much higher degree the changes in citrate and malate content in the seedling's axis (shoot and root) than in the cotyledons. 2. During seed soaking, citrate is used by the germ. 3. In the period of protrusion of the germ and its further development, the content of citrate in the seedling's axis increases and then decreases, whereas the content of malate increases steadily. 4. Malate is partly transferred from the cotyledons to the seedling's axis during the first stage of germination. After the beginning of photosynthesis, the increase in malate in the roots derives from the photosynthesis products. 5. In the cotyledons, the content of citrate and malate in relation to the dry weight is almost unaltered during germination.

The energy required for germination and growth of the seedling is supplied mainly by oxidation of organic acids, which are also utilized for synthesis of a number of organic compounds. However, the accumulation in all the plants, at all stages of their development and in all tissues, of large quantities of some organic acids, particularly citrate and malate, may indicate their special function which so far has not been elucidated.

The daily rhythm of changes in the content of citrate and malate, and their dependence on light, were examined in detail in leaves, mainly of succulent plants, tobacco and *Papilionaceae*. However, the reports concerning the changes in the content of these compounds occurring in germinating seeds, and their influence on the process of germination, are rather scarce and somewhat divergent.

In ripe seeds of the papilionaceous plants, rather large quantities of citrate are present (0.3 - 2% of dry weight); the content of malate is 4 - 20 times lower (Täufel & Krusen, 1952; Soldatenkov & Mazurova, 1956a; Dupéron, 1958; Schramm, 1961), but no other compounds of the tricarboxylic acid cycle have been detected (Dupéron, 1958). In addition to citrate and malate, sugar acids (products of primary oxidation of sugars, different from the onic acids) are present in much higher quantities (Soldatenkov & Mazurova, 1956b; Schramm, 1961).

The presence of citrate seems to be essential for germination of seeds. The higher

content of citrate (Täufel & Pohloudek-Fabini, 1955b; Täufel & Behnke, 1956; Sabala, 1962) or soaking of seeds in a citrate solution (Cotrufo, 1963) stimulated germination in many plants, whereas malate does not seem to affect this process (Sabala, 1962). In seeds of the *Papilionaceae*, the total content of organic acids, particularly of citrate and malate, undergoes considerable changes during germination. However, the reported data are divergent, especially those for citrate; some authors observed a decrease (Täufel & Pohloudek-Fabini, 1955a, b; Soldatenkov & Mazurova, 1956a; Dupéron, 1958), whereas others, an increase (Munch-Petersen, 1944; Buruiana, Pop, Pop & Popa, 1959). As regards malate, its content was usually found to increase (Holton & Noll, 1955; Soldatenkov & Mazurova, 1956a; Dupéron, 1958), although under certain conditions its presence could not be detected (Schramm, 1961).

The aim of the present work was to examine the effect of light on the changes in the content of citrate and malate in different parts of germinating seeds of horsebean.

MATERIALS AND METHODS

Horsebean seeds (*Vicia faba* L. vel *Faba vulgaris* Moench, *minor*), variety "Nadwiślańska", obtained from the Plant Culture Station in Orłowo (Wąbrzeźno, Poland), were used. The seeds were stored for one year. They were well developed, sound, weighing 0.45 - 0.50 g., with germination activity of about 95%.

The seeds were soaked in distilled water for 24 hr., then placed on wet filter paper in Petri dishes and aerated for 10 min. twice a day. The cultures were kept in thermostates and, beginning with the second day, one part was kept in the dark at 24°, and another at 22° in the light of an electric discharge lamp; the radiation intensity under the glass of Petri dishes was 800 luxes, and on the germination beds 1000 luxes. On the fourth day, the germinating seeds were transferred to germination beds containing water which was supplemented every day and aerated for 7 min. In the cultures kept in light, the cotyledons were distinctly growing green, even while in seed coats, and after about 10 days they turned black and the top part of epicotyl became flabby.

The air-dried seeds (0 day), the seeds soaked for 24 hr. (1st day) and the seeds and seedlings on the 3rd, 5th, 7th, 9th, 12th and 15th day of growth were taken for analysis. The material was collected always at the same time in the morning and immediately freeze-dried. The whole seeds or the isolated cotyledons (without the seed coat) and the germs were analysed at zero time and on the 1st day. Upon the differentiation of the axis, beginning with the 5th day, the cotyledons, the roots and the shoots in total (epicotyl) were analysed separately. For analysis, samples were taken from varying number of seedlings (from 20 for shoots to 150 for germs), depending on the dry weight and expected content of citrate and malate.

Citrate and malate were extracted with water after Pohloudek-Fabini & Wollmann (1961) with some modifications. The pulverized sample of the freeze-dried material not exceeding 2 g. (most frequently 0.3 - 1.0 g.) was treated in a centrifuge

tube with 15 ml. of water, three times for 15 min. at 20° and twice at 60°. The supernatants were pooled, 2 g. of Wofatit F cation-exchange resin (H^+ form) was added, the mixture stirred for 10 min. and centrifuged. The resin was washed with 10 ml. of water, and the combined supernatants left in the cold till the next day. This procedure ensured efficient deproteinization, not interfering with further purification; a large amount of protein was precipitated immediately upon the contact with the resin and the rest precipitated in the cold, giving a clear, protein-free solution. Only in the case of the cotyledons, which are very rich in protein, some of the coagulated protein did not sediment, therefore the turbid solution was cleared by filtration through Schott G5 filter. The protein-free extract was applied to the Wofatit L-150 column (OH^- form) and the acids adsorbed were eluted with2N-NaOH.

Citric acid was determined by the pentabromoacetone method according to Taylor (1953), except that sodium metavanadate instead of ammonium metavanadate was used for preparation of the bromination mixture. To an aqueous suspension of 8.6 g. of V_2O_5, 10.6 g. of NaOH *in substantia* was added and stirred until a clear solution of sodium metavanadate was obtained; subsequently 19.826 g. of KBr and 5.44 g. of $KBrO_3$ were added and the solution made up to one litre. The accuracy of determinations in our hands was $\pm 2.5\%$.

Malic acid was determined by the method of Reifer (1955), the accuracy in our hands being $\pm 0.4\%$.

The results are average values from three experiments; the differences did not exceed $\pm 18\%$ (usually less than 10%) for citrate determinations, and $\pm 4\%$ for malate determinations.

RESULTS

The changes in the total content of citrate together with malate in the germinating seeds and young seedlings, were dependent on light (Fig. 1). Within the first 24 hr., during imbibition, the content of the two compounds decreased. In the seeds which further germinated in light, a steady decrease was maintained during the following days till the appearance of leaves, and then an unchanged content was maintained. In darkness, during the period of 3 - 5 days, the content of the acids strongly increased; protrusion of the shoot was accompanied by a rapid decrease which, however, was not as great as the decrease in light. The decrease was maintained for several days, and on the 15th day of growth an increase was observed.

The drop in the content of the two acids during the first day was caused by the decrease in citrate (Fig. 2), the amount of malate at this time being virtually unchanged. The decrease in light beginning with the 7th day, and the increase beginning in darkness after the 12th day, were also due mainly to changes in citrate content. However, malate contributed to the transient increase observed in darkness from the 3rd to the 5th day.

Changes in the content of citrate and malate were different in various parts

of the seedling (Fig. 3). In the cotyledons, the content of malate, after a transient increase, decreased, the respective changes being more pronounced in darkness than in light. As regards the content of citrate in the cotyledons of the seeds germinating in light, a decrease on the 1st day, and a second decrease beginning with

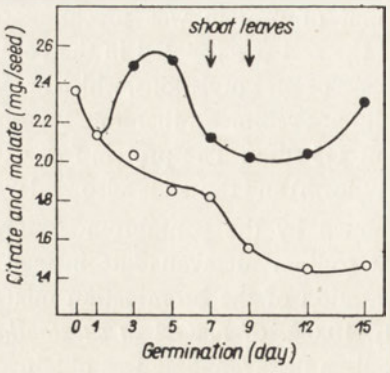

Fig. 1. Total content of malate together with citrate in the whole seedling during germination (●), in darkness and (○), in light.

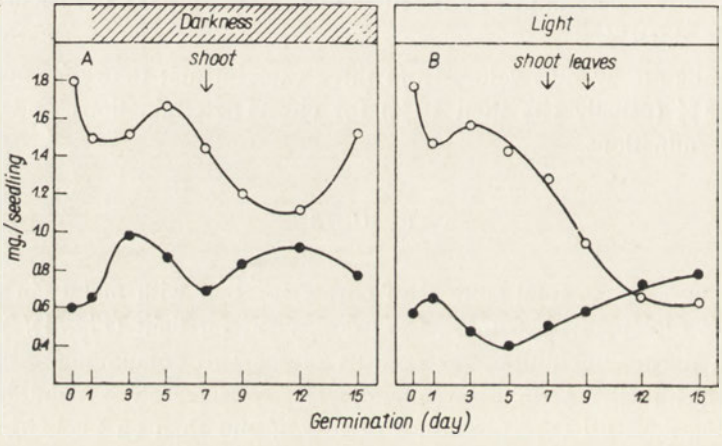

Fig. 2. Content of (○), citrate and (●), malate in the whole seedling during germination in darkness and in light.

the 3rd day were observed, reaching on the 12th day 0.6 mg., an amount corresponding to about 1/3 of the initial value. In the seeds germinating in darkness, the decrease was much smaller and a distinct increase was observed on the 15th day. In the germ, after the period of imbibition a decrease in citrate to half the initial value was observed. Subsequently, the content of citrate in the root slightly increased and then remained practically unaltered. On the other hand, in the shoot the changes in citrate, although quantitatively smaller than those of malate, were much more characteristic. In the light, a strong increase was observed which lasted till the protrusion of the shoot; then appeared a rapid decrease which stopped at the time

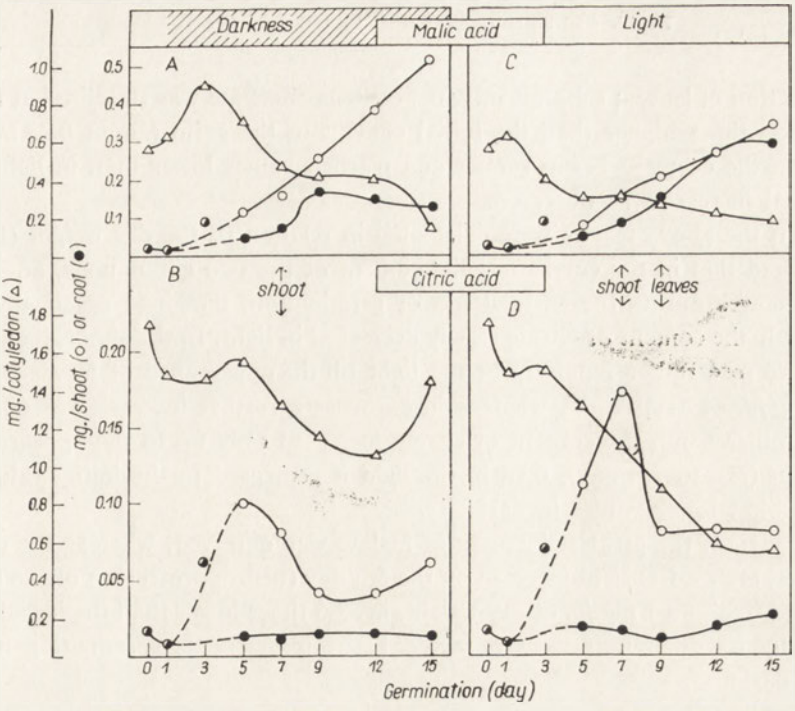

Fig. 3. Content of malate and citrate in particular parts of the seedling during germination in darkness and in light. (△), Cotyledons (pair); (○), shoot; (●), root; half-filled-in circles correspond to germs.

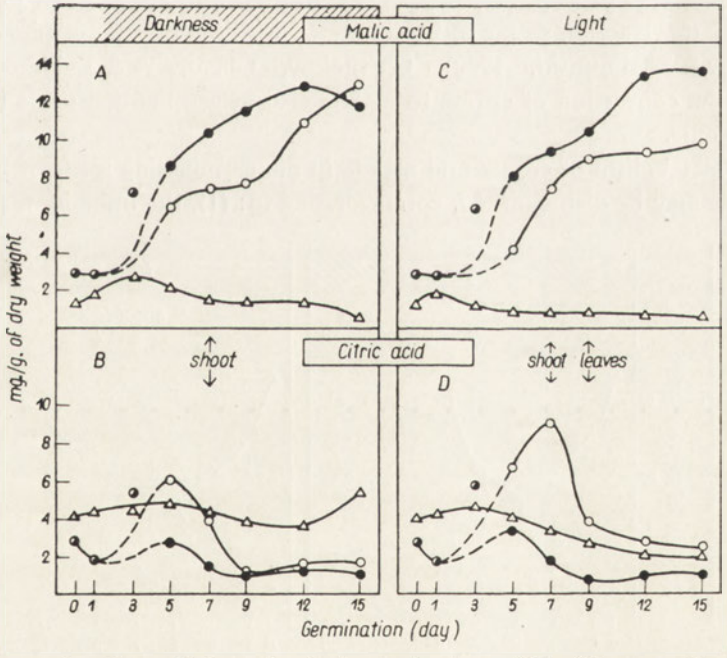

Fig. 4. Content of malate and citrate in relation to the dry weight of the respective parts of the seedling during germination in darkness and in light. (△), Cotyledons (pair); (○), shoot; (●), root; half-filled-in circles correspond to germs.

of formation of leaves; subsequently, the content of citrate was stabilized at a value about five times higher than the initial content in the germ (about 0.08 mg.). In darkness, the decrease began sooner and reached values lower than in light; then a gradual increase was observed.

When the above results were calculated in relation to the dry weight (Fig. 4), it appeared that in the cotyledons the content of the two compounds, after some small changes during the early stages of germination, remained practically unaltered, only the content of citrate slowly decreased in light. In the shoot, the content of malate rose rather regularly in the light till the appearance of leaves reaching 9 - 10 mg./g. of dry weight, whereas in darkness a further increase was observed. On the other hand, the content of citrate increased only up to the appearance of the shoot (3rd to 7th day), then in the light it decreased to the initial value, and to somewhat lower values in darkness.

In the root, the greatest increase of malate was observed, whereas the content of citrate, after a transient increase on the 5th day (before protrusion of the shoot), decreased to about 1 mg./g. of dry weight, i.e. 2.5 times lower than the initial value. Practically no differences were observed between the seeds germinating in light and in darkness.

DISCUSSION

In leaves of many species of plants, the interconversion of citrate and malate is light-dependent. In darkness, malate is converted into citrate, and in light *vice versa*; the latter reaction seems to be connected with the process of photosynthesis (Chesnokov & Zhabotinski, 1960). Chesnokov, Glagolyeva & Lubimova (1955) suggested the conversion of citrate to malate also in germinating seeds of papilionaceous plants.

In the metabolism of citrate and malate in the germinating seeds of horsebean, three stages may be distinguished, corresponding to: (1), the imbibition phase; (2),

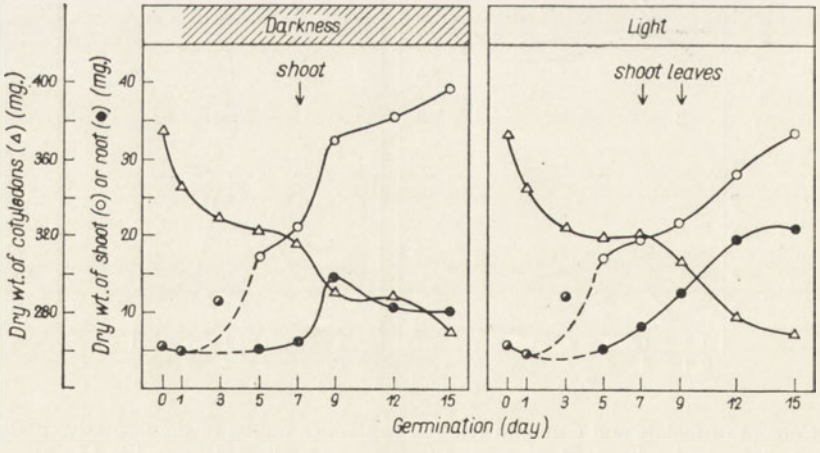

Fig. 5. Dry weight of the parts of the seedling during germination in darkness and in light. (△), Cotyledons (pair); (○), shoot; (●), root; half-filled-in circles correspond to germs.

germination up to the beginning of photosynthesis (as judged by the appearance of leaves); and (3), later growth of the seedling.

During the imbibition phase, the total amount of citrate in the cotyledons decreased (Fig. 3), but its content remained unaltered in relation to the dry weight (Fig. 4) which decreased simultaneously (Fig. 5), mainly due to the loss of large amounts of amino acids (H. Mazurowa & R. W. Schramm, in preparation). In the germ, which showed only small loss in dry weight, the content of citrate decreased. This seems to indicate that during imbibition, even before the beginning of the catabolic processes in the cotyledons, citrate is utilized by germ. This observation seems to be related to the reported effect of citrate on the germination of seeds (Täufel & Pohloudek-Fabini, 1955b; Täufel & Behnke, 1956; Sabala, 1962). So far, the process of utilization of citrate during the imbibition phase has not been elucidated. Most probably, citrate does not serve as respiratory substrate. In the seeds in which, up to the piercing of the seed coat, anaerobic conditions are known to prevail (Frietinger, 1927), anaerobic processes predominate, and the tricarboxylic acid cycle becomes operative only after a few more days (Poljakoff-Mayber & Evenari, 1958; Mayer & Poljakoff-Mayber, 1963). Besides, citrate is not so readily oxidized in the germinating seeds as the other metabolites of the tricarboxylic acid cycle (Poljakoff-Mayber & Evenari, 1958). However, it cannot be excluded that from the beginning of germination citrate is used, similarly as in animal tissues, for extramitochondrial synthesis of fatty acids (Bhaduri & Srere, 1963; Kornacker & Lowenstein, 1965) necessary for lipid layers of intracellular membranes, particularly for mitochondria which are formed at this time in large quantities (Hackett, 1963).

The second stage, which begins with the breaking of the seed coat, and includes protrusion and growth of the shoot and differentiation of the seedling's axis, is characterized by an increase in the content of citrate and malate, especially in the shoot.

During the third stage, the changes appearing in various parts of the seedling were somewhat different but in general a stabilization was observed; in seedling axis, the content of citrate was always lower than that of malate, and in the cotyledons citrate predominated.

During the second and third stages, light played an important role. In darkness, the sum of citrate and malate was by about 25% higher than in light (Fig. 1), due in the second stage mainly to the higher amount of malate (Fig. 2). Further changes, both in darkness and in light, were due to the changes in citrate content. The decrease till the 9th day was connected with the beginning of photosynthesis (in horsebean 7th - 9th day). This seems to indicate that the seeds, both those germinating in darkness and in light, follow the same biological rhythm leading to the appearance of photosynthesis. However, beginning from the 9th day, differences were found to occur. In darkness, the decrease of citrate was arrested, and then an increase appeared in the cotyledons and the shoot (Fig. 3B). In light, citrate continued to decrease, and it may be assumed that its function was taken over, at least in part, by the products of photosynthesis.

Probably, in the course of differentiation of the seedling's axis, before the appearance of chlorophyll and beginning of photosynthesis, light and citrate act synergetically. The increase in the content of citrate in the shoot in light is greater, and its decrease on the 9th day is arrested at a level higher than in darkness (Fig. 3B and D).

In the seeds germinating in darkness, metabolic disturbances appear at the time when photosynthesis should normally begin. The rapid growth of the shoot at the cost of the cotyledons, as estimated by an increase in dry weight, is stopped whereas the dry weight of the root begins even to decrease (Fig. 5). At this time, in contrast to the increase of malate in the root in light, there is no such increase in darkness. This seems to indicate that the increase in malate accumulating in the root in light originates from the process of photosynthesis and not from the cotyledons' reserves.

The appearance in light of chlorophyll in the hypogeal cotyledons of horsebean seems not to be connected with photosynthesis and with supplying of assimilates to root, in contrast to the cotyledons of epigeal seeds which early begin to supply the root with these products (Tuichibayev & Kruzhilin, 1965).

The steady increase in the content of malate in the seedling's axis (Fig. 4) supports the view on the direct participation of this compound in the metabolism of the rapidly developing plant, mainly as a source of carbon skeleton (Rautanen, 1948; Kursanov, 1957a, b). Citrate increases temporarily in the seedling's axis in the periods of protrusion of the germ and of the shoot (3rd to 7th day). In contrast to the increase of malate and citrate in seedling's axis, their content in the cotyledons decreases, but in relation to the dry weight it remains almost unaltered. Only in the period directly preceding germ protrusion, at the time of storage food mobilization, first a transient increase of malate takes place, and then a smaller increase in citrate. The role of citrate and malate remaining in the cotyledons seems to be connected with food mobilization. Probably malate in this period passes to the seedling's axis, which is not yet provided with the products of photosynthesis. Citrate may act as a regulator of metabolic processes in the cotyledons, stimulating respiration and oxidative phosphorylation. This assumption might explain the presence of virtually constant amounts of citrate in the cotyledons in relation to their dry weight, the presence of large quantities of citrate in the cotyledons in the seeds germinating in darkness, as well as its increase (Fig. 3B) at the time when leaves should have appeared and photosynthesis should have begun. On the other hand malate, due to its ability to pass through the mitochondrial membrane, and to the activity of malate dehydrogenase, may regulate the intra- and extramitochondrial level of $NADH_2$ (Krebs, 1966), participating, together with the pentose phosphate cycle, in the generation and utilization of $NADH_2$ and $NADPH_2$. The role of citrate and malate in germinating seeds is discussed in greater detail in another paper (Schramm, 1967).

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WPŁYW ŚWIATŁA NA ZAWARTOŚĆ CYTRYNIANU I JABŁCZANU W KIEŁKUJĄCYCH NASIONACH I MŁODYCH SIEWKACH BOBIKU (*VICIA FABA* L. *MINOR*)

Streszczenie

1. Światło wpływa w znacznie większym stopniu na zmiany zawartości cytrynianu i jabłczanu w osi siewki (pęd, korzeń) niż w liścieniach.

2. W okresie pęcznienia nasienia cytrynian jest zużywany przez zarodek.

3. W okresie wybijania kiełka i dalszego jego rozwoju, w osi siewki cytrynian przejściowo wzrasta, jabłczan natomiast wzrasta regularnie.

4. Jabłczan z liścieni zostaje w pierwszym okresie kiełkowania częściowo przeprowadzony do osi siewki. Po rozpoczęciu fotosyntezy przyrost jabłczanu w korzeniu pochodzi z procesu fotosyntezy.

5. W liścieniach zawartość cytrynianu i jabłczanu w stosunku do suchej wagi prawie nie zmienia się podczas kiełkowania.

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AMINO ACID-ACTIVATING ENZYMES IN YELLOW LUPIN SEEDS, AND PURIFICATION OF LEUCYL-sRNA SYNTHETASE

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1. In extracts obtained from seeds of yellow lupin (*L. luteus* L.), an enzyme system catalysing the amino-acid dependent exchange of [^{32}P]pyrophosphate into ATP is present. Of the protein amino acids, leucine was activated to the greatest extent, methionine, valine, isoleucine and alanine somewhat less, while other amino acids were activated but slightly or not at all. *N*-Acetyl derivatives of some amino acids were also activated. 2. Leucyl-sRNA synthetase was purified 270-fold. Its properties were similar to those reported for synthetases from other sources. The molecular weight of the enzyme, determined by the gel-filtration procedure, was 170 000.

The first step in the synthesis of protein, the activation of amino acid carboxyl group, has been demonstrated in higher plants, among others in spinach leaves and pea epicotyls (Clark, 1958), in pea seeds and *Convallaria majalis* rhizomes (Peterson & Fowden, 1963) and in wheat germs (Moustafa & Lyttleton, 1963). The methods of isolation and purification of some plant aminoacyl-sRNA synthetases have been described. From wheat germs, a synthetase specific for valine (Moustafa, 1963) and for lysine and methionine (Moustafa, 1964) was isolated; a corresponding enzyme for alanine was isolated from tomato roots (Attwood & Cocking, 1965), and for proline from mung bean seeds (Peterson & Fowden, 1965).

The aim of the present work was to demonstrate the presence of amino acid-activating enzymes in seeds of yellow lupin (*Lupinus luteus* L., var. Express) and to isolate leucyl-sRNA synthetase [L-leucine : sRNA ligase (AMP), EC 6.1.1.4].

MATERIALS AND METHODS

Reagents. ATP (disodium salt) was a product of Nutr. Biochem. Corp. (Cleveland, Ohio, U.S.A.); tris(hydroxymethyl)aminomethane of B.D.H. (Poole, Dorset, England). Protein amino acids were obtained from Nutr. Biochem. Corp. or Sigma Chem, Co. (St. Louis, Mo., U.S.A.). Yeast RNA (Merck, Darmstadt, Germany) was additionally purified in the following way: 2 g. was dissolved in 25 ml. of 0.05 M-potassium phosphate buffer, pH 7.3, then 2.5 ml. of 20% solution of potassium acetate was added, and RNA was precipitated with 2.5 volumes of 95%

ethanol. The precipitate was filtered, washed with ethanol and ether, then dried. Glutathione was obtained from Sigma Chem. Co., 2-mercaptoethanol from Koch-Light Lab., Ltd. (Colnbrook, England), streptomycin sulphate from Tarchomińskie Zakłady Farmaceutyczne Polfa (Warszawa, Poland). DEAE-cellulose DE 11 was a product of Balston (England), Sephadex G-200, DEAE-Sephadex and Dextran-blue were from Pharmacia (Uppsala, Sweden).

Ox albumin, 5 times crystallized was from B.D.H. (England), yeast alcohol dehydrogenase from C.F. Boehringer & Soehne (Mannheim, Germany); myoglobin was prepared according to Walters & Taylor (1961).

N-Acetyl derivatives of DL-valine, DL-leucine, DL-alanine, DL-glutamic acid and DL-histidine were synthesized according to Greenstein & Winitz (1961), *N*-acetylglycine according to Herbst & Shemin (1959) and *N*-acetyl-DL-methionine according to du Vigneaud & Meyer (1932). sRNA was isolated from yellow lupin seeds according to Legocki, Szymkowiak, Pech & Pawełkiewicz (1967).

Non-protein amino acids and other reagents used in this work were from Fabryka Odczynników Chemicznych (Gliwice, Poland).

Radioactive chemicals. [^{32}P]Pyrophosphate was prepared from ^{32}P-labelled potassium orthophosphate (sp. act. about 3 mc/mg. P, Institute of Nuclear Research, Warszawa, Poland). The solution of [^{32}P]orthophosphate (1 mc) was evaporated at 40°, 6.1 mg. of K_2HPO_4 dissolved in 4 ml. of water was added, and pH of the mixture adjusted to 9 with 2 N-KOH. After evaporation, the dry residue was submitted to pyrolysis in an electric oven at 400 - 500° for 7 hr. The obtained [^{32}P]pyrophosphate was purified by paper chromatography in a solvent system of *tert*-butanol - formic acid - water (80 : 5 : 20, by vol.), the spot being located by the method of Hanes & Isherwood (1949). The yield of the synthesis of [^{32}P]pyrophosphate was about 95%. L-[U-^{14}C]Leucine (sp. act. 35 mc/m-mole) and L-[U-^{14}C]valine (sp. act. 6.9 mc/m-mole) were obtained from the Radiochemical Centre (Amersham, England); DL-[1-^{14}C]methionine (sp. act. 9.8 mc/m-mole) from Service Molecules Marquées CEA (Gif-sur-Yvette, France); and L-[U-^{14}C]isoleucine (sp. act. 36.4 mc/m-mole) from the Institute of Radioisotopes (Prague, Czechoslovakia).

Analytical procedures. Radioactivity of ^{32}P was measured in AAH-55 end-window counter (PIE, Warszawa; mica window, 1.5 mg./cm^2) using electronic scale PEL-5A (Biuro Urządzeń Techniki Jądrowej, Warszawa, Poland). Radioactivity of ^{14}C was measured in liquid scintillator of Bray (1960) in Pyrex vessels with the scintillation counter SE 2 (Biuro Urządzeń Techniki Jądrowej, Warszawa, Poland) with an efficiency of 40%.

Orthophosphate was assayed by the method of Fiske & Subbarow in the modification of Bartlett (1959). Protein was determined turbidimetrically by the tannin method of Mejbaum-Katzenellenbogen (1955).

Exchange assay. The standard reaction mixture contained in a volume of 1 ml.: 100 μmoles of tris-HCl buffer, pH 7.5; 5 μmoles of ATP (disodium salt) neutralized to pH 7 with 1 N-KOH; 5 μmoles of [^{32}P]pyrophosphate (about 50 000 counts/min./μmole); 10 μmoles of $MgCl_2$; 10 μmoles of one amino acid or a mixture

of L-amino acids, 10 μmoles of each; and 3.5 mg. of protein of the crude enzyme extract, or 25 - 300 μg. (depending on the degree of purification) of the leucyl-sRNA synthetase preparation. After incubation for 20 min. at 37°, the reaction was stopped by adding 2 ml. of ice-cold 5% trichloroacetic acid. ATP was separated according to DeMoss & Novelli (1956) by adsorption on 50 mg. of activated charcoal (Carbopol HZ, pH 7). Then ATP was hydrolysed in 2 ml. of 1 N-HCl in a boiling-water bath for 15 min. and the radioactivity and orthophosphate were determined in 0.5 ml. supernatant portions. In the control incubation samples, amino acid(s) was omitted.

The results are expressed as percentages of exchange and in μmoles of [^{32}P]pyrophosphate exchanged/1 hr./1 mg. of protein.

Aminoacyl-sRNA formation. The incubation mixture contained in a final volume of 0.5 ml.: 50 μmoles of tris-HCl buffer, pH 7.3; 5 μmoles of $MgCl_2$; 5 μmoles of KCl; 2 μmoles of ATP (disodium salt) neutralized to pH 7 with KOH; 1 μmole of reduced glutathione; 0.1 μmole of L-[^{14}C]amino acid (0.25 μc), except DL--methionine: 0.2 μmole (0.7 μc); 0.5 mg. of sRNA and 50 - 500 μg. of the enzyme. The sample was incubated for 20 min. at 37°, then 0.35 mg. of carrier yeast RNA (0.1 ml.) and 3 ml. of a solution of 0.5 M-NaCl in 67% ethanol (cooled to -10°) were added (Berg, Bergmann, Ofengand & Dieckmann, 1961). The mixture was left for 10 min. at -10°, then the precipitate was centrifuged, washed three times with 3 ml. portions of the above solution, then with 96% ethanol and finally with ether. The traces of ether were evaporated at 40° and the residue dissolved in 0.3 ml. of formic acid, added with 1 ml. of 2-methoxyethanol and 10 ml. of liquid scintillator (Bray, 1960), cooled to 2 - 4°, and the radioactivity was measured. In each experiment, control samples were run with boiled enzyme.

Crude enzyme extract. Seeds of *L. luteus*, var. Express, harvested in 1965 and kept at 10°, were ground in a laboratory mill (Brabender, Duisburg, Germany), the finest fractions being collected. All further manipulations were carried out at 2 - 4°.

The flour, 70 g., was ground in a mortar with 210 ml. of 0.4 M-saccharose - - 0.04 M-potassium phosphate buffer, pH 7.3, containing mercaptoethanol (1 mM), and left for 30 min. The slurry was pressed through gauze and adjusted to pH 7.3 with 1 N-KOH. The dark-yellow solution was centrifuged at 60 000 *g* for 35 min. and the supernatant, after rejection of the upper layer of fat, was dialysed for 10 hr. against 1 litre of the starting buffer. The content of protein in the crude extract amounted to 40 - 60 mg./ml., and the enzyme activity did not change during one week at -10°.

Purification of leucyl-sRNA synthetase. The crude extract, 160 ml., was treated with 12 ml. of 5% solution of streptomycin sulphate neutralized with 1 N-KOH. After 15 min., 98 ml. of saturated ammonium sulphate solution (neutralized with 1 N-KOH) was added, and after 20 min. with stirring the sediment was centrifuged off. To the supernatant (250 ml.), 55 ml. of ammonium sulphate solution was added and the sediment (the 36 - 48% sat. fraction) collected by centrifugation. This protein fraction could be stored at -10° for several weeks without loss of activity.

For further purification, the sediment was dissolved in 15 ml. of 0.02 M-potassium phosphate buffer, pH 7.3, containing 1 mM-$MgCl_2$ and 1 mM-2-mercaptoethanol, and dialysed for 4 hr. against 1 litre of the same buffer. A solution containing 650 mg. of protein was applied to a DEAE-cellulose column (40×1.3 cm.) equilibrated previously with the starting buffer. Proteins were eluted with a gradient of increasing concentration of KCl in 0.02 M-phosphate buffer, pH 7.3. Fractions of 3 ml. were collected, the elution rate being 0.5 ml./min. The activity of leucyl-sRNA synthetase was found in the fraction eluted at 0.06 - 0.16 M-KCl concentration. At this stage, the enzyme kept at 4° retained full activity not longer than 18 hr.

The active fraction from the DEAE-cellulose column, 2 mg., was applied to a column of DEAE-Sephadex A-25 Medium (45×0.8 cm.) equilibrated previously with 0.02 M-potassium phosphate buffer, pH 7.3, containing 1 mM-$MgCl_2$, 1 mM--mercaptoethanol and 0.06 M-KCl. The elution was carried out with the same buffer but containing 0.16 M-KCl. The elution rate was 5 ml./hr. and fractions of 1 ml. were collected. The enzyme was usually found in fractions no. 21 - 25 (0.32 mg. of protein/ml.). When kept for 4 hr. at 4°, it lost more than 50% of the activity.

Molecular weight determination. The molecular weight of leucyl-sRNA synthetase was determined according to Whitaker (1963); 2 ml. of the DEAE-cellulose fraction (5 mg. of protein) was applied on the Sephadex G-200 column (2×50 cm.) equilibrated with 0.2 M-potassium phosphate - 1 mM-mercaptoethanol buffer, pH 7.3. The effluent was collected in 1 ml. fractions, in which leucyl-sRNA synthetase assays were performed.

The calibration of Sephadex G-200 was carried out with 4 mg. of ox albumin (mol. wt. 67 000), 4 mg. of myoglobin (17 800), and 5 mg. of yeast alcohol dehydrogenase (151 000), the proteins being dissolved in 2 ml. of 0.2 M-phosphate buffer, pH 7.3. The bed volume of the column was determined with Dextran blue (mol. wt. 2×10^6).

RESULTS

Activation of amino acids by the crude enzyme preparation. The optimum conditions for the amino-acid dependent exchange of [^{32}P]pyrophosphate into ATP were: 37°, 20 min. incubation at pH 7.3. The extract which had been heated for 3 min. at 100° exhibited practically no catalytic properties, similarly as the system containing no ATP (Table 1). Magnesium ion could be partly replaced by manganese or cobalt ions, but then the activity was only 28 and 49%, respectively. EDTA at 0.01 M concentration almost completely inhibited the exchange reaction. A supplementary addition of Mg^{2+} ion in an amount equivalent to EDTA, restored only 10% of the activity.

The results of activation assays with 20 protein amino acids are shown in Table 2. Leucine was activated to the greatest extent (50% of the value found for the mixture of all amino acids). The activation of methionine, valine, isoleucine and alanine was also very high (24 - 33% of the value for the mixture). Cysteine and glutamic acid were activated to a somewhat smaller degree, whereas the remaining 13 amino acids only slightly or not at all.

Table 1

General conditions for the amino acid-dependent [^{32}P]pyrophosphate-ATP exchange reaction in the crude enzyme preparation

The complete incubation mixture contained in a final volume of 1 ml.: 100 μmoles of tris-HCl buffer, pH 7.5; 5 μmoles of ATP; 5 μmoles of pyrophosphate (about 50 000 counts/min./μmole); 10 μmoles of $MgCl_2$; mixture of L-amino acids, 10 μmoles of each; and 3.5 mg. protein of the crude enzyme extract. The incubation was carried out at 37° for 20 min. The results have been corrected for endogenous activation.

Sample	ATP (counts/min./μmole)	Exchange (%)
Complete system	2740	11.9
Complete system with enzyme boiled (control)	17	0.05
ATP omitted	38	0.1
Mg^{2+} omitted	75	0.2
EDTA (10 μmoles) added	109	0.3
EDTA (10 μmoles) and Mg^{2+} (10 μmoles) added	312	1.0

Table 2

Effect of individual amino acids on the [^{32}P]pyrophosphate-ATP exchange reaction in the crude enzyme preparation

Composition and incubation conditions as described in Table 1, except that instead of the mixture of amino acids, 10 μmoles of the L-amino acid indicated was added. The results have been corrected for endogenous activation.

Amino acid	^{32}P incorporated in ATP (μmole/hr./mg. protein)	Percentage of activation of amino acid mixture
Alanine	0.063	14.1
Arginine	0	0
Aspartic acid	0	0
Asparagine	0	0
Cysteine	0.084	18.8
Glutamic acid	0.063	14.1
Glutamine	0	0
Glycine	0	0
Histidine	0.013	2.9
Isoleucine	0.105	23.5
Leucine	0.225	50.3
Lysine	0.015	3.3
Methionine	0.146	32.6
Phenylalanine	0.008	1.6
Proline	0.017	3.8
Serine	0.021	4.5
Threonine	0	0
Tyrosine	0.015	3.3
Tryptophan	0.013	2.9
Valine	0.126	28.2
Mixture of 20 amino acids	0.447	100.0

Activation of *N*-acetylamino acids and some non-protein amino acids is presented in Table 3. All these compounds stimulated the reaction of exchange, but to a rather small extent. However, the *N*-acetyl derivative of histidine had a greater effect than histidine itself.

Table 3

Effect of N-*acetylamino acids and non-protein amino acids on the* [^{32}P]*pyrophosphate--ATP exchange reaction in the crude enzyme extract*

Composition and incubation conditions as in Table 1. The results have been corrected for endogenous activation.

Compound	^{32}P incorporated in ATP (μmole/hr./mg. protein)
N-Acetyl-DL-leucine	0.017
N-Acetyl-DL-methionine	0.053
N-Acetyl-DL-valine	0
N-Acetyl-DL-alanine	0.007
N-Acetyl-DL-glutamic acid	0
N-Acetyl-DL-histidine	0.043
DL-Norleucine	0
DL-Norvaline	0.033
DL-Homoserine	0
DL-Allothreonine	0.023

Table 4

Comparison of exchange raection with the formation of aminoacyl-sRNA

The experiments were carried out with the 36 - 48% ammonium sulphate sat. enzyme fraction. Formation of aminoacyl-adenylates was assayed by the exchange reaction (see Table 1), with 350 μg. of enzyme protein. Formation of aminoacyl-sRNA was assayed in a mixture containing in a final volume of 0.5 ml.: 50 μmoles of tris-HCl buffer, pH 7.3; 5 μmoles of $MgCl_2$; 5 μmoles of KCl; 2 μmoles of ATP (disodium salt); 1 μmole of reduced glutathione; 0.1 μmole (0.25 μc) of the indicated L-[^{14}C]amino acid (except DL-methionine, 0.2 μmole, 0.7 μc); 0.5 mg. of sRNA; and 350 μg. of the enzyme protein. The incubation was carried out for 20 min. at 37°. The results have been corrected for endogenous values.

Amino acid	Formation of aminoacyl-adenylates (counts/min./μmole ATP)	Formation of aminoacyl-sRNA (counts/min./mg. sRNA)
Leucine	1360	2246
Methionine	777	1888
Isoleucine	718	1322
Valine	282	922
Leucine+methionine+isoleucine+ +valine	1005	6476
Calculated sum	3137	6380

The specific activity of the exchange reaction measured with the mixture of amino acids was always much smaller than the sum of the activities with separate amino acids (Table 2). This was found also with the 36 - 48% ammonium sulphate saturation fraction for leucine, methionine, isoleucine and valine, and their mixture (Table 4). On the other hand, when the enzyme activity was measured not by the exchange reaction but by the aminoacyl-sRNA assay, the activity of the mixture of the four amino acids was the same as the sum of the activities of the individual amino acids (Table 4).

Purification of the leucyl-sRNA synthetase preparation. The course of purification of leucyl-sRNA synthetase from *L. luteus* seeds is presented in Table 5. The enzyme, as measured by the pyrophosphate-ATP exchange reaction, was purified 270-fold, the yield being about 7%. The preparation could be further purified by Sephadex G-200 gel filtration, even up to 350-fold, but then the yield became very low, due probably to increased instability of the enzyme. The molecular weight of leucyl--sRNA synthetase determined by the gel-filtration technique was about 170 000.

A sharp pH optimum for the purified enzyme was found at pH 7.5 (Fig. 1). At a pH lower than 5.5 the enzyme was irreversibly inactivated. This precluded utilization of the isoelectric precipitation technique for isolation of the enzyme. The rate of the pyrophosphate-ATP exchange reaction increased with the concentration of the purified leucyl-sRNA synthetase (Fig. 2) up to about 40 μg. enzyme/ml. The effect of leucine concentration on the rate of exchange, is shown in Fig. 3; the K_m value was found to be 0.2 mM.

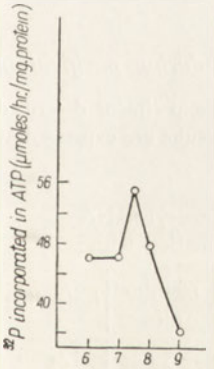

Fig. 1

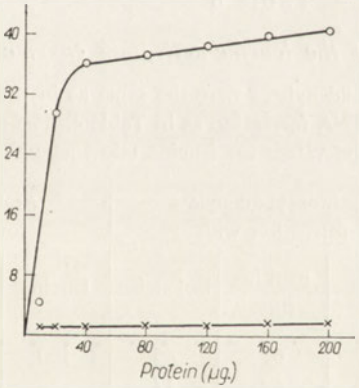

Fig. 2

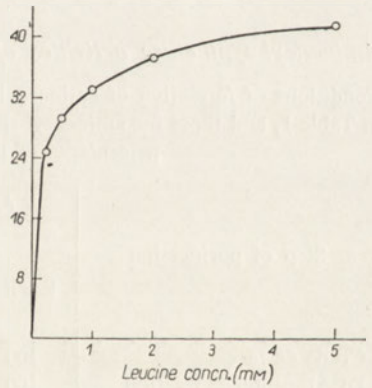

Fig. 3

Fig. 1. The effect of pH on the activity of leucyl-sRNA synthetase. Composition and incubation conditions as described in Table 1, 30 μg. of purified enzyme preparation being used.

Fig. 2. The effect of concentration of leucyl-sRNA synthetase on the rate of [^{32}P]pyrophosphate--ATP exchange reaction. Composition and incubation conditions as described for Table 1. (○), Complete system; (×), control, without leucine.

Fig. 3. The effect of leucine concentration on the rate of [^{32}P]pyrophosphate-ATP exchange reaction, catalysed by purified leucyl-sRNA synthetase preparation. Composition and incubation conditions as described for Table 1, except that increasing amounts of leucine were applied.

Leucyl-sRNA synthetase proved to be highly sensitive to inhibitors of thiol groups. The enzyme activity was inhibited completely by 1 mM-*p*-chloromercuribenzoate and 3 mM-iodoacetate, and by 90% by 1 mM-iodoacetate, whereas 0.1 mM- and 0.3 mM-*N*-ethylmaleimide inhibited it, respectively, by 67 and 83%. These results, together with instability of the enzyme in the absence of glutathione or mercaptoethanol, indicate its thiol nature.

Table 5

Purification of leucyl-sRNA synthetase

Activity of the enzyme was measured by the [^{32}P]pyrophosphate-ATP exchange reaction. Composition of the incubation mixture, procedure and details of purification are described under Materials and Methods. Specific activity is expressed as μmoles [^{32}P]pyrophosphate incorporated in ATP/hr./mg.protein.

Step of purification	Protein (mg.)	Specific activity	Degree of purification	Yield (%)
Crude enzyme extract	7200	0.245	1	100
36 - 48% $(NH_4)_2SO_4$ sat. ppt.	578	2.17	8.9	71.0
DEAE-cellulose fraction	25.5	17.6	71.8	25.3
DEAE-Sephadex fraction	1.9	65.8	269.0	7.1

Table 6

Aminoacyl-synthetase activities of the leucine-activating enzyme during purification

Conditions of formation of aminoacyl-adenylates were the same as in the experiment described in Table 1, and those for aminoacyl-sRNA formation as in Table 4. The results are expressed as percentages of the values for leucine taken as 100.

Step of purification	Aminoacyl-adenylate formation with			Aminoacyl-sRNA formation with		
	leucine	methionine	isoleucine	leucine	methionine	isoleucine
Crude extract	100	65	46	100	45	57
DEAE-cellulose fraction	100	40	17	—	—	—
DEAE-Sephadex fraction	100	10	5	100	10	8.5

The preparations of leucyl-sRNA synthetase purified on DEAE-Sephadex, showed some activity toward methionine and isoleucine, both in the exchange assay and in aminoacyl-sRNA formation, but no activity toward other amino acids was observed. The comparison of specificity of leucyl-sRNA synthetase preparations at successive stages of purification, toward these three amino acids is shown in Table 6.

DISCUSSION

The results of the present work indicate that in extracts from *L. luteus* seeds the general conditions for the amino acid-dependent exchange of pyrophosphate into ATP are similar to those reported so far. Non-dialysed extracts gave higher percent exchange due to the presence of endogenous amino acids and some other compounds. In experiments not presented here, sulphate activation was found; the same observation with other plant material was reported by Marcus (1958).

The activation of *N*-acetylamino acids by crude lupin-seed extract (Table 3) seems to be of interest in relation to the occurrence in some proteins of *N*-terminal acetylated amino acids. Activation of *N*-acetylamino acids has been reported by Pearlman & Bloch (1963) who suggested that they start the synthesis of polypeptide chain. This, however, was not supported by Marchis-Mouren & Lipmann (1965) who demonstrated that the acetyl group in foetal haemoglobin is introduced after formation of the polypeptide chain. Moreover, according to Adams & Capecchi (1966), Webster, Engelhardt & Zinder (1966) and Noll (1966), the chain is initiated by *N*-formylmethionine. Therefore the slight activation of *N*-acetylamino acids observed in the present work, can be regarded as a non-specific reaction.

The crude enzyme extract from *L. luteus* seeds exhibited marked activation of seven out of twenty protein amino acids (leucine, methionine, valine, isoleucine, alanine, cysteine and glutamic acid). Similarly to the observations of DeMoss & Novelli (1956) and Moustafa & Proctor (1962), also in the present work the sum of activation of individual amino acids was higher than the activation of their mixture. The purified leucyl-sRNA synthetase exhibited a similar behaviour, a marked decrease in activation of leucine being observed in the presence of methionine or isoleucine, or of both those amino acids added together. Although in the present work the nature of this inhibition has not been studied, it seems possible to explain it either by competition for the active site of leucyl-sRNA synthetase, or by allosteric transition of the enzyme. On the other hand, the sum of transfer of particular amino acids to sRNA corresponded to the transfer of the amino acid mixture, in agreement with the concept of individual synthetases possessing independent sites for specific sRNA (Berg *et al.*, 1961).

The leucyl-sRNA synthetase purified 270-fold, showed both in the exchange reaction and in incorporation to sRNA some activity toward methionine and isoleucine, this activity being respectively 10 and 5 - 8.5% of the activity toward leucine. The reaction of transfer of amino acids to sRNA is known to be more specific than the exchange reaction (Norris & Berg, 1964). Therefore the transferase activity toward methionine and isoleucine may indicate that the preparation of leucyl-sRNA synthetase was not sufficiently purified. However, attempts at further purification were unsuccessful. Although Sephadex G-200 gel filtration permitted to increase the specific activity of the synthetase, the yield was so low that the method was abandoned.

The studied properties of leucyl-sRNA synthetase from *L. luteus* seeds did not differ from those of the enzyme isolated from other sources.

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ENZYMY AKTYWUJĄCE AMINOKWASY W NASIONACH ŁUBINU ŻÓŁTEGO I OCZYSZCZANIE LEUCYLO-sRNA SYNTETAZY

Streszczenie

1. W wyciągach z nasion łubinu żółtego *L. luteus* L. stwierdzono obecność układu enzymatycznego katalizującego zależną od aminokwasów wymianę między nieorganicznym [^{32}P]pirofosforanem i ATP. Z białkowych aminokwasów najsilniej aktywowana była leucyna, nieco słabiej metionina, walina, izoleucyna i alanina, natomiast inne aminokwasy prawie nie były aktywowane. Wykazano również aktywację niektórych *N*-acetylopochodnych aminokwasów.

2. Leucylo-sRNA syntetazę oczyszczono 270-krotnie. Przebadane własności enzymu były zbliżone do opisanych własności syntetaz innego pochodzenia. Ciężar cząsteczkowy syntetazy oznaczony metodą sączenia żelowego wynosił 170 000.

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ISOLATION AND PROPERTIES OF SOLUBLE RIBONUCLEIC ACID FROM YELLOW LUPIN SEEDS

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1. sRNA isolated from seeds of yellow lupin (*Lupinus luteus* L.) by the phenol extraction procedure, precipitation with cetyltrimethylammonium bromide and purification by Sephadex G-200 gel filtration, retained the ability to accept amino acids. 2. Chromatography of sRNA on DEAE-cellulose column at 72° gave sharp peaks of acceptor activities specific for leucine, isoleucine and valine. The activity for methionine was found in three fractions. 3. [^{14}C]Aminoacyl-sRNA was fractionated on protamine-coated kieselguhr. Heterogeneity of leucyl-, isoleucyl-, methionyl- and valyl-sRNA was demonstrated.

The properties and function of transfer ribonucleic acid (sRNA) have been extensively studied, but mainly on material from micro-organisms and animal tissues. In the present work, the isolation and amino acid acceptor activity of sRNA from seeds of yellow lupin is presented.

MATERIALS AND METHODS

Lupinus luteus seeds (var. Express) were ground in a Brabender type mill and the flour used for experiments.

Special reagents. ATP, disodium salt (Reanal, Budapest, Hungary); reduced glutathione (Sigma Chem. Co., St. Louis, Mo., U.S.A.); CTP, disodium salt (Calbiochem, Los Angeles, Cal., U.S.A.); L-amino acids (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. or Sigma Chem. Co.); tris(hydroxymethyl)aminomethane and protamine sulphate (British Drug Houses, Poole, Dorset, England); 2-mercaptoethanol (Koch-Light Ltd., Colnbrook, Bucks, England); streptomycin sulphate (Tarchomińskie Zakłady Farmaceutyczne, Polfa, Warszawa, Poland); DEAE-cellulose (Balston, England); celite Hyflo Supercel (C. Roth, Karlsruhe, Germany); 8-hydroxyquinoline (T. Schuchardt, Munich, Germany); cetyltrimethylammonium bromide (Chemapol, Prague, Czechoslovakia); 2-methoxymethanol (IOBA-Fischeman, Vienna, Austria) distilled over 2,4-diaminophenol hydrochloride before use; yeast RNA (Merck, Darmstadt, Germany) purified by precipi-

tation with ethanol from 2% solution of potassium acetate, pH 5; Sephadex G-200 (Pharmacia, Uppsala, Sweden); L-[U-^{14}C]leucine (35 mc/m-mole) and L-[U-^{14}C]valine (6.9 mc/m-mole) were from the Radiochemical Centre (Amersham, England), DL-[1-^{14}C]methionine (9.8 mc/m-mole) from Service Molecules Marquées CEA (Gif-sur-Yvette, France); L-[U-^{14}C]isoleucine (36 mc/m-mole) from Inst. Radioisotopes (Prague, Czechoslovakia). Scintillators PPO (2,5-diphenyloxazole) and POPOP (*p*-bis[2-(5-phenyloxazolyl)]-benzene) were products of Pilot Chem. Inc. (Watertown, Mass., U.S.A.).

Analytical methods. Spectrophotometric determinations were performed in a Hilger H 700 instrument. Protein was determined by the tannin method of Mejbaum-Katzenellenbogen (1955). Phosphorus was assayed by the method of Fiske & Subbarow in the modification of Bartlett (1959). DNA was determined by the diphenylamine method of Dische (1930).

To determine amino acids and protein in the purified preparation of sRNA, a sample was hydrolysed in 6 N-HCl for 18 hr. at 100°. The hydrolysate was submitted to chromatography on Whatman no. 1 paper in butan-1-ol - acetic acid - - water (100 : 45 : 125, by vol.) and the spots located with ninhydrin. The amount of the amino acids in the hydrolysate was estimated by comparison with arginine standards.

The content of polysaccharides in the purified sRNA preparation was assayed by the method described by Brubacker & McCorqodale (1963). A sample of 10 mg. was hydrolysed for 18 hr. at 37° in 0.5 N-KOH, then the nucleotides were removed by exhaustive dialysis against distilled water. The dialysis residue was hydrolysed in 0.6 N-HCl for 2 hr. at 100°, and after neutralization the reduction was determined by the method of Somogyi (1945).

Preparation of aminoacyl-sRNA synthetases from lupin seeds. The seeds were extracted and enzymes isolated as described previously (Legocki & Pawełkiewicz, 1967). The procedure consisted of removal of nucleic acids from the crude extract with streptomycin sulphate, fractionation with ammonium sulphate, and chromatography on DEAE-cellulose. On ammonium sulphate fractionation, leucyl-sRNA synthetase was precipitated by 0.36 - 0.48 saturation, isoleucyl-sRNA and valyl- -sRNA synthetases by 0.29 - 0.46 sat., and the enzyme for methionine at 0.31 - 0.45 sat. From DEAE-cellulose the enzymes were eluted with a gradient of increasing concentration of KCl in 1 mM-$MgCl_2$ - 1 mM-mercaptoethanol - 0.05 M-potassium phosphate buffer, pH 7.3. The enzyme for leucine emerged at 0.06 - 0.16 M-KCl, that for isoleucine and valine at 0.045 - 0.20 M-KCl, and for methionine at 0.05 - - 0.19 M-KCl. The aminoacyl-sRNA synthetases obtained according to this procedure, active toward leucine, isoleucine, valine and methionine, were purified respectively 70-, 60-, 75- and 40-fold in relation to the crude extract.

Isolation of sRNA

Purified RNA preparation. This was obtained by the method of Ralph & Bellamy (1964). To 2 kg. of ground lupin seeds was added 12 l. of a mixture of equal volumes of 0.5% solution of sodium naphthalene-1,7-disulphonate and 90% aqueous phenol

containing 0.1% of 8-hydroxyquinoline. The mixture was mechanically shaken for 1 - 2 hr. at 5° and left for 30 min.; then the upper water phase was decanted. This procedure, although it resulted in a somewhat lower yield, was found to be more rapid and convenient, especially for large-scale preparation, than the separation of the two phases by centrifugation. The separated supernatant fluid was shaken with half its volume of phenol solution. The mixture was centrifuged for 20 min. at 3000 *g* and the aqueous layer was shaken again with half its volume of ether to remove phenol. The ether layer was discarded and, to remove residual ether, nitrogen was bubbled through the aqueous solution. Then RNA was precipitated with 2.5 vol. of 95% ethanol cooled to -15°, centrifuged, washed successively with ethanol, acetone and ether, and air-dried. The sediment (about 30 g.) was treated three times with 500 ml. portions of 0.025 M-NaCl - 0.05 M-tris-HCl buffer, pH 7.6 at 0°. The insoluble material was removed and to the extract were added equal volumes of 2.5 M-potassium phosphate buffer, pH 7.6, and 2-methoxyethanol, both cooled to -3°. The mixture was shaken and centrifuged. The supernatant layer was withdrawn, from the interphase the insoluble material was removed and to the joint clear solutions an equal volume of 0.2 M-sodium acetate was added. Then RNA was precipitated by half a volume of 1% cetyltrimethylammonium bromide solution. After 30 min. at 0°, the mixture was centrifuged for 10 min. at 3000 *g*. The sediment was washed three times with cold 70% ethanol containing 0.1 M--sodium acetate to convert the cetyltrimethylammonium-RNA into the sodium salt. Then the sedimented RNA was dissolved in 100 ml. of 0.05 M-tris-HCl, pH 8.9, and incubated for 40 min. at 37° to remove the amino acids linked to sRNA. Then the solution was adjusted to pH 7.6 by 0.1N-HCl, dialysed overnight against distilled water, and freeze-dried. At this stage, about 700 mg. of RNA was obtained from 2 kg. of flour from lupin seeds.

Purified sRNA preparation. To remove high-molecular nucleic acids, gel filtration according to Delihas & Staehelin (1966) and Schleich & Goldstein (1966) was applied. About 400 mg. of the RNA preparation was dissolved in 8 ml. of 0.01 M solution of potassium acetate, pH 7.0, and applied to a column of Sephadex G-200 (120×7 cm.) equilibrated with the same solution. The column was eluted with 0.01 M-potassium acetate, fractions of 17 ml. being collected. To the pooled fractions containing sRNA (about 200 ml.), solid potassium acetate was added to final concentration of 0.2 M, then 2.5 vol. of 95% ethanol cooled to -15°. The mixture was left overnight. Then the sediment of sRNA was dissolved in a small amount of water, dialysed and freeze-dried. The yield was 200 - 300 mg.

Determination of the acceptor activity of sRNA

The incubation mixture contained in a final volume of 0.5 ml.: 50 μmoles of tris--HCl buffer, pH 7.3; 5 μmoles of $MgCl_2$; 5 μmoles of KCl; 2 μmoles of ATP (disodium salt); 0.1 μmole of one of the L-[U-^{14}C]amino acids (0.25 μc), except DL--[1-^{14}C]methionine (0.2 μmole, 0.7 μc); 1 μmole of reduced glutathione; 0.5 mg.

of sRNA preparation and 0.5 mg. of protein of the appropriate aminoacyl-sRNA synthetase fraction. After 20 min. incubation at 37°, 0.35 mg. of yeast RNA (0.1 ml.) and 3 ml. of a chilled solution of 0.5 M-NaCl in 67% ethanol, were added and centrifuged at 0°. The sediment was washed three times with 3 ml. portions of the above solution, then with 95% ethanol and ether, dried at 40°, dissolved in 0.3 ml. of formic acid, and 5 ml. of liquid scintillator was added (Bray, 1960); the radioactivity was measured in the scintillation counter SE2 (Biuro Urządzeń Techniki Jądrowej, Warszawa, Poland). As control, samples containing boiled enzyme were prepared.

To determine the total sRNA acceptor capacity of the particular sRNA fractions, the samples were incubated for different time intervals, up to 60 min., in the presence of excess enzyme and with smaller amounts of sRNA (0.15 - 0.30 mg./sample). The acceptor capacity was calculated from the values of maximum incorporation of the amino acid studied.

Chromatography of sRNA on DEAE-cellulose at different temperatures

This was carried out after Baguley, Bergquist & Ralph (1965). sRNA purified by Sephadex gel filtration, 20 - 150 mg., was applied to a DEAE-cellulose column (1.5×45 cm.) provided with a constant temperature water jacket. The column was equilibrated with 0.25 M-NaCl - 2 mM-EDTA - 20 mM-sodium acetate buffer, pH 6, and eluted with a constant gradient of NaCl from 0.25 to 2.0 M, in the same buffer. Fractions of 5 ml. were collected at a flow rate of 1 ml./min., and extinction at 258 mμ was determined. Two successive fractions containing sRNA were pooled, made 2% in respect to potassium acetate and sRNA precipitated by 2.5 vol. of ethanol. After centrifuging, the sediment was dissolved in 2 ml. of water; in 0.2 ml. portions, the acceptor activities for the four amino acids studied were assayed.

Charging of sRNA with [^{14}C]amino acids

This was carried out as presented by Moldave (1963). sRNA purified by gel filtration, 3 mg., was incubated with a mixture consisting of: 450 μmoles of tris-HCl buffer, pH 7.3; 15 μmoles of $MgCl_2$; 15 μmoles of KCl; 6 μmoles of reduced glutathione; 15 μmoles of ATP; 1.5 μmoles of CTP; 0.6 μmole of one of the L-[^{14}C]amino acids (4 μc, for DL-methionine 8 μc); 0.6 μmole of each of the 19 remaining non-radioactive amino acids, and 5 mg. protein of the appropriate aminoacyl-sRNA synthetase fraction. After 20 min. incubation at 37°, an equal amount of water-saturated phenol was added. The mixture was vigorously shaken for 30 min. at 5°, then centrifuged for 10 min. at 6000 *g*. The aqueous layer was separated and the phenol was shaken, once with an equal volume of water and once with half its volume. To the combined water phases, 0.1 volume of 20% potassium acetate, pH 5, and 2.5 vol. of chilled 95% ethanol were added. The sediment was left for 1 hr. at -10°, then centrifuged, dissolved in 1.5 ml. of water, dialysed overnight against distilled water and then submitted to chromatography on protamine-coated kieselguhr.

Chromatography of [^{14}C]*aminoacyl-sRNA on protamine-coated kieselguhr*

Protaminated kieselguhr was prepared according to the procedure given by Legaut-Démare, Rebeyrotte, Leprieur & Roussaux (1964). Hyflo Supercel, 15 g., was suspended in 100 ml. of 0.2 M-NaCl - 0.05 M-potassium phosphate buffer, pH 6.7, the air was removed under vacuum, then 3 ml. of 1% solution of protamine sulphate was added dropwise with constant stirring. The suspension was poured into a tube (1.1 × 24 cm.) and excess protamine was removed by washing the column with 150 ml. of the starting buffer. Onto the column, ^{14}C-labelled aminoacyl-sRNA, usually about 3 mg., was applied. The separation was carried out at room temperature at an NaCl gradient from 0.2 to 1.6 M. Fractions of 3 ml. were collected at a rate of 0.8 ml./min., and extinction at 258 mμ was measured. To each fraction were added 0.35 mg. of yeast RNA (as carrier) in 0.1 ml. of water and then 3 ml. of 10% trichloroacetic acid. After centrifuging, the sediment was washed with 2 ml. of ethanol, then with 1.5 ml. of ether, and dried at 40°. The dried sediment was dissolved in 0.3 ml. of formic acid, 5 ml. of liquid scintillator (Bray, 1960) was added and radioactivity of [^{14}C]aminoacyl-sRNA measured.

RESULTS

The preparation of RNA from lupin seeds obtained by the method of Ralph & Bellamy (1964) contained, in addition to sRNA, high-molecular-weight nucleic acids. To separate sRNA, gel filtration on Sephadex G-200 columns according

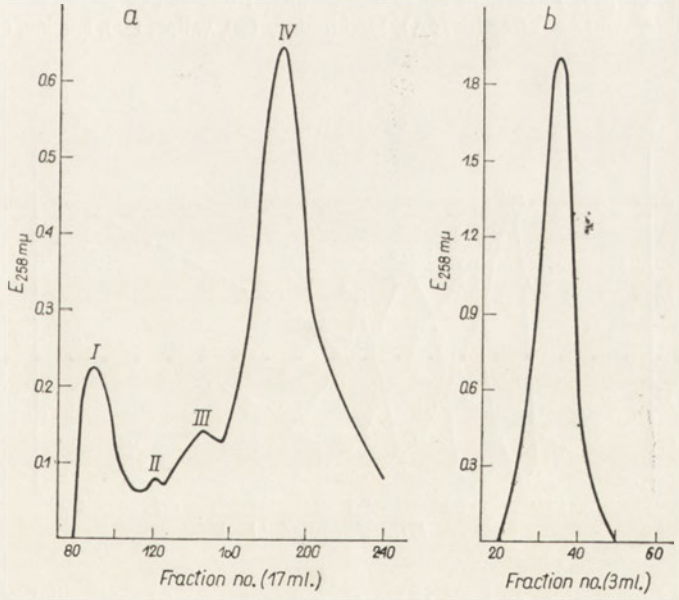

Fig. 1. Elution profiles of lupin sRNA from Sephadex G-200 column. (*a*), 500 mg. of RNA in 0.01 M-potassium acetate, pH 7.0, was applied on the column (120 × 7 cm.); (*b*), refiltration of 3 mg. of the sRNA peak *IV* on a 194 × 1.1 cm. column.

to Delihas & Staehelin (1966) and Schleich & Goldstein (1966) was applied. Figure 1a represents the elution profile, in which peaks *I*, *II* and *III* correspond to high-molecular RNA and peak *IV* to sRNA. Refiltration of peak *IV* through a large column (195 × 1.1 cm.) of Sephadex G-200 gave a homogeneous preparation (Fig. 1b). It should be added that varying amounts of high-molecular RNA were found to

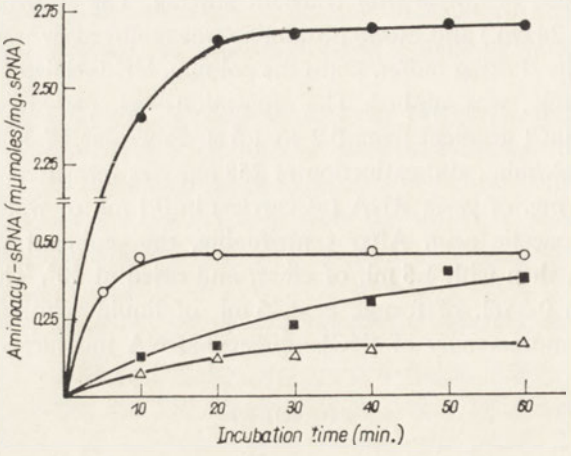

Fig. 2. Rate of formation of aminoacyl-sRNA. The incubation mixture contained in a final volume of 0.5 ml.: 50 μmoles of tris-HCl buffer, pH 7.3; 5 μmoles of $MgCl_2$; 5 μmoles of KCl; 2 μmoles of ATP (disodium salt); 0.1 μmole of one of the L-[^{14}C]amino acids (0.25 μc), except DL-methionine (0.2 μmole, 0.7 μc); 1 μmole of reduced glutathione; 0.25 mg. of sRNA and 0.5 mg. of the appropriate sRNA synthetase fraction. (△), Methionine, (■), valine, (○), leucine, (●), isoleucine.

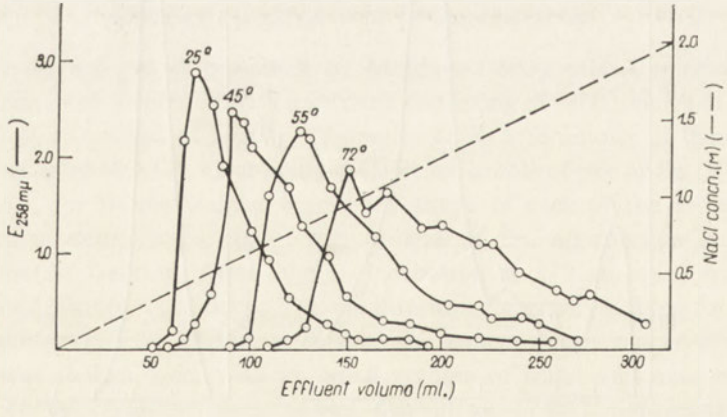

Fig. 3. Effect of temperature on lupin sRNA elution profile from DEAE-cellulose column (45 × × 1.5 cm.). The column was equilibrated with 0.25 M-NaCl - 2 mM-EDTA - 20 mM-Na-acetate buffer, pH 6.0. sRNA was eluted with NaCl gradient in the same buffer, at the temperature indicated. Fractions of 4.7 ml. were collected.

be present in the obtained preparations of RNA, despite apparently identical conditions of isolation.

The purified sRNA contained trace amounts of DNA (0.5%), protein and amino acids (0.05%) and polysaccharides (0.5%). The content of phosphorus in the preparation dried over P_2O_5 amounted to 7.5%. The absorption spectrum showed a maximum at 258 mμ and a minimum at 230 mμ. The E_{258}/E_{230} and E_{258}/E_{280} ratios were 2.08 and 2.09, respectively. The $E^{1\%}_{258}$ value of the solution in 0.15 M-NaCl - 0.015 M-potassium phosphate buffer, pH 7.0, was 192.

The acceptor activity of sRNA for isoleucine, leucine, valine and methionine in relation to time of incubation, is shown in Fig. 2. The amounts of amino acids linked to 1 mg. of sRNA after 60 min. were 2.8, 0.5, 0.3 and 0.15 mμmoles, respectively.

The sRNA purified by Sephadex gel filtration was submitted to chromatography on the DEAE-cellulose column. The effect of temperature on the elution profile of sRNA by NaCl concentration gradient, is presented in Fig. 3. With increasing temperature, the elution profiles became broader and elution required higher salt concentration. Since the broadest elution profile was obtained at 72°, the fractions eluted at this temperature by 0.8 - 2.0 M-NaCl concentration gradient were assayed for acceptor activity for the individual amino acids. Sharp peaks were obtained for leucine, isoleucine and valine, whereas the sRNA specific for methionine was found in three separate fractions (Fig. 4).

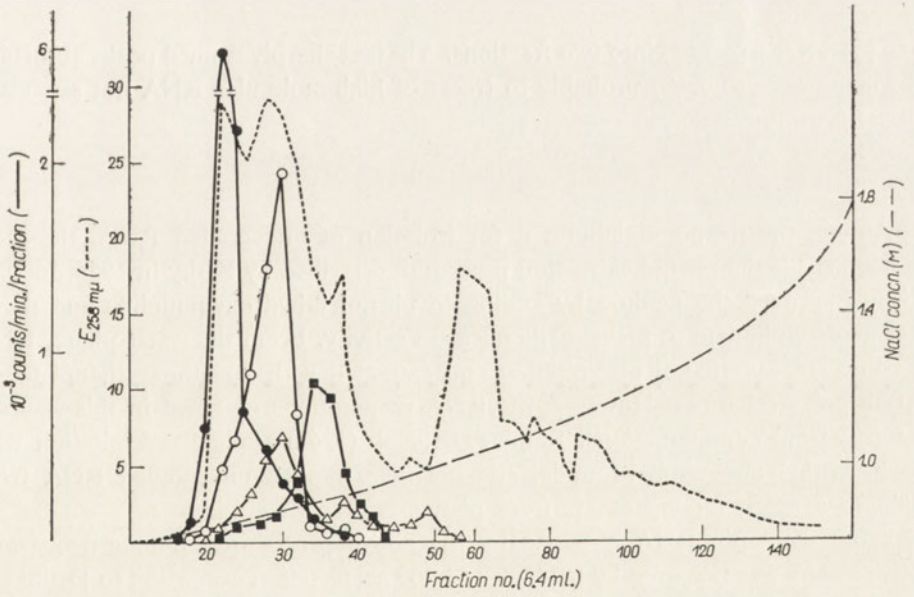

Fig. 4. Elution profile of lupin sRNA from DEAE-cellulose column (45 × 1.5 cm.) at 72°. The column was equilibrated with 0.5 M-NaCl - 2 mM-EDTA - 20 mM-Na-acetate buffer, pH 6.0, and sRNA (150 mg.) was eluted with NaCl gradient in the same buffer. The fractions (6.4 ml.) were assayed for acceptor activity for (●), isoleucine, (○), leucine, (△), methionine and (■), valine.

The elution of [^{14}C]aminoacyl-sRNA from a column of protamine-coated kieselguhr is shown in Fig. 5. Chromatographic profiles for the labelled leucyl-, isoleucyl-, methionyl- and valyl-sRNA differed from each other, and every [^{14}C]ami-

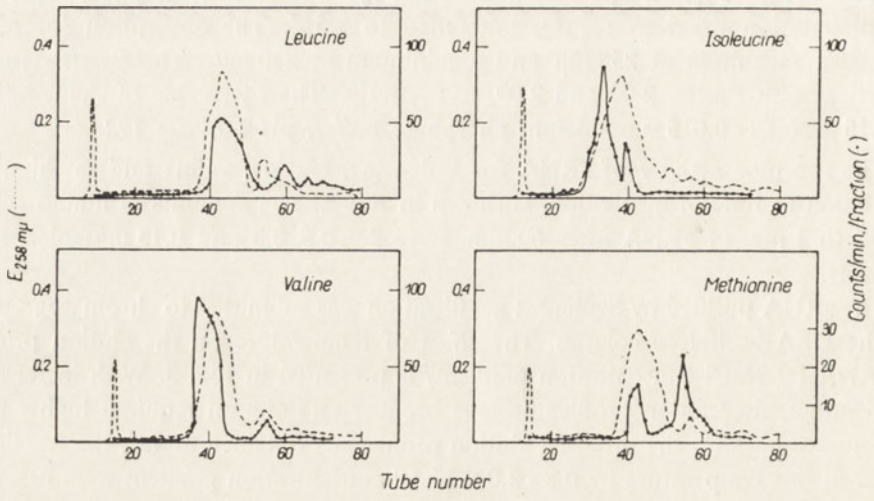

Fig. 5. Fractionation of ^{14}C-labelled aminoacyl-sRNA on protamine-coated kieselguhr. About 3 mg. of the preparation dissolved in 50 mM-potassium phosphate buffer, pH 6.7, was applied on a column (24×1.1 cm.) and eluted with NaCl gradient from 0.2 to 1.6 M in the same buffer. In fractions of 3 ml., extinction at 258 mμ and radioactivity were determined.

noacyl-sRNA separated into two fractions. The first sharply defined peaks absorbing at 258 mμ corresponded probably to traces of high-molecular RNA not separated by gel filtration.

DISCUSSION

Among the methods elaborated for isolation of undegraded RNA, the most widely used is the phenol extraction procedure described by Kirby in 1956. In 1962 Kirby improved the method by adding to phenol 8-hydroxyquinoline and naphthalene disulphonate to prevent ribonuclease activity. From the water phase, RNA is recovered by ethanol precipitation and extracted with 2-methoxyethanol to separate polysaccharides. However, the RNA preparations obtained in this way are not free from contaminations. Ralph & Bellamy (1964) applied precipitation with cetyltrimethylammonium bromide, and obtained purified undegraded RNA from different sources, among others from plants.

From the lupin RNA obtained by the above procedures, homogeneous and active sRNA was prepared by Sephadex G-200 gel filtration according to Delihas & Staehelin (1966) and Schleich & Goldstein (1966). Gel filtration was found to be an essential step in sRNA purification. Similarly as it was reported by the above cited authors, also *E. coli* sRNA (Calbiochem., Los Angeles, Cal., U.S.A.) was found to contain ribosomal RNA, and yeast sRNA prepared according to Holley

et al. (1961) was rather heavily contaminated by low-molecular UV-absorbing compounds.

In the present work, from 2 kg. of ground lupin seeds about 30 g. of crude RNA was obtained, and from this amount 600 - 800 mg. of partly purified RNA and only 300 - 700 mg. of sRNA after gel filtration. Thus the content of sRNA in the crude preparation amounted to only 1 - 2%. The purified sRNA contained only traces of polysaccharides, amino acids, protein and DNA. The absorption spectrum was characteristic for pure nucleic acids with the maximum at 258 mμ, minimum at 230 mμ, and $E_{258}^{1\%}$ equal to 192, or $\varepsilon_{(p)}$ 7900. The content of phosphorus in our preparation (7.5%) and the $E_{258}^{1\%}$ value differ but slightly from the values reported by Lindahl, Henley & Fresco (1965) for a pure preparation of yeast sRNA, 9.0% and 215, respectively.

The total acceptor capacity calculated from the data given in Fig. 2, taking the molecular weight of sRNA as equal to 27 000 (Lindahl *et al.*, 1965), was for leucine 0.014, for isoleucine 0.076, for methionine 0.0041 and for valine 0.008 mole of the amino acid per mole of sRNA, and corresponded to the values for undegraded unfractionated sRNA from other sources (see e.g. Lindahl, Adams & Fresco, 1966).

The suitability of the method of Baguley *et al.* (1965) for fractionation of yeast sRNA on DEAE-cellulose at elevated temperature, was confirmed for lupin sRNA. In our experiments a temperature of 72° was applied whereas Baguley *et al.* (1965) used 65°. In the effluent, the acceptor activity for leucine, isoleucine and valine appeared each in one peak, whereas the activity for methionine was found in three fractions. This last observation may suggest the existence of several forms of the sRNA specific for methionine, differing in affinity toward the anionic exchanger, or may indicate its heterogeneity.

For fractionation of aminoacyl-sRNA, Sueoka & Yamane (1962) employed chromatography on methylated albumin. In the present work, sRNA charged with amino acid was fractionated on protamine-coated kieselguhr, an adsorbent which had been applied for chromatography of nucleic acids by Legault-Démare *et al.* (1964). The elution profiles of the four aminoacyl-sRNA's from lupin indicate that the separation obtained was more efficient than that achieved by Sueoka & Yamane (1962) with methylated albumin. Each of the [^{14}C]aminoacyl-sRNA's studied in the present work, separated into two fractions. This is in agreement with the generally known heterogeneity of specific sRNA's.

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Note added in proof. After this manuscript was submitted, a paper describing the tRNA fractionation on a protamine-coated kieselguhr column was published by Hayashi (1966).

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IZOLOWANIE I NIEKTÓRE WŁASNOŚCI ROZPUSZCZALNEGO KWASU RYBONUKLEINOWEGO Z NASION ŁUBINU ŻÓŁTEGO

Streszczenie

1. Z nasion łubinu żółtego (*Lupinus luteus*) izolowano sRNA posługując się metodą ekstrakcji fenolowej, wytrącaniem bromkiem cetylotrójmetyloamoniowym i sączeniem żelowym na kolumnie Sephadex G-200. Preparaty sRNA wykazywały normalną czynność akceptorową dla aminokwasów.

2. Chromatografia sRNA na kolumnie DEAE-celulozowej w temp. 72° dawała ostro zaznaczone maksima aktywności akceptorowej dla leucyny, izoleucyny i waliny. Aktywność akceptorową dla metioniny znajdowano w trzech frakcjach.

3. [^{14}C]Aminoacylo-sRNA frakcjonowano na protaminowanej ziemi okrzemkowej. Wykazano heterogenność leucylo-, izoleucylo-, metionilo- i walilo-sRNA.

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RIBONUCLEIC ACID ACCOMPANYING DEOXYRIBONUCLEIC ACID IN THE COURSE OF EXTRACTION FROM RAT BRAIN

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1. [^{32}P]Phosphate was given to rats by intracranial injection, and after 1.5 and 24 hr. DNA was extracted from brain homogenate, successively by the method of Kay and by the phenol procedure. 2. The isolated DNA fibres always contained soluble RNA and ribosomal RNA. DNA purified by chromatography on a column of methylated albumin contained some ribonuclease-resistant RNA. 3. In the protein fraction, rapidly labelled RNA with the G+C/A+U ratio close to 1 was found to be present.

By phenol extraction, Kirby (1958) obtained from liver tissue a pure preparation of RNA. Kay (1964) isolated pure DNA from Ehrlich ascites cells by extracting nucleic acids with 1 M-NaCl in the presence of sodium dodecyl sulphate, and precipitating DNA fibres with 1 volume of ethanol. Our preliminary observations with brain tissue indicated that in the RNA preparation obtained by the phenol method some DNA was always present (Borkowski & Borkowska, 1965); on the other hand, DNA fibres obtained according to Kay (1964) from brain homogenate, contained RNA (Borkowski & Borkowska, 1966). In the present paper more detailed studies on the RNA's accompanying DNA isolated from rat brain, are described.

MATERIAL AND METHODS

Animals. Adult white Wistar rats weighing 150 - 170 g. were used for experiments. Radioactive phosphate ([^{32}P]Na_2HPO_4, sp. act. 0.25 mc/μmole, Institute of Nuclear Research, Świerk, Poland) was given by intracranial injection in the region of frontal lobes, in a dose of 33 μc ^{32}P in 20 μl. per 100 g. of rat body weight. For experiments, rats were killed by decapitation 1.5, 12 or 24 hr. after the injection. Each brain, together with the cerebellum, was homogenized in a Potter-Elvehjem type homogenizer in 30 ml. of 0.9% NaCl at 0°.

Extraction of nucleic acids. To combined homogenates of 8 brains was added 0.1 vol. of 5% solution of sodium dodecyl sulphate (SDS) in 45% ethanol, and NaCl *in substantia* to 1 M concentration. The mixture was stirred for 1 hr. at 0°, centrifuged for 30 min. at 0° at 2500 *g*, and to the supernatant 1 vol. of ethanol was added. The formed fibres were collected from the solution with a glass rod and homo-

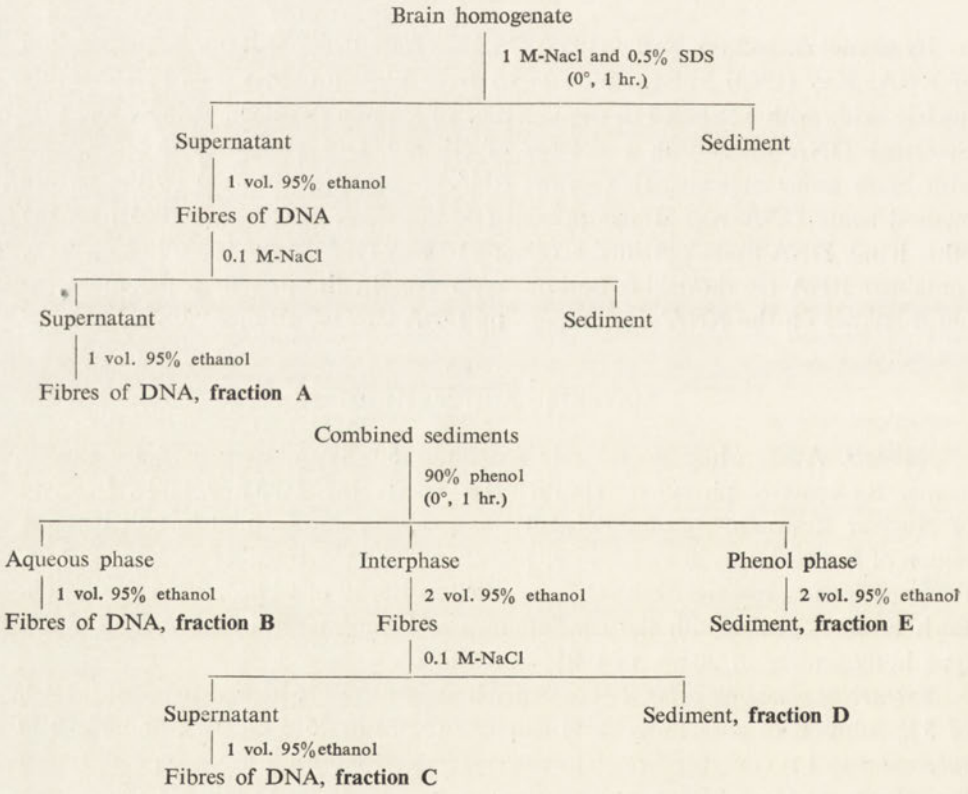
genized in 0.1 M-NaCl. After centrifuging down the insoluble material, to the supernatant 1 vol. of 95% ethanol was added. DNA fibres were obtained, which are subsequently referred to as fraction A.

The sediments remaining after the first and second centrifugation were homogenized again in 0.7 M-NaCl containing 0.3% SDS, and extracted with 1 vol. of 90% phenol for 1 hr. at 0°, with constant stirring. After centrifuging, the aqueous phase was extracted three times with ether to remove phenol and the remaining ether was removed by flushing air. Sodium acetate was added to a concentration of 2%, then 1 vol. of 95% ethanol, and the fibres which formed were collected. This preparation is referred to as fraction B.

To the interphase separated from the phenol phase, 2 vol. of 95% ethanol was added. The fibres were collected from the solution, homogenized in 0.1 M-NaCl and centrifuged at 20 000 *g*. To the clear supernatant, 1 vol. of 95% ethanol was added; the fibres which were collected are referred to as fraction C. The sediment, insoluble in 0.1 M-NaCl, is referred to as fraction D.

To the phenol phase was added 2 vol. of 95% ethanol, centrifuged, and the obtained sediment delipidated according to Bloor (1929). The resulting powder is referred to as fraction E.

The essential steps of the extraction procedure are shown in Scheme 1.



Scheme 1. Isolation of nucleic acids from rat brain

Analytical methods. Fractionation of nucleic acids was carried out on methylated albumin-kieselguhr columns (Mandell & Hershey, 1960). Enzymic hydrolysis of RNA was performed after Marmur (1961) using crystalline pancreatic ribonuclease (EC 2.7.7.16; British Drug Houses, Poole, Dorset, England) in citrate buffer. The ribonuclease was purified before use by heating at 80° for 10 min. (Marmur, 1961). Quantitative determinations of RNA and DNA were carried out on samples submitted to alkaline hydrolysis according to Schmidt & Thannhäuser (1945). RNA was estimated spectrophotometrically by determining the difference in extinction at 260 and 286 mμ (Tsanev & Markov, 1960) and by ribose determination (Mejbaum, 1939). DNA was estimated by the diphenylamine reaction (Dische, 1939; Borkowski & Sikorska, 1964). The amounts of RNA and DNA were expressed in μg. P. The base composition of RNA was determined after fractionation of alkaline hydrolysis products on Dowex 50 and Dowex 1 columns (Katz & Comb, 1963). The activity of the samples was measured on a wet layer with a Geiger-Muller mica window counter (4 mg./cm.^{2}).

RESULTS

Determinations of RNA and DNA were carried on the fractions which precipitated as fibres on addition of 1 vol. of ethanol, and on the nucleic acids remaining in the protein sediment. The content of RNA and DNA in particular fractions is shown in Table 1. The distribution of RNA in the analysed fractions, excepting fraction C, was rather similar, whereas DNA was present mainly in fraction B. Consequently, the RNA/DNA ratio for particular fractions exhibited rather large differences.

Fraction A obtained from rat brain by the method of Kay (1964) contained a considerable amount of RNA, the RNA/DNA ratio being 1.7. Separation of fraction A on the methylated albumin-kieselguhr column gave three peaks of absorption, which were eluted with NaCl solutions of the following concentrations: peak *I*, 0.4 M; peak *II*, 0.6 M; peak *III*, 0.7 - 0.8 M (Fig. 1a). According to Mandell & Hershey (1960), peak *I* corresponds to soluble RNA (sRNA), peak *II* to DNA and peak *III* to ribosomal RNA (rRNA). The particular fractions were precipitated with 95% ethanol, then submitted to alkaline hydrolysis and analysed (Table 2). In peak *I*, no DNA was present, and the base composition of RNA corresponded to the composition of brain sRNA reported by Mahler, Moore & Thompson (1966). Peak *II*, in addition to DNA, contained a RNA possessing a base composition corresponding to brain rRNA (Mahler *et al.*, 1966); peak *III*, as judged from its base composition, also contained rRNA. No significant differences were observed in the activities of the particular RNA's, either at 1.5 or 24 hr. after the injection of [^{32}P]phosphate.

Attempts to purify DNA by twofold reprecipitation of fraction A with 1 vol. of ethanol, were unsuccessful, and all three kinds of RNA were always present. Therefore digestion by ribonuclease was applied to remove RNA. After 30 min. incubation, the nucleic acids were precipitated and submitted to column chro-

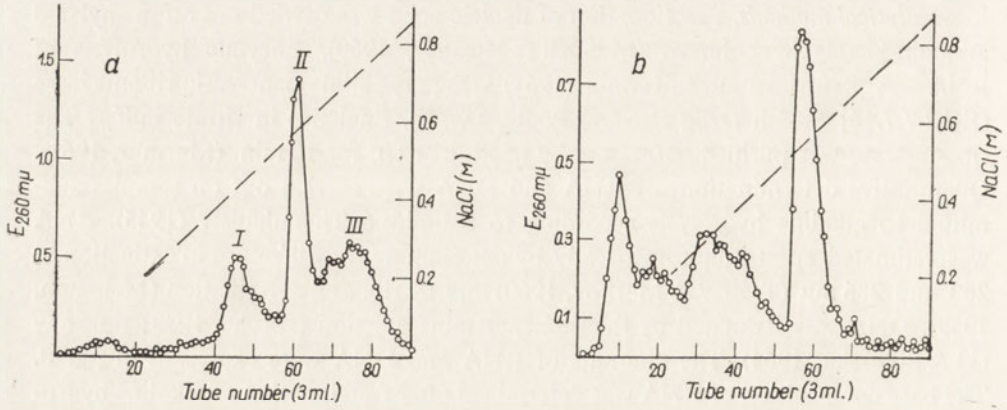


Fig. 1. Chromatography on methylated albumin-kieselguhr column (23 mm. × 120 mm.) of (*a*), fraction A of brain nucleic acids and (*b*), fraction A after ribonuclease digestion. Nucleic acids, 1 - 3 mg., dissolved in 0.14 M-NaCl - 0.01 M-tris-HCl buffer, pH 7.1, were applied to the column and eluted with an NaCl gradient from 0.14 to 1.5 M in tris buffer at 20°.

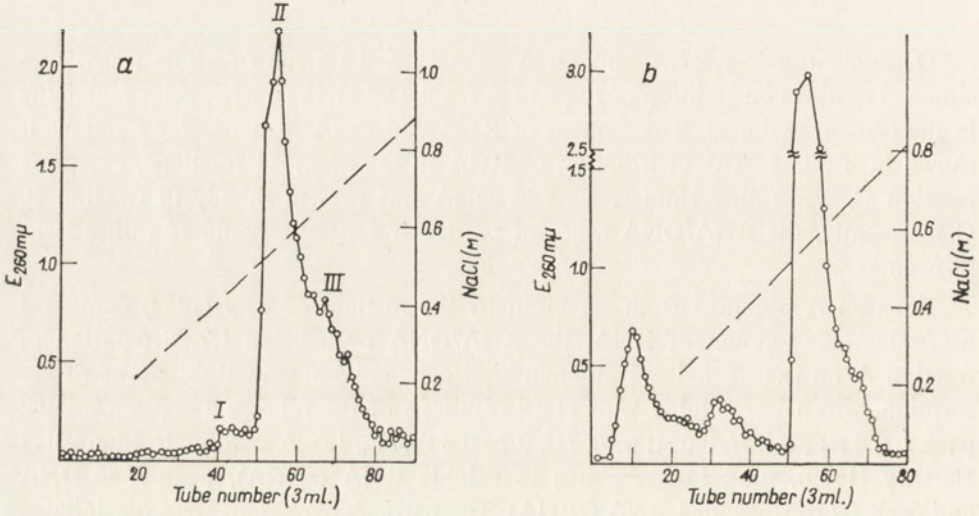


Fig. 2. Chromatography on methylated albumin-kieselguhr column (23 mm. × 120 mm.) of (*a*), fraction B of brain nucleic acids and (*b*), fraction B after ribonuclease digestion. Nucleic acids, 1 - 3 mg., dissolved in 0.14 M-NaCl - 0.01 M-tris-HCl buffer, pH 7.1, were applied to the column and eluted with an NaCl gradient from 0.14 to 1.5 M in tris buffer at 20°.

matography on methylated albumin-kieselguhr (Fig. 1b). The first two peaks contained core RNA remaining after ribonuclease digestion; DNA emerged at 0.6 M--NaCl concn., whereas there was no peak corresponding to high-molecular RNA. In the DNA fraction precipitated with ethanol after alkaline hydrolysis, ribonucleotides were found to be present. The specific activity of ribonuclease-resistant RNA bound to DNA did not differ much from the activities of the other RNA's.

Fraction B, isolated after phenol extraction from the aqueous phase, contained more DNA than fraction A, and had the RNA/DNA ratio of 0.3 (Table 1). Se-

paration on the column of methylated albumin-kieselguhr (Fig. 2a) gave, in addition to a small peak of low-molecular RNA, a high peak eluted with 0.6 M-NaCl, and a weakly resolved fraction emerging at 0.7 M-NaCl. The analysis showed that peak *I* contained only RNA, peak *II* DNA with a small amount of RNA, and peak *III* equal amounts of the two nucleic acids (Table 2). The base composition of peak *III* was characteristic of rRNA. The specific activities of particular RNA's did not differ much from each other, either at 1.5 or 24 hr. after [^{32}P]phosphate injection. After ribonuclease digestion of fraction B, DNA separated by column chromatography (Fig. 2b) was found to contain a small amount (0.78%) of ribonuclease-resistant RNA.

In the small fraction C isolated from the interphase, the RNA/DNA ratio was 0.4 (Table 1). After digestion with ribonuclease the total ribonuclease-resistant RNA amounted to 7 - 20% of DNA. Its specific activity resembled that of RNA of fractions A and B, being respectively 75 and 248 counts/min./μg. P-RNA at 1.5 and 24 hr. after [^{32}P]phosphate injection.

Table 1

The content of RNA and DNA in isolated fractions from rat brain

The fractions were isolated as described in Materials and Methods, and Scheme 1. The results are average values, ± S D., of 5 experiments with 8 brains each.

Fraction	μg. P of		RNA/DNA ratio
	RNA	DNA	
A	187 ± 20	112 ± 18	1.7
B	238 ± 30	785 ± 43	0.3
C	14 ± 5	35 ± 7	0.4
D	120 ± 40	40 ± 12	3.0
E	135 ± 24	0 ± 6	—

Table 2

Analysis of fractions A and B of brain nucleic acids after separation on methylated albumin-kieselguhr column

The results are average values of 5 experiments with 8 brains each. The radioactivity of ribonucleotides isolated 1.5 and 24 hr. after [^{32}P]phosphate injection, was determined.

Preparation	μg. P of		Activity of RNA (counts/min./μg. P)		Base composition of RNA (molar percentage)				$\frac{G+C}{A+U}$	$\frac{A+G}{U+C}$
	RNA	DNA	1.5 hr.	24 hr.	U	G	C	A		
Fraction A										
peak *I*	44	0	62	345	23	29.4	26.5	21.1	1.27	1.02
peak *II*	14	58	72	300	16.9	37	29.4	16.7	1.98	1.16
peak *III*	57	8	41	350	17.8	35	29	18.2	1.80	1.13
Fraction B										
peak *I*	14	0	73	326	—	—	—	—	—	—
peak *II*	20	167	74	350	—	—	—	—	—	—
peak *III*	25	22	61	400	21	35.4	26.9	16.7	1.65	1.11

Table 3

Analysis of RNA from fractions D and E

The results are average values of 5 experiments with 8 brains each. The radioactivity of ribonucleotides isolated 1.5 and 12 hr. after [^{32}P]phosphate injection, was determined.

Fraction	Activity of RNA (counts/min./μg. P)		Base composition of RNA (molar percentage)				$\frac{G+C}{A+U}$	$\frac{A+G}{U+C}$
	1.5 hr.	12 hr.	U	G	C	A		
D	485	203	26.7	30.5	24.1	18.7	1.2	0.97
E	211	383	29.9	30.7	22.5	16.9	1.13	0.9

Fractions D and E contained nucleic acids which could not be separated from protein. The amount of RNA in these fractions was much higher than that of DNA (Table 1). The nucleotide composition of the two fractions was very similar (Table 3) and it should be noted that the ratio G+C/A+U was close to 1. The radioactivity determinations showed that 1.5 hr. after administration of labelled phosphate, the specific activity of RNA present in fraction D exceeded sevenfold, and in fraction E threefold, the activity of RNA in fractions A and B. After 12 hr., the activity of RNA in fraction E increased whereas in fraction D it decreased (Table 3).

DISCUSSION

The presence of RNA-DNA complexes of a hybrid character has been demonstrated in ascites tumour cells by Mandel & Borkowska (1964), in HeLa cells by Krsmanovic, Kanazir & Errera (1965) and in rat liver mitochondria by Nass, Nass & Hennix (1965).

In our attempt to isolate the RNA-DNA hybrid from rat brain, it seemed that the method of Kay (1964) for DNA isolation would be the most convenient. It was found, however, that DNA fibres obtained by this procedure from brain homogenate (fraction A) are contaminated with large amounts of sRNA and rRNA. Moreover, only 10 - 15% of DNA present in the brain was extracted. Therefore for the extraction of the DNA remaining in the sediment, the phenol procedure was applied (fraction B). By the two procedures, 80 - 96% of the total brain DNA could be extracted, the rest being present in the protein sediment.

The fibres of DNA obtained by precipitation with 1 vol. of ethanol, were consistently found to contain three kinds of RNA, namely sRNA, rRNA and ribonuclease-resistant RNA bound to DNA. The base composition of sRNA and rRNA were in agreement with the data reported by Mahler *et al.* (1966) for the respective brain RNA's. Determinations of specific activity of the ribonuclease-resistant RNA bound to DNA did not yield sufficient information on its metabolic nature. Different amounts of RNA bound to DNA found in fractions A (25%) and B (12%) indicate that the yield of this form of RNA is dependent on the method of DNA extraction.

It seems of interest that the protein sediment, in addition to DNA, contained also RNA. This non-extracted RNA differed in base composition from sRNA and

rRNA; it had a high content of uridine and the G+C/A+U ratio close to 1. Similar base composition was reported by Egyhazi (1966) for RNA non-extractable by the phenol method from isolated glial and neuronal cells.

Most of the rapidly labelled RNA is known to remain in the interphase on phenol extraction (Georgiev & Mantieva, 1962; Kimura, Tomoda & Sibatani, 1965). In our experiments, the rapidly labelled fraction of brain RNA was also found in the interphase and bound with protein (fraction D). The simultaneous presence of DNA may permit to assume the presence of a hybrid of messenger RNA with DNA template.

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KWAS RYBONUKLEINOWY TOWARZYSZĄCY KWASOWI DEZOKSYRYBONUKLEINOWEMU PODCZAS JEGO EKSTRAKCJI Z MÓZGU SZCZURA

Streszczenie

1. Szczurom podano doczaszkowo [^{32}P]fosforan i po 1,5 i 24 godz. z mózgu ekstrahowano DNA bezpośrednio metodą Kay'a i następnie metodą fenolową.

2. Izolowane włókna DNA zawierały zawsze rozpuszczalny RNA i rybosomalny RNA. DNA oczyszczony chromatografią na kolumnie z metylowanej albuminy zawierał niewielkie ilości RNA niewrażliwego na działanie rybonukleazy.

3 We frakcji białkowej obecny był RNA szybko znakujący się i wykazujący stosunek G+C/A+U zbliżony do jedności.

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RIBONUCLEASES IN DEVELOPING RAT LIVER

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1. In the nuclear and mitochondrial liver fractions, ribonuclease activity is the highest on the seventh day of life. 2. Optimum pH values for alkaline ribonucleases of sulphosalicylic acid extracts from nuclear and mitochondrial liver fractions of adult and 7-day-old rats, are 7.7 and 8.5, respectively. 3. 0.1 M-Mg^{2+} inhibited by 50% the enzymic activity only in the extracts from liver mitochondria of adult rats.

Ribonucleases have been shown to occur in all so far examined animal and plant tissues. They play an important role in the metabolism of nucleic acids and in this way influence protein biosynthesis. As it has been established that the content of nucleic acids in rat liver increases during development (Fukuda & Shibatani, 1953), a study of ribonucleolytic activity in the liver of both young and adult rats was undertaken. While the experiments were in progress there appeared a communication by Bresnick, Sage & Lanclos (1966), who found that the ribonucleolytic activity per mg. protein was the highest in liver nuclei of rats weighing 5 - 10 g. and then decreased with the age of the animal.

MATERIALS AND METHODS

Preparation of subcellular fractions. White Wistar rats from 2 to 120 days old were used. The animals were killed by decapitation, the livers removed and washed with ice-cold 0.25 M-sucrose. A 10% homogenate in 0.25 M-sucrose was prepared in a motor-driven Potter-Elvehjem glass homogenizer. The nuclei were isolated by centrifugation at 1000 *g* for 10 min., and mitochondria by centrifugation at 17 000 *g* for 10 min. Nuclei and mitochondria were suspended in a volume of 0.25 M-sucrose corresponding to that of the homogenate.

All these manipulations, as well as preparation of sulphosalicylic acid extracts, were carried out at 0 - 4°. The obtained subcellular fractions were stored at -20°; before activity determinations they were thawed and frozen four times.

Preparation of 0.2 M-*sulphosalicylic acid extracts.* To 9 volumes of the suspension of nuclei or mitochondria, 1 vol. of 2 M-sulphosalicylic acid was added with stirring. After 30 min., the extracts were filtered through Macherey, Nagel & Co. (Düren,

Germany) no. 61 filter paper, the clear solutions were adjusted to pH 7 with 4 N-NaOH and dialysed for 24 hr. against two changes of 100 volumes of water.

Purification of ribonucleic acid. Commercial yeast RNA (B.D.H., Poole, England), 10 g., was suspended in 50 ml. of 0.1 mM-EDTA, adjusted to pH 7.0 with 4 N-NaOH, and dialysed for 24 hr. against two changes of 2.5 liters of 1 M-NaCl, then for 48 hr. against four changes of water. The purified product was freeze-dried and stored in a desiccator at 0 - 4°.

Determination of ribonuclease activity. This was carried out after Roth (1958a). The incubation mixture contained in 1 ml.: 0.8 or 0.6 ml. of universal buffer of Davies (1959) of appropriate pH; 1.5 mg. of yeast RNA; and 0.1 ml. of a suitably diluted suspension of nuclei, mitochondria or 0.2 ml. of the sulphosalicylic acid extract. The reaction was allowed to proceed for 30 min. at 37°, then undigested RNA was precipitated with 1 ml. of 1 N-HCl in 76% ethanol. The supernatant was diluted with 2 vol. of water and the increase in extinction at 260 mμ was measured in 1 cm. quartz cuvettes in a Unicam SP 500 spectrophotometer. A blank was prepared in the same way except that the enzyme preparation was added after precipitation of RNA. The activity was expressed as $\Delta E_{260m\mu}$ after 30 min. incubation.

Protein determination. Protein in the nuclear and mitochondrial fractions was determined by the biuret method as described by Layne (1957), and in the sulphosalicylic acid extracts by the tannin method (Mejbaum-Katzenellenbogen, 1955).

RESULTS

The ribonucleolytic activity in adult rats was found to have two pH optima, at pH 6.0 and 7.7, both in the nuclear and mitochondrial fraction. In the young animals (2, 7, 14 and 28 days old) the optimum at pH 6.0 was also observed, whereas the optimum at pH 7.7 not always did appear. Moreover, a very distinct optimum was found in all young rats at pH 8.5 (Fig. 1).

Changes in the ribonucleolytic activity per mg. protein during development of the animals, at pH 6.0, 7.7 and 8.5 are presented in Table 1. On the second day of life, the activity both at alkaline and acid pH values was relatively low and did not differ significantly from that in adult rats. On the seventh day, however, the activity was found to increase to values 3 - 4 times higher, and then it decreased gradually.

Roth (1957) and Gordon (1965) demonstrated that alkaline ribonuclease, in contrast to acid ribonuclease, can be extracted with 0.25 N-sulphuric acid. In our experiments, 0.2 M-sulphosalicylic acid was used, and in the extracts from nuclei and mitochondria of young rats the pH optimum was 8.5 while in the extracts from nuclei and mitochondria of adult rats the optimum was at pH 7.7, and additionally in mitochondria a second optimum appeared at pH 6.5 (Fig. 1).

Table 2 shows the amount of protein and the activity of ribonuclease extracted with sulphosalicylic acid. The experiments were performed on 7- and 120-day-old

rats. From liver nuclei of both groups of animals as well as from mitochondria of 7-day-old rats, 0.6 - 1.2% of protein was extracted, whereas for mitochondria of adult rats the extraction amounted to 3 - 8% of protein. About 20% of the ribonucleolytic activity was extracted with sulphosalicylic acid from either of the two

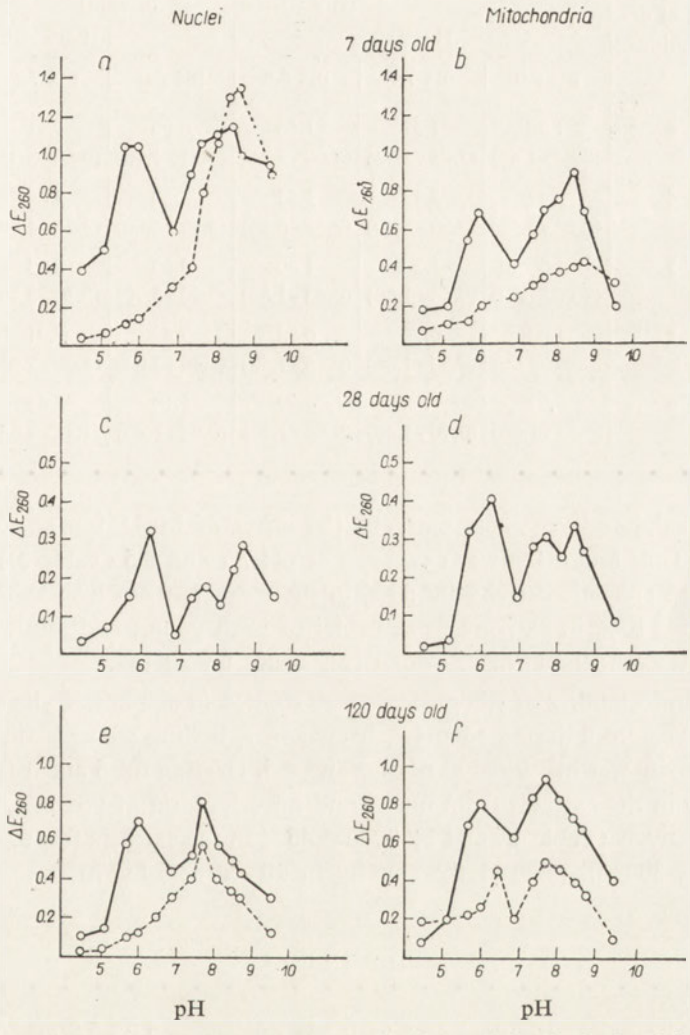

Fig. 1. Ribonucleolytic activity at different pH values in liver nuclei and mitochondria of 7-, 28- and 120-day-old rats. Enzyme activity was assayed as described in Methods. ΔE_{260} values for (———) the whole fraction, and (- - - -), the sulphosalicylic acid extract are presented.

subcellular fractions studied, both in young and adult rats. The activity per mg. protein in extracts from nuclei of young as well as adult rats, and from mitochondria of young rats was purified 20 - 40-fold. However, in liver mitochondria of adult rats, from which a large amount of non-enzymic protein was extracted, the ribonuclease activity was purified only 3 - 8-fold.

Table 1

Ribonucleolytic activity in liver nuclei and mitochondria of growing rats

Enzyme activity was determined as described in Methods, and protein by the biuret method (Layne, 1957). In parentheses the limit values are given.

Age (days)	No. of determinations	Activity ($\Delta E_{260\,m\mu}$/mg. protein)					
		Nuclei			Mitochondria		
		pH 6.0	pH 7.7	pH 8.5	pH 6.0	pH 7.7	pH 8.5
2	6	1.26 (0.84 - 1.68)	1.51 (1.23 - 1.92)	1.56 (1.00 - 2.25)	0.99 (0.52 - 1.70)	1.51 (0.82 - 2.35)	1.62 (0.87 - 2.30)
7	6	2.57 (2.00 - 3.12)	3.13 (2.20 - 5.49)	3.53 (2.52 - 6.54)	3.20 (3.38 - 4.00)	3.48 (3.20 - 4.80)	4.57 (3.77 - 6.40)
14	3	1.30 (0.86 - 1.70)	1.20 (0.54 - 1.68)	1.40 (0.63 - 1.80)	3.55 (3.30 - 3.70)	2.93 (2.30 - 3.70)	3.10 (2.60 - 3.70)
28	3	1.03 (0.40 - 1.40)	0.74 (0.35 - 1.08)	0.88 (0.46 - 1.30)	2.40 (1.60 - 3.00)	1.91 (1.08 - 2.50)	2.13 (1.20 - 2.60)
120	6	0.77 (0.51 - 1.00)	1.12 (0.70 - 1.40)	0.99 (0.64 - 1.30)	1.10 (0.86 - 1.40)	1.65 (1.32 - 2.04)	1.32 (1.10 - 1.64)

In the sulphosalicylic acid extracts, the effect of Mg^{2+} ion at concentrations of 5 mM, 50 mM and 0.1 M was examined at pH 7.7 and 8.5 (Table 3); 0.1 M-Mg^{2+} inhibited the enzymic activity by 50% in the extracts from liver mitochondria of adult and by 10% in extracts from nuclei of both young and adult animals, the extracts from mitochondria of young rats being unaffected.

The thermostability of ribonuclease was studied in sulphosalicylic acid extracts. The extracts were adjusted to pH 7, heated in a boiling water bath, then cooled and the activity was measured at pH 7.7 and 8.5. Heating for 5 and 10 min. reduced the activity in the extracts from nuclei and mitochondria of young rats by 10 and 20%, respectively (Table 4), and by 20 and 45% in the extracts from adult animals. Heating for 30 min. reduced the activity in all extracts by 70%.

DISCUSSION

Acid and alkaline ribonucleases with pH optima at 5.2 - 5.8 and 7.8 - 8.2, respectively, have been found to be present in animal tissues (De Lamirande, Allard, Da Costa & Cantero, 1954; Roth, 1954; Beard & Razell, 1964). In our experiments, a third peak of enzymic activity at pH 8.5 was observed in nuclei and mitochondria during development of rats; the activity of both acid and alkaline ribonucleases was the highest on the seventh day of life.

The specific activity determinations showed large differences between the animals of the same age. Bresnick *et al.* (1966) studied alkaline ribonuclease activity in hepatic nuclei during development, taking weight of the animals as the criterion

of development. Although our experiments were carried out with a fraction of the homogenate centrifuged at 1000 *g* whereas Bresnick *et al.* (1966) used purified nuclei, our results seem to agree with those of Bresnick as far as the change of specific activity in nuclei during development is concerned. Our 7-day-old rats which weighed 8 - 11 g. corresponded probably to the 5 - 10 g. group of Bresnick. The nuclei of these animals showed the highest ribonucleolytic activity.

It has been previously established that alkaline ribonuclease may be extracted from animal tissues with diluted acids (Roth, 1957; Gordon, 1965). The present results indicate that another ribonuclease with the optimum at pH 6.5 may be also extracted from liver mitochondria of adult rats using 0.2 M-sulphosalicylic acid.

Alkaline ribonucleases are known to be heat-stable (Roth, 1958b; Maver & Greco, 1962). Our results indicate that the ribonucleases from mitochondria and nuclei of young rats exhibit greater heat stability than those from adult animals.

Table 2

Ribonucleolytic activity extracted with sulphosalicylic acid from liver nuclei and mitochondria of young and adult rats

Enzyme activity was determined as described in Methods, at pH 7.7. Protein in whole fractions was determined by the biuret method (Layne, 1957) and in the extracts by the tannin method (Mejbaum-Katzenellenbogen, 1955). In the experiments with 7-day-old rats, livers from all the litter were pooled. Total activity is expressed as $\Delta E_{260m\mu}$ and specific activity as $\Delta E_{260m\mu}$/mg. protein.

Age (days)	Expt. no.	Whole fraction			0.2 M-Sulphosalicylic acid extract					
		Protein (mg.)	Total activity	Specific activity	Protein (mg.)	Total activity	Specific activity	Purification	Extracted protein (%)	Yield of activity (%)
Nuclei										
7	1	225	517	2.30	1.32	138.6	105	45	0.58	26
	2	150	823	5.49	1.93	330	171	31	1.28	40
	3	180	396	2.20	1.84	101.2	55	25	1.02	25
120	1	303	281	0.93	2.97	47.5	16	17	0.98	16
	2	212	282	1.33	1.40	60.2	43	30	0.66	21
	3	405	421	1.04	3.88	73.7	19	18	0.95	17
Mitochondria										
7	1	76.5	336.6	4.4	0.50	22	44	10	0.65	6
	2	37.5	180.0	4.8	0.43	40.4	94	19	1.1	22
	3	56.3	180.1	3.2	0.63	40.9	65	20	1.1	23
120	1	123	175.9	1.43	9.45	47.2	5	3.5	7.68	27
	2	112	179.2	1.6	3.20	38.4	12	7.5	2.85	21
	3	112	228.5	2.04	2.94	44.1	15	7.3	2.62	19

Table 3

Effect of Mg^{2+} *ion on ribonucleolytic activity in the sulphosalicylic acid extracts from liver nuclei and mitochondria of young and adult rats*

Enzyme activity is expressed as percentage in relation to the sample without added Mg^{2+} ion. The mean values from 3 experiments are given, with limit values in parentheses.

Mg^{2+} ion concn. (M)	Activity (%)							
	Nuclei				Mitochondria			
	pH 7.7		pH 8.5		pH 7.7		pH 8.5	
	7 days old	120 days old	7 days old	120 days old	7 days old	120 days old	7 days old	120 days old
0	100	100	100	100	100	100	100	100
5×10^{-3}	85 (77 - 94)	110 (105 - 120)	106 (100 - 110)	112 (106 - 119)	105 (95 - 120)	66 (58 - 80)	103 (95 - 113)	75 (71 - 80)
5×10^{-2}	93 (85 - 99)	99 (85 - 112)	107 (100 - 114)	111 (106 - 116)	114 (97 - 140)	72 (62 - 82)	108 (100 - 119)	62 (50 - 70)
10^{-1}	79 (73 - 84)	89 (80 - 104)	90 (85 - 95)	90 (74 - 116)	103 (100 - 110)	55 (50 - 63)	102 (100 - 105)	55 (48 - 62)

Table 4

Effect of heating on ribonucleolytic activity in sulphosalicylic acid extracts from liver nuclei and mitochondria of young and adult rats

The extracts were heated in a boiling-water bath, then cooled, and enzyme activity was determined. The results are expressed as percentages in relation to the non-heated sample. The mean values of 3 experiments are given, with limit values in parentheses.

Heating (min.)	Activity (%)							
	Nuclei				Mitochondria			
	pH 7.7		pH 8.5		pH 7.7		pH 8.5	
	7 days old	120 days old	7 days old	120 days old	7 days old	120 days old	7 days old	120 days old
0	100	100	100	100	100	100	100	100
5	93 (90 - 98)	76 (64 - 87)	92 (87 - 97)	72 (66 - 82)	96 (92 - 99)	73 (66 - 83)	89 (87 - 92)	74 (68 - 80)
10	84 (74 - 92)	69 (61 - 75)	82 (74 - 89)	61 (53 - 68)	85 (79 - 90)	55 (47 - 60)	75 (69 - 77)	58 (53 - 63)
30	30 (24 - 38)	32 (28 - 36)	35 (29 - 46)	29 (23 - 38)	36 (33 - 40)	31 (26 - 35)	32 (28 - 38)	31 (25 - 39)

The observation that Mg^{2+} ion inhibited alkaline ribonuclease only in the sulphosalicylic acid extracts from liver mitochondria of adult rats and had no effect on the enzyme in mitochondrial extracts from 7-day-old rats, may suggest that during development the intracellular ribonucleases are subject not only to quantitative changes but may undergo qualitative alterations as well.

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RYBONUKLEAZY WĄTROBY SZCZURA W CZASIE ROZWOJU

Streszczenie

1. Aktywność rybonukleazy we frakcji jądrowej i mitochondrialnej wątroby szczura jest najwyższa w 7-mym dniu życia.

2. Optimum pH alkalicznej rybonukleazy w ekstraktach kwasem sulfosalicylowym frakcji jądrowej i mitochondrialnej z wątroby dorosłych i 7-miodniowych szczurów jest, odpowiednio, pH 7.7 i 8.5.

3. 0.1 M-Mg^{2+} hamował w 50% enzymatyczną aktywność tylko w ekstraktach z mitochondriów wątroby dorosłych szczurów.

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STREPTOCOCCAL GROUP H TRANSFORMING SYSTEM WITH REPRODUCIBLE HIGH TRANSFORMATION YIELDS

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1. A detailed description is presented of the transforming system for two strains of group H hemolytic streptococci: Challis and Wicky. 2. Reproducible and high yields of transformants have been obtained by a suitable choice and adjustment of the following experimental conditions: the initial density of the recipient culture; the presence of an excess of the competence factor during growth; the ratio of transforming DNA concentration to the number of recipient cells and the time of contact between them; and time of plating. It has been possible in this way to obtain about 50% transformants in the case of the Challis strain and about 60% for the Wicky. These values, in terms of viable units, represent the sum of transformants for two non-linked markers. 3. It is furthermore shown that, with these systems, an excess of DNA above the initial "saturation" value may lead to an appreciable decrease in the number of transformants. 4. The significance of the associated experimental conditions is discussed and the possibility is considered of increasing these yields, and of their evaluation in terms of the fractional number of cells transformed.

It is probably not at all unreasonable to ask ourselves at the present time whether the usually encountered low transformation frequencies in bacterial transformation systems are merely a reflection of the mechanism of this process, or whether they are the natural consequence of non-optimal reaction conditions between recipient cells and the transforming DNA molecules.

A perusal of the literature shows that there are at least several instances where the yields of transformants are relatively high. For example, with the *Diplococcus pneumoniae* system, Guild & DeFilippes (1957) reported on one occasion a transformation frequency of 28% for a single marker; and Hotchkiss (1954), employing a synchronous culture, obtained a yield of 17% for the streptomycin resistance marker. However, under normal conditions, the transformation yield for this system does not exceed 5%. Another illustration of an exceptionally high transformation frequency is that of Goodgal & Herriott (1961), who reported with the *Haemophilus influenzae* system an overall figure of about 40% as the calculated sum for six non-linked markers, the maximum for a single marker being about 10%. This result is also unique, the usual yields with the foregoing system being less than 4% (Spencer & Herriott, 1965).

The foregoing and additional examples (see below) demonstrate that transformation yields may be appreciably increased by a suitable choice of conditions, from which it logically follows that appropriate conditions may conceivably be found leading to a nearly quantitative yield of transformants. If such a situation could be approximated with a given transforming system, it would *a priori* make available an improved tool for studying the mechanism and nature of the transformation process.

Our interest in this problem was stimulated by the streptococcal group H system originally described by Pakuła, Fluder, Hulanicka & Walczak (1958) and subsequently studied in greater detail by Pakuła, Piechowska, Bańkowska & Walczak (1962). With this system it proved possible to attain consistent yields of transformants of the order of 15% with a single marker, and occasionally 20% and higher. It was therefore considered of interest to examine whether some modifications of this system could lead to any appreciable increase in the efficiency of the transformation process, the search for optimal conditions being based in part on previously reported results (Pakuła *et al.*, 1962).

MATERIALS AND METHODS

Bacterial strains

The Challis donor strain of group H hemolytic streptococci (Pakuła *et al.*, 1962) was resistant to dihydrostreptomycin (2000 μg./ml.), cathomycin (30 μg./ml.) and erythromycin (4 μg./ml.). The corresponding recipient strain was sensitive to all of the foregoing antibiotics at the following concentrations: dihydrostreptomycin, 25 μg./ml.; cathomycin, 12.5 μg./ml.; erythromycin, 0.05 μg./ml.

The Wicky strain of group H hemolytic streptococci was obtained from Dr. H. Osowiecki. This strain was used as a recipient and was sensitive to all of the above antibiotics.

Isolation of DNA

Challis transforming DNA was isolated by two methods, that of Marmur (1961); and by means of a procedure based on the methods of Saito & Miura (1963) and Berns & Thomas (1965).

The initial steps in both procedures involved collection of the bacterial cells from Todd-Hewitt culture medium, lysis of the cells by means of an extract from a culture of *Streptomyces albus* (McCarty, 1952), and precipitation of the crude DNA from the lysate with 1.5 volumes ethanol.

In applying the Marmur procedure, the crude DNA precipitate was dissolved in 0.1 M-sodium versenate containing 0.15 M-NaCl at pH 8, and the published procedure then followed as for other bacterial lysates.

In the second method, the crude DNA was dissolved in SSC (0.15 M-NaCl - - 0.015 M-sodium citrate), Duponol added to a concentration of 2%, and the mixture heated at 60° for 10 min., following which it was treated with pronase at a con-

centration of 0.5 mg./ml. at 37° for 24 to 40 hr. The solution was then deproteinized with water-saturated phenol at pH 7.5 to 8.0, the DNA precipitated by addition of 1.5 volumes ethanol, dissolved in SSC, and exhaustively dialysed against SSC to remove traces of phenol. The solution was digested with pancreatic ribonuclease at a concentration of 50 μg./ml. for 1 hr. at 37°, again subjected to deproteinization with phenol, and the DNA finally precipitated with ethanol. The purified DNA was then dissolved either in physiological saline or SSC and subjected to dialysis against the same solvent to remove traces of phenol.

The yield of DNA by both the above procedures was similar and amounted to about 3 mg. per gram of wet bacteria.

The DNA content of the solutions was measured spectrally, and by the method of Burton (1955). The difference between these two was taken as a measure of the RNA content, which was estimated to be well below 5%. Protein was determined by the method of Lowry (see Layne, 1957), which gave values ranging from 0.8 to 1.6% in different preparations. The low protein content was further testified to by the ratio of extinction at 260 mμ to that at 230 mμ, which ranged from 2.27 to 2.35.

Transformation medium

The transformation medium, somewhat modified from that previously described (Pakuła *et al.*, 1962) was prepared as follows. To an extract of fresh ox heart (1 kg. per liter water) was added Difco yeast extract to a concentration of 1% and Difco neopeptone to a concentration of 1.5%, following which the pH was adjusted to 8.3 - 8.5. To this was added 2.2 ml. of 10% $CaCl_2$ per liter, the mixture brought first to the boiling point, then to room temperature, and filtered. The filtrate was brought to pH 7.4 - 7.6, and glucose added to a concentration of 0.033%; the medium was then sterilized at 0.75 atmospheres for 15 min. To this medium, immediately prior to use, was added one-tenth the volume of filtered (Seitz) swine serum heated to 62° for 30 min. to destroy deoxyribonuclease activity.

RESULTS

Transformation with DNA prepared by two different procedures. It has been reported by Saito & Miura (1963) that the yield of transformants in the *Bacillus subtilis* system, which is normally quite low, can be increased by a factor of 8 with the use of DNA which has been deproteinized by the phenol method, as compared to that subjected to deproteinization by the Sevag procedure. This prompted us to compare the transforming activity of Challis DNA isolated with the aid of pronase and phenol, as compared to that prepared by the method of Marmur. However, both for the dihydrostreptomycin resistance marker, as well as that for cathomycin, the transformation frequencies were identical for both DNA preparations, as were also the minimum DNA concentrations to give transformants, viz. 5×10^{-6} μg./ml. in a cell population of 1×10^6 cells/ml.

While our findings do not necessarily invalidate those of Saito & Miura (1963), one possible interpretation for the difference in results can be advanced. It has previously been shown that the Sevag chloroform deproteinization technique may lead to some fractionation of a DNA preparation (Piechowska & Shugar, 1965) which, if it occurred, would almost certainly lead to differences in transformation efficiency of a given marker, or between several markers.

Selection of density of recipient cell population. Previous experiments (Pakuła *et al.*, 1962) on the transformation of the Challis strain had already pointed to the significance of the initial density of the cell population on the maximal attainable degree of competence. An increase in the initial density of a culture from a range of $0.2 - 0.5 \times 10^5$ viable units/ml. to a range of $1.5 - 9.0 \times 10^5$ viable units/ml. led to a twofold increase in the maximum transformation yield, and simultaneously accelerated the appearance of the competent state. Subsequent, unpublished, experiments have shown that a further increase in density of the initial cell culture leads to a decrease in the yield of transformants during the appearance of the competent state. This finding limited us to a selection of the above-mentioned optimal initial density of a Challis culture, for maximum competence of the recipient cells, to about $4 - 9 \times 10^5$ viable units/ml.

Influence of growth phase and competence factor(s) on yield of transformants. In a culture of Challis recipient cells with an initial count of about 9×10^5 viable units/ml., competence manifests itself about 15 min. after initiation of growth, and persists up to the end of the logarithmic phase. However, maximum competence prevails only in the early logarithmic growth phase. It is known from the results of Pakuła *et al.* (1962) and Dobrzański & Osowiecki (1966) that at least one of the factors responsible for streptococcal competence is a protein-like substance which appears in the medium during growth of a Challis culture, and that the concentration of this factor is higher in old cultures. It follows that, to obtain the maximum number of competent cells, both the concentration of competent factor and the stage of growth of the culture must be taken into account. Consequently a culture of competent recipients was prepared by the addition, to a fresh culture in the early logarithmic phase (after about 2 hr. incubation), of one-fourth the volume of a filtrate from a 5 hr. culture of Challis recipient cells, so as to ensure an adequate concentration of competence factor during the growth phase most advantageous for formation of the competent state. [It is, perhaps, worth noting that an excess of the competence factor is without adverse effect on the transformation frequency (Osowiecki, personal communication; Dobrzański & Osowiecki, 1966)]. Following 20 min. incubation in the presence of the competence factor, samples of the culture medium were withdrawn at successive 10 min. intervals and frozen at -70°. The competence of these samples was measured and those showing the highest yields of transformants were stored for subsequent use. The density of the recipient cultures in the phase of maximum competence varied from 1 to 4.8×10^6 viable units/ml.

Concentration of DNA and transformation yield. It is implicitly assumed that the competence of a recipient strain is characterized by the number of transformants obtained at a saturation concentration of DNA, i.e. where a further increase in DNA concentration does not increase the number of transformants. This concept is not fully applicable to our Challis system since an increase in DNA concentration beyond a certain level actually leads to a decrease in the number of transformants, as illustrated by the calibration curve shown in Fig. 1. It will be seen from the figure

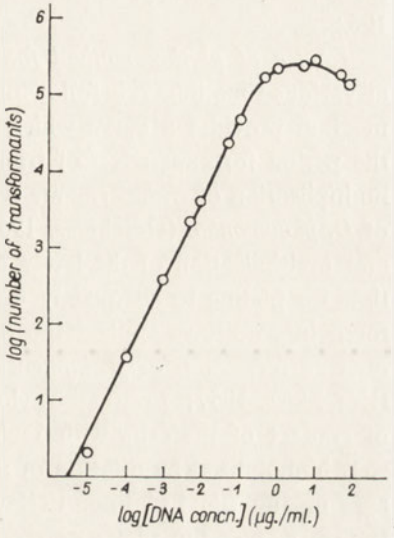

Fig. 1. Influence of DNA concentration on number of dihydrostreptomycin-resistant transformants in Challis strain.

that the dependence of the number of transformants on DNA concentration is linear to about 0.5 μg./ml., then levels off at about 3 to 5 μg./ml. DNA. Above this concentration the number of transformants begins to decrease so that at 50 μg./ml. it is 78% of the plateau value, and at 80 μg./ml. about 54%.

Attention should be drawn to the fact that the density of the recipient culture employed for the calibration curve in Fig. 1 was 2×10^6 colony-forming units/ml. For a recipient culture density eightfold higher than this, no decrease in the number of transformants below the plateau level was observed even at a DNA concentration of 100 μg./ml. (Pakuła *et al.*, 1962). It is consequently clear that the ratio of the number of DNA molecules to the number of recipient cells is of primary importance. We shall revert in the discussion, below, to the possible reasons for the decrease in transformation yield at high DNA concentrations. On the basis of the foregoing, however, a DNA concentration of 3 to 5 μg./ml. was subsequently employed in evaluations of the transformation yields of Challis recipients.

Time of contact of recipient cells with DNA. Appearance of transformants may be observed after about 1 min. contact of recipient cells with DNA, but about 10 min. contact is required to attain the maximum number of transformants (Pa-

kuła *et al.*, 1962). This suggests that the increase in number of transformants, about threefold, resulting from prolonged contact of recipient cells with DNA, must be due to the appearance of additional competent cells during this period. If a thawed, freshly diluted competent culture is employed for transformation, cell multiplication commences after about 20 min. of lag phase, so that the new competent cells must of necessity be the initial ones, which entered the competent state during the lag phase. It is at the moment not feasible to establish the appearance of new competent cells by other methods, since the proportion of competent cells in a culture decreases rapidly, about 20-fold during 20 min. of the lag phase (Pakuła *et al.*, 1962).

Choice of plating method for transformants. Plating of Challis transformants at various time intervals following contact of the recipient cells with DNA in fluid medium provides a curve which does not exhibit a clear differentiation between the period for expression of transformants, the plateau level, and the period for multiplication of transformants (Pakuła *et al.*, 1962) as is observed, e.g. in the case of *D. pneumoniae* (Hotchkiss, 1957). The periods for expression of transformants and multiplication of the latter overlap, so that it is difficult to select the proper time for plating to adequately evaluate the yield of transformants. In view of the foregoing, we have applied the overlay technique previously employed in studies on transformation of *H. influenzae* (Alexander & Leidy, 1953) and *D. pneumoniae* (Hotchkiss, 1957; Ephrussi-Taylor, 1959). Several minutes following termination of contact of the cells with DNA (by addition of deoxyribonuclease), the cells were immobilized by dilution of samples of the fluid cell suspension with a blood-agar medium, and plating. Following 3 hr. incubation at 37° for expression of transformants, the plates were coated with a second layer of blood-free agar containing the antibiotic required for selection of transformants, and incubation then continued at 37°. For transformation to dihydrostreptomycin resistance, incubation was for 30 - 40 hr.; for cathomycin and erythromycin resistance, about 60 hr.

Routine conditions for transformation of Challis and Wicky strains. (a) Preparation of competent cultures. Warmed transforming medium was inoculated from an 18-hr. culture in broth containing 3% lamb's blood, using 5 ml. of 100-fold diluted culture per 100 ml. transforming medium. The initial density of such a culture in the transforming medium was $4 - 8 \times 10^5$ viable units per ml. Following 2.25 hr. incubation at 37°, there was added 25% by volume of a filtrate from a 5-hr. culture of Challis recipients (the filtrate was prepared the previous day, stored at 4°, and warmed to room temperature before use). At intervals of 20, 30 and 40 min. incubation at 37°, samples were removed and rapidly brought to -70°. These frozen samples were then tested for transformation efficiency, and those with the highest yields set aside for use in subsequent experiments.

It must be emphasized that the high transformation yields reported in this paper are attainable only if the frozen competent cultures are employed within a period of 24 hr. from the time of freezing. Prolonged storage of these competent cultures, even at -70°, leads to a progressive decrease of competence (as has also been observed in the case of *D. pneumoniae*), as will be shown below.

(b) Transformation reaction. A sample of a competent Challis culture was thawed and diluted 3 to 5-fold with fresh transformation medium at a temperature of 0 - 4°, to give a density of about 5×10^5 viable units per ml. At this temperature, thawing and dilution do not provoke any loss in competence. To 1.8 ml. portions of the diluted recipient culture, in test tubes maintained at 0 - 4°, were added 0.2 ml. portions of a DNA solution to give a final concentration of 3 to 5 μg./ml. DNA.

The tubes containing the recipient strain and DNA were then all transferred simultaneously to a water bath at 37°. After 18 min., 0.1 ml. of Worthington deoxyribonuclease solution was added to each tube to give a concentration of 2 μg./ml., and the tubes left for an additional 5 min. During these manipulations the recipient culture does not multiply, since it is in the lag phase. After 5 min. hydrolysis with deoxyribonuclease, the tube contents were diluted with agar in broth containing 3% lamb's blood and spread on agar dishes. Following 3 hr. incubation at 37° for expression of transformants, the dishes were plated with a second layer of agar containing, in place of blood, an antibiotic at such a concentration that, after diffusion throughout the entire volume of agar, the antibiotic concentrations were: 125 μg./ml. for dihydrostreptomycin; 13 μg./ml. for cathomycin; 0.5 μg./ml. for erythromycin. Controls were prepared without addition of antibiotics, and also without added DNA in order to check for possible mutations.

Transformation yields. Of the three frozen cultures of Challis recipients, prepared as above, a 50% yield of transformants was normally obtained from one or two. This figure is the sum of the yields for the dihydrostreptomycin resistance marker, about 28%; and for cathomycin resistance, about 33%; the sum being corrected by subtraction of double transformants (since the yield for the erythromycin marker was only about 1%, it was not employed in these experiments). This is permissible since the two markers are non-linked, as is proven by Fig. 2, which

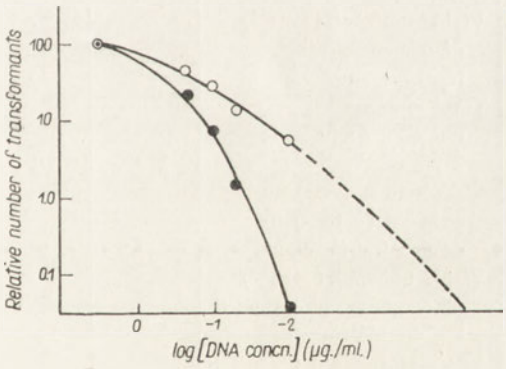

Fig. 2. Effect of DNA concentration on the relative number of double transformants in Challis strain: (○), relative number of transformants to dihydrostreptomycin-resistance; (●), relative number of double transformants (dihydrostreptomycin and cathomycin resistance).

shows the relative number of double transformants, compared to singles, with decreasing DNA concentrations; note that the slope of the curve for the decrease in double transformants is about 2 as compared to 1 for the singles (Goodgal, 1961). Not shown in the figure is the observation that the number of double transformants observed experimentally was less than that to be expected theoretically for two independent processes.

Table 1

Determination of double transformants in Challis strain by the replica plating technique

The numbers in the no. columns should be multiplied by 10^3 to give the actual number of viable units/ml. culture. In the column for cathomycin transformants, these are the actual numbers of cathomycin transformants on a single plate, which were subsequently replicated on agar with dihydrostreptomycin.

Expt. no.	Culture density	Cathomycin transformants		Dihydrostreptomycin transformants		Double transformants (no.)	
	(no.)	(no.)	(%)	(no.)	(%)	calculated	found
1*	4450	357	8	300	7	24	13
2	1300	372	28	290	22	78	24
3*	4660	260	6	158	3.4	9	10

* The low transformation frequencies in these two experiments were due to the use of recipient cultures stored at -70° for two weeks (see text).

Table 2

Quantitative data on transformation of group H streptococci

Recipient ...	Challis strain				Wicky strain	
Experiment no. ...	1	2	3	4	5	6
Viable units/ml. ($\times 10^{-3}$)	519	632	544	284	292	272
Viable units/ml. ($\times 10^{-3}$) of dihydrostreptomycin transformants	136	138	150	73	99	107
Viable units/ml. ($\times 10^{-3}$) of cathomycin transformants	181	233	178	96	120	104
Total viable units/ml. ($\times 10^{-3}$) for 2 markers	317	371	328	169	219	211
Calculated no. viable units/ml. ($\times 10^{-3}$) for double transformants	47.5	51.2	49.5	24.4	41.0	41.0
Viable units/ml. ($\times 10^{-3}$) of transformants corrected for double transformants	270	320	279	145	178	170
% transformation (viable units)	52	51	51	51	61	63

The foregoing result was independently controlled by plating the bacteria (following 2 hr. incubation in fluid medium after reaction with DNA for expression of transformants) on agar containing one antibiotic; following growth, the colonies were transferred by the replica technique (Lederberg & Lederberg, 1952) on

agar containing the second antibiotic. The replica plating technique was employed for this purpose in order to exclude the possibility of some synergetic action of two antibiotics which may occur in the procedure widely applied in evaluation of double transformants with the help of agar containing two antibiotics (as above).

Replicas were made of cathomycin-resistant transformants to plates with dihydrostreptomycin. Saturation concentrations (3 - 5 μg./ml.) of DNA were employed to increase the probability of the appearance of double transformants. Results of three experiments are shown in Table 1. Two of these were carried out with a recipient culture which had been stored at -70° for two weeks, with consequent low transformation frequencies (see above). It will be noted from Table 1 that the calculated yields of double transformants tend to be higher than the experimentally observed ones, as already observed above with the use of agar containing both antibiotics.

Typical transformation frequencies, utilizing recipients which had been kept at -70° for not more than 24 hr., are illustrated for the two non-linked markers in Table 2. The overall yields, after correction for statistically expected double transformants, are about 50%.

The foregoing transformation frequencies are in terms of viable units, since the majority (99%) of the cells grow in clusters of which 50% consist of 2 cells, 20% of 3 cells, 12% of 4, and 17% with more than 4 cells.

Table 2 also presents two typical examples of high transformation frequencies with a recipient Wicky strain, which can take up DNA and undergo transformation only when cultured in the presence of a competence factor obtained from a Challis culture filtrate (Pakuła & Walczak, 1963; Osowiecki & Łancow, 1965). The recipient cultures were prepared in the same manner as those for the Challis strain. Table 2 shows that the corrected transformation frequencies for the two non-linked markers are even higher than for the Challis strain, about 62%.

DISCUSSION

The attainment of high transformation frequencies with a given transforming system requires the establishment of well-defined conditions, since no general procedures as yet exist applicable to more than one system. Furthermore the relatively high yields reported occasionally for some systems (and listed in the introductory paragraphs, above) do not appear to be easily reproducible. In view of the exceptionally high yields obtained in this study for two strains of hemolytic streptococci, as well as the reproducibility of these yields (over a period of two years), it seems appropriate to briefly summarize and discuss the factors which make this possible.

It now seems well established that one of the factors involved in bacterial transformation is the so-called "competence factor" which, in whole or in part, is a protein-like substance (Dobrzański & Osowiecki, 1966; Tomasz & Mosser, 1966; Charpak & Dedonder, 1965). In the present investigation the presence of an excess

of this factor was maintained in the early logarithmic growth phase of the cultures, since in this growth stage a maximum proportion of the population possesses the ability to transfer to the competent state.

As was shown some years ago by Hotchkiss (1954), synchronized growth of the recipient population appreciably increases the yield of transformants. An analogous situation prevailed for our Challis recipients culture, the freezing and subsequent thawing of which, prior to reaction with transforming DNA, led to partial synchronization of growth, as described in an earlier publication (Piechowska & Shugar, 1967).

Furthermore, in the above experiments, the competence of recipient cultures was profited from to the fullest possible extent by maintaining the cells in contact with DNA for a period during which new competent cells appeared, without any simultaneous cell division (i.e. during the lag phase). Contact between cells and DNA was interrupted at the point where the yield of transformants no longer increased. Plating, for evaluation of yields, was carried out a few minutes after DNA uptake, but prior to commencement of growth of the recipient population. Finally, immobilization of the cells prior to expression of transformation, and addition of the selective agent several hours later, made possible an unequivocal evaluation of transformation frequency and elimination of errors which might have resulted from multiplication of a fraction of the transformant population during the period for expression of transformants in fluid media (in those instances where the colony count, at the time of addition of DNA, is taken to be 100%).

Another factor of some significance in increasing the yield of transformants is the initial density of the recipient culture prior to formation of the competent state, as well as the ratio of the DNA concentration to the number of competent recipient cells during the transformation reaction. It should be noted, in this connection, that the evaluation of maximum transformation frequencies in all transformation systems is usually carried out with the use of DNA concentrations equivalent to "saturation" or higher. However, as shown above, with the Challis system the DNA concentration cannot be raised much above the "saturation" value, since at a concentration above 10 μg./ml., in the presence of 2×10^6 viable units/ml., the number of transformants actually begins to decrease. The source of this effect remains to be clarified, but is vividly illustrated by the adverse influence of high DNA concentrations on the growth and viability of recipient cells, described in detail in an earlier publication (Piechowska & Shugar, 1967). A similar decrease in number of transformants (about 50%) at high DNA concentrations is shown by a calibration curve for the streptococcal system (Chen & Ravin, 1966), although this is not commented upon by the authors.

From a study of the influence of DNA on the growth and viability of Challis recipients (Piechowska & Shugar, 1967), it was estimated that the number of cells capable of DNA uptake exceeded 70%. Since the experimentally observed yield of transformants is about 50% (in viable units), it follows that the two non-linked markers which gave this result by no means exhaust the possibility of increasing the transformation frequency. However, one cannot exclude the possibility that

DNA is taken up by some cells which are incapable, for some reason, of undergoing transformation (Perry & Slade, 1962; Germaine & Anderson, 1966; Young, 1967).

Since transformation yields, or frequencies, are normally evaluated in terms of colony-forming units, it is of interest to see what these figures mean in relation to the degree of competence of a given cell population. For simplicity let us assume that 50% of the cells are competent and capable of giving rise to transformants. If the population grows in clusters consisting of 2 cells each, then the probability of finding a cluster containing 2 non-competent cells is 1/4, so that 75% of the clusters will give rise to colonies, i.e. the apparent transformation frequency will be 75%. If the number of cells per cluster is 3, the probability of finding a cluster free of competent cells will be 1/8, so that 87.5% of the clusters will give transformant colonies. For 4 cells per cluster, the number of transformant colonies will be 94%.

The above reasoning is readily extended to cell populations with degrees of competence from very low to very high values, and this will be done in a separate publication together with a discussion of its implications. It may appear curious, at first sight, that this has not previously been taken into account in calculations of transformation frequencies. Closer examination suggests that it has not been considered because of the low transformation frequencies normally encountered. However, as we shall subsequently show, it may be of considerable significance even with cell populations containing only a few percent of competent cells.

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POWTARZALNA, WYSOKA WYDAJNOŚĆ TRANSFORMACJI PACIORKOWCÓW Z GRUPY H

Streszczenie

1. Przedstawiono szczegółowy opis transformacji dwóch hemolizujących szczepów paciorkowców z grupy H: Challis i Wicky.

2. Otrzymano odtwarzalną i wysoką wydajność transformacji przez ustalenie i dobór odpowiednich warunków doświadczalnych. Najważniejszymi czynnikami były wyjściowa gęstość hodowli biorcy, obecność nadmiaru czynnika kompetencji w czasie wzrostu, stosunek stężenia transformującego DNA do ilości komórek biorcy, czas kontaktu pomiędzy nimi, jak również czas posiewu transformantów. W ten sposób możliwe było otrzymanie 50% transformantów w przypadku szczepu Challis i około 60% w przypadku Wicky. Wartości te, wyrażone w ilości kolonii, reprezentują sumę transformacji dwóch niezależnych cech.

3. Wykazano, że w układzie transformacji paciorkowców nadmiar DNA, powyżej początkowej wartości „nasycającej", może prowadzić do znacznego zmniejszenia liczby transformantów.

4. W dyskusji omówiono znaczenie różnych warunków doświadczalnych, możliwość zwiększenia wydajności transformacji oraz możliwość oceny ilości komórek transformowanych.

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BINDING OF A NATURAL TEMPLATE TO *ESCHERICHIA COLI* RIBOSOMES IN THE PRESENCE OF SOLUBLE RNA

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1. Soluble RNA (sRNA) isolated from *Escherichia coli*, free from high-molecular RNA, reduced the binding of rapidly labelled [^{14}C]RNA to ribosomes. 2. sRNA affected also the formed template-ribosome complex. 3. The effect of sRNA was unaltered by chlortetracycline, which blocks the binding of sRNA to ribosomes.

As a result of rapid progress in the studies on the amino acid code, investigations aimed at elucidating the mechanism of the interaction of ribosomes with messenger RNA (mRNA) and soluble ribonucleic acid (sRNA), have been undertaken in many laboratories. Nirenberg & Leder (1964) demonstrated that the binding of phenylalanine-sRNA (Phe-sRNA) to *E. coli* ribosomes is proportional to the concentration of polyuridylate (poly U), that of proline-sRNA to polycytidylate (poly C), and of lysine-sRNA to polyadenylate (poly A). Free sRNA was bound to the template-ribosome complex as well as aminoacyl-sRNA. Similar results were reported by Kurland (1966) for highly purified *E. coli* ribosomes.

Spyrides & Lipmann (1962) and Takanami & Okamoto (1963) demonstrated that poly U binds to the 30s subunits of ribosomes. Gilbert (1963) and Takanami & Okamoto (1963) found by sucrose gradient centrifugation that poly U associates with ribosome to form polysomes. Similar results were obtained by Takanami & Okamoto (1963) with other homopolymers and copolymers, e.g. polyuridylcytidylate (poly UC). The extent and tightness of the binding appeared to differ. Poly U was able to displace poly C (Moore, 1966), as well as RNA of turnip yellow mosaic virus (Haselkorn & Fried, 1964). In contrast to the synthetic polynucleotides, the RNA of phage f2 bound only with one *E. coli* ribosome, forming a monosome (Takanami, Yan & Jukes, 1965).

Studies on the effect of sRNA on the binding of template to ribosomes so far have not given conclusive evidence, and the reported results are often contradictory. Hatfield (1965) demonstrated that Phe-sRNA stimulates binding to ribosomes of trinucleotides: uridyluridylcytidylate (UUC) and uridyluridyluridylate (UUU), whereas Moore (1966) has not found any effect of sRNA on the binding of poly U.

In the present paper, the effect of sRNA on the binding of rapidly labelled [^{14}C]RNA to *E. coli* ribosomes is described. Preliminary report of these investigations has been published (Perzyński & Szafrański, 1967).

MATERIALS AND METHODS

Reagents. [8-^{14}C]Adenine sulphate, spec. act. 28.9 mc/m-mole (The Radiochemical Centre, Amersham, England); deoxyribonuclease (DNase), electrophoretically pure, free from RNase (Worthington, Freehold, N.J., U.S.A.); Sephadex G-100 (Pharmacia, Uppsala, Sweden); yeast extract and acid casein hydrolysate (Difco Lab., Detroit, Mich., U.S.A.); tris, ATP and sodium dodecylsulphate (SDS) (Sigma Chemical Co., St. Louis, Mo., U.S.A.); guanosine triphosphate (GTP), sodium salt (Pabst. Lab., Milwaukee, Wisc., U.S.A.); glycine, DL-alanine, L-asparagine, L-cysteine, L-hydroxyproline, DL-phenylalanine, L-proline, L-tryptophan, DL-tyrosine, L-glutamic acid, L-leucine, DL-valine (Fabryka Odczynników Chemicznych, Gliwice, Poland); L-glutamine (Schwarz Lab., N.Y., U.S.A.); L-histidine (Schuchardt, München, West Germany); DL-isoleucine (Toscat, England); L-lysine (La Roche, Basel, Switzerland); DL-serine (Chemapol, Praha, Czechoslovakia); DL-threonine and β-mercaptoethanol (British Drug Houses, Poole, Dorset, England); L-aspartic acid (Riedel, Germany); L-arginine and DL-methionine (Fluka A. G., Buchs, Switzerland). Chlortetracycline chlorohydride was a gift from Dr. Zuzanna Kowszyk-Gindifer of the Institute of Antibiotics (Warszawa). Cellulose nitrate H. A. millipore filters were from the Millipore Filter Corp. (Bedford, Mass., U.S.A.).

Analytical methods. The growth of the *E. coli* culture was followed by measuring the turbidity of the medium at 600 mμ in the Spectronic 20 colorimeter (Bausch & Lomb). The ribosomes concentration was determined by measuring the extinction at 260 mμ, taking $E^{1\%}_{1cm}$ to be 150 (Petermann, 1964) and molecular weight 2.6×10^6 (Tissieres, Watson, Schlessinger & Hollingworth, 1959). sRNA and RNA were determined spectrophotometrically assuming that $E^{1\%}_{260}$ is 240 (Nathans & Lipmann, 1961) and molecular weight of sRNA 26.5×10^3 (Lindahl, Adams & Fresco, 1966). The rate of [^{14}C]RNA binding to ribosomes was determined by the Millipore filter technique of Nirenberg & Leder (1964) or by centrifuging the ribosomes in a discontinuous sucrose gradient (Wettstein, Staehelin & Noll, 1963) using "Superspeed 40" MSE ultracentrifuge. Radioactivity was determined with a G. M. window counter.

E. coli B. was grown at 32° in a medium containing per 1 litre: 5 g. of yeast extract, 5 g. of casein hydrolysate, 21.8 g. of K_2HPO_4, 17.0 g. of KH_2PO_4, 0.12 g. of $MgSO_4$, and 5 g. of glucose; pH of the medium was 7. At the logarithmic phase of growth ($E_{600} = 0.76$) the culture was cooled rapidly by adding pieces of solid CO_2 and centrifuged in a Sharpless centrifuge. The separated cells were washed twice by suspension and centrifugation in standard buffer solution (0.014 M-magnesium acetate - 0.06 M-KCl - 0.006 M-β-mercaptoethanol - 0.01 M-tris, pH 7.8, according to Nirenberg & Matthaei, 1961); then the cells were distributed in portions suitable for separate experiments and stored at -60°.

Preparation of ribosomes. A portion of *E. coli* cells was suspended in three volumes of standard buffer solution and disrupted in the Eaton press (Eaton, 1962). The homogenate was treated with DNase (1 μg./ml.), centrifuged successively at 14 000 *g* and 30 000 *g*, and non-disrupted cells and cell debris were discarded. To remove mRNA, the supernatant was incubated for 90 min. at 37° with amino acids, ATP and GTP according to Nirenberg & Matthaei (1961), except that phosphoenolpyruvate and pyruvate phosphotransferase were omitted; then the mixture was centrifuged for 30 min. at 30 000 *g*. The supernatant was centrifuged in "Superspeed 40" MSE ultracentrifuge for 3 hr. at 105 000 *g* at 2°. The sediment which contained ribosomes was suspended in standard buffer solution and centrifuged for 15 min. at 20 000 *g*. The supernatant was centrifuged again at 105 000 *g*, the pellet of ribosomes suspended in a small volume of the standard buffer solution and centrifuged at 20 000 *g*. The suspension of ribosomes was distributed in portions suitable for separate experiments and stored at -60° in sealed tubes. In some experiments ribosomes kept for a few days at 0°, were used.

Preparation of sRNA. Total RNA was isolated from *E. coli* cells disrupted in the Eaton press, by the phenol method of Scherrer & Darnell (1962). The crude RNA preparation was digested with DNase (2 μg./ml.) at room temperature for 30 min., then phenol was added to 45% concentration. The separation of the two phases was facilitated by centrifuging, then the aqueous layer was made 1.5 M in respect to NaCl, and left for a few hours at 0°. The sediment of high-molecular

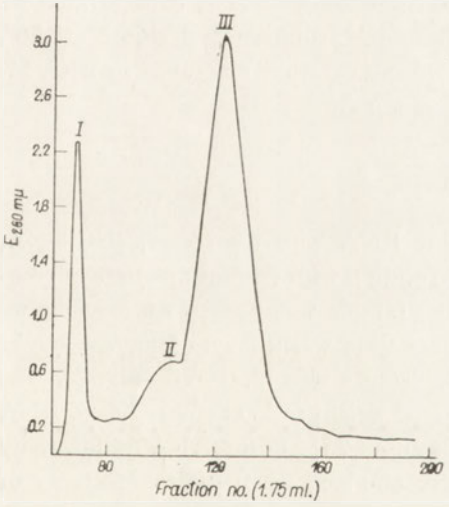

Fig. 1

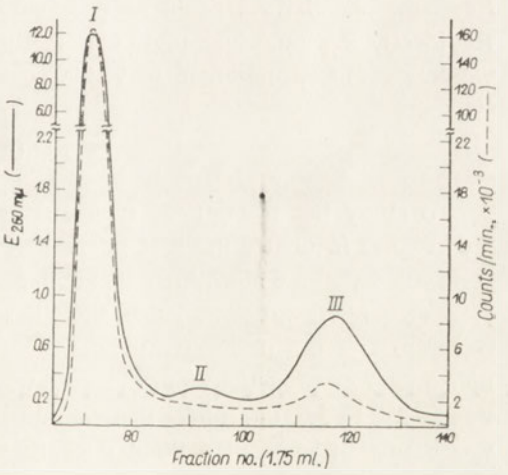

Fig. 2

Fig. 1. Fractionation of sRNA on Sephadex G-100. The preparation, 10 mg., was applied to a column (120×2 cm.) equilibrated with 1 M-NaCl and eluted with the same solvent. The extinction was measured at 260 mμ in 1 cm. light-path cells.

Fig. 2. Fractionation of [^{14}C]RNA on Sephadex G-100. To [^{14}C]RNA preparation, 6.4 mg., [^{12}C]sRNA was added to visualize the sRNA fraction, and applied onto the column (120×2 cm.) equilibrated with 0.1 M-NaCl. The elution was carried out with the same solvent. The extinction was measured at 260 mμ in 0.5 cm. light-path cells, and samples from each fraction were taken, evaporated on planchettes and radioactivity was determined with a G. M. window counter.

RNA was centrifuged off. To the supernatant, 2.5 volumes of cold absolute ethanol was added and left overnight at -15°. The precipitated sRNA was spun down, dissolved in a small volume of water, residual phenol was extracted with ether, and the ether was removed by flushing nitrogen. The solution was adjusted to pH 8.8 by adding tris *in substantia*, and incubated at 37° for 60 min. to deacetylate sRNA. The preparation was dialysed overnight against 1 M-NaCl at 3° and purified on Sephadex G-100 to separate sRNA from residual high-molecular RNA (Schleich & Goldstein, 1966). The profile of separation is shown in Fig. 1. Fraction *III* which contained sRNA accounted for 80% of the total material applied to the column. It was dialysed against water and concentrated by freeze-drying to 5.6 mg./ml. Then the preparation was dialysed again for 4 hr. against standard buffer solution and stored at -20°.

Preparation of rapidly labelled [^{14}C[*RNA. E. coli* B was grown on the glucose H. I. medium of Baldwin & Shooter (1963) at 32° (in 150 ml.). At the logarithmic phase of growth ($E_{600} = 0.91$) the cells were harvested by rapid centrifuging, suspended in 30 ml. of fresh medium, and [8-^{14}C]adenine was added to 0.12 mM concentration. After 4 min. of incubation non-radioactive adenine (4 mM) was added, the culture rapidly cooled and centrifuged at 2°. RNA was isolated by phenol method of Scherrer & Darnell (1962), dialysed overnight against water, and after adding NaCl to 0.1 M concentration, fractionated on a Sephadex G-100 column (Fig. 2). Fraction *I* containing high-molecular RNA accounted for 90% of the radioactivity applied to the column. This fraction was dialysed against water, concentrated by freeze-drying, dialysed again against standard buffer solution and stored at -20°. Radioactivity of the high-molecular [^{14}C]RNA preparation measured in a G. M. window counter, amounted to 95 000 counts/mg./min.

RESULTS

To study the reaction of rapidly labelled RNA with ribosomes, [^{14}C]RNA, 5.6 μg., was incubated in the standard buffer solution with various amounts of ribosomes at 23° for 6 min. After incubation, the sample was poured on a Millipore filter (Nirenberg & Leder, 1964), the ribosomes were washed with standard buffer solution, and the radioactivity bound to ribosomes was measured (Fig. 3). The maximum of radioactivity was observed when the weight ratio of ribosomes to RNA was as 220 to 5.6, and under these conditions about 25% of the RNA radioactivity was bound to ribosomes. Similar results were obtained when the radioactivity of ribosomes isolated by discontinuous sucrose gradient centrifugation according to Wettstein *et al.* (1962), was determined.

The effect of sRNA on the interaction of [^{14}C]RNA with ribosomes is shown in Fig. 4. The amount of [^{14}C]RNA bound to ribosomes decreased with increasing sRNA concentration. The inhibiting effect was observed already when 12 μg. of sRNA per 220 μg. of ribosomes was added. The inhibition was not due to degradation of the [^{14}C]RNA, as on incubation of this preparation with sRNA the presence of nucleolytic enzymes has not been detected.

The effect of sRNA on the binding of [^{14}C]RNA to ribosomes was the same at 15°, 23°, 36° and 45°. The results obtained by the Millipore filter technique and discontinuous sucrose gradient centrifugation were in close agreement.

The order in which the components were added had a marked influence on the binding of RNA to ribosomes (Table 1). When [^{14}C]RNA and ribosomes were incubated together with sRNA, the binding decreased by more than 50% in relation to the sample incubated without sRNA. A similar result was observed when ribosomes were first incubated with sRNA, and [^{14}C]RNA was added later; on the

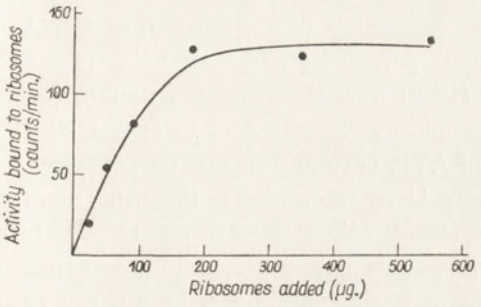

Fig. 3

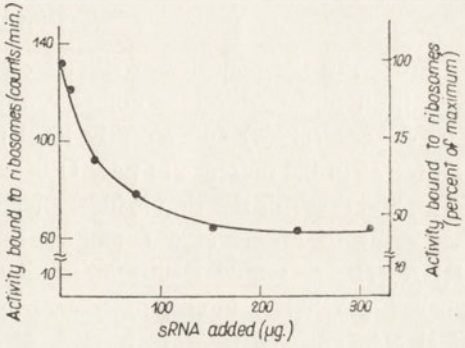

Fig. 4

Fig. 3. Binding of rapidly labelled RNA to ribosomes. [^{14}C]RNA, 5.6 μg., was incubated in standard buffer solution with increasing amounts of ribosomes for 6 min. at 23°; final volume 0.3 ml. After incubation, the mixture was diluted to 3 ml. with standard buffer solution, filtered through the Millipore filter, washed with 15 ml. of the same solution, and the radioactivity remaining on the filter was determined with a G. M. window counter.

Fig. 4. The effect of sRNA concentration on the interaction of [^{14}C]RNA with ribosomes. The components of the incubation mixture were added in the following order: 5.6 μg. of [^{14}C]RNA, various amounts of sRNA, and 220 μg. of ribosomes. After 6 min. incubation at 23°, the ribosomes were isolated by the Millipore filter technique and radioactivity determined.

Table 1

The effect of sRNA on the binding of [^{14}C]*RNA to ribosomes and on the* [^{14}C]*RNA-ribosome complex*

The incubation mixture (final volume 0.3 ml.) contained standard buffer solution, 180 μg. of ribosomes, 5.6 μg. of [^{14}C]RNA, 120 μg. of sRNA. Preincubation as well as incubation was carried out at 23° for 5 min. Binding of [^{14}C]RNA to ribosomes was determined by the Millipore filter technique.

Incubation mixture	Radioactivity bound to ribosomes	
	(counts/min.)	(%)
[^{14}C]RNA+ribosomes	95	100
[^{14}C]RNA+sRNA+ribosomes	45	47
Ribosomes preincubated with sRNA; after 5 min. [^{14}C]RNA added	40	42
Ribosomes preincubated with [^{14}C]RNA; after 5 min. sRNA added	65	68

other hand, when sRNA was added to ribosomes which had been incubated with [^{14}C]RNA, about 70% of the radioactivity remained bound to ribosomes.

In further experiments on the mechanism of action of sRNA on the [^{14}C]RNA--ribosome complex formation, chlortetracycline was applied to block the sRNA--binding sites (Suarez & Nathans, 1965; Hierowski, 1965). However, chlortetracycline was found to have no influence either on binding of [^{14}C]RNA to ribosomes or on its detachment induced by sRNA. Thus it seems that binding of sRNA to ribosomes is not essential for the release of [^{14}C]RNA.

DISCUSSION

So far, in the majority of studies on the mechanism of the reaction of sRNA with the template-ribosome complex, synthetic polynucleotides were used. Nirenberg & Leder (1964) and recently Kurland (1966) have demonstrated that *E. coli* ribosomes in the presence of poly U bind specifically Phe-sRNA or its deacetylated form, and that this reaction is independent of ATP, GTP and supernatant enzymes. According to Moore (1966), binding of poly U to ribosomes is independent of sRNA. The presented results indicate that sRNA affects the amount of rapidly labelled [^{14}C]RNA bound to ribosomes. Since [^{14}C]RNA preparation was free of sRNA, and the ribosomal RNA present in the preparation is known not to react with ribosomes (Ishihama, Mizuno, Takai, Otaka & Osawa, 1962), it might be supposed that radioactivity bound to ribosomes corresponds to mRNA. When sRNA was present in the incubation medium, the amount of ribosome-bound [^{14}C]RNA decreased with increasing sRNA concentration. The decrease in radioactivity was not due to dilution of the [^{14}C]RNA, as the sRNA preparation was free of high-molecular, non-radioactive RNA. Nor was it due to the action of nucleolytic enzymes, as [^{14}C]RNA was not degraded in the presence of sRNA. The maximum inhibition of binding was achieved in the presence of about 100 mμmoles of sRNA per 1 mμmole of ribosomes, but even at a molar relation as low as 5 : 1 the inhibitory effect of sRNA was observed. In the experiments of Moore (1966), the binding of poly U to ribosomes was not affected by sRNA in a 30-fold molar excess. On the other hand, in our experiments sRNA not only reduced the binding capacity of [^{14}C]RNA to ribosomes but also affected, although to a smaller extent, the template-ribosome complex after it had been formed. The action of sRNA on the binding of template to ribosomes is independent of the binding to ribosomes of sRNA itself, as chlortetracycline, which is known to block by 50% the binding of sRNA to ribosomes (Suarez & Nathans, 1965; Hierowski, 1965), had no effect on the reaction studied.

The mechanism of the interaction of sRNA, template and ribosomes has not yet been clarified. According to Kaji, Suzuka & Kaji (1966), binding of specific sRNA to 30s subunits of *E. coli* ribosomes takes place in the presence of appropriate polynucleotides. The binding to 50s subunits seems to be non-specific. However, the 50s subunits play an important role, as specific binding of sRNA to 30s subunits of non-dissociated (70s) ribosomes was greater than to 30s subunits alone. Nirenberg & Leder (1964) demonstrated that the template-ribosome complex

occurs throughout the pH 5.5 - 7.8 range, and the optimum temperature for studying this reaction in the *E. coli* system is 23°. The accuracy of stoicheiometric calculations for binding of sRNA to ribosomes is rather low. Nirenberg & Leder (1964) estimated the Phe-sRNA ribosome ratio to be 1 : 5.8 at saturating poly U concentration. Kurland (1966) found a ratio of 1 : 4, whereas Warner & Rich (1964) reported that one ribosome may bind two molecules of sRNA.

Approximately 25 - 30 nucleotides are known to participate in the association of template with ribosome (Takanami & Zuby, 1964; Takanami *et al.*, 1965). In the attachment, 3′ and 5′ OH groups do not seem to be involved (Dahlberg & & Haselkorn, 1966). Magnesium or other divalent cations are required in this reaction. The optimum concentration of Mg^{2+} was found to be 10 mM, and it can be replaced by Ca^{2+} or Mn^{2+}, whereas 1 mM-Mg^{2+} inhibits completely the reaction. Recently Moore (1966) reported that spermine in 1 mM concentration can replace 10 mM-Mg^{2+}. Secondary structure of the template plays a role in the formation of the complexes. Synthetic polynucleotides possessing random structure, for instance poly U, interact effectively with ribosomes. Polymers exhibiting some degree of secondary structure, e.g. poly A or poly UA, show little affinity to ribosomes (Takanami & Okamoto, 1963). Ribosomal RNA and RNA isolated from *E. coli* cells infected with phage T_2, were unable to bind ribosomes; however, after formaldehyde treatment to block the amino groups of bases, the two RNA preparations showed considerable interaction with ribosomes (Okamoto & Takanami, 1963). Thus the secondary structure of RNA determined the possibility of, and probably also the site for, the binding of template with ribosomes.

Recently, Furano, Bradley & Childers (1966) demonstrated that in intact ribosomes of *E. coli* 90% of RNA phosphate groups are free, as judged by their ability to react with methyl orange. This indicates that RNA in ribosomes has essentially single-stranded structure. On the other hand, RNA isolated from the ribosomes contains double-helical character, and this may explain its inability to bind ribosomes. Furano *et al.* suggested the binding of ribosomes to mRNA through magnesium bridges between phosphate groups of ribosomal RNA and mRNA. sRNA was postulated to be bound to mRNA by hydrogen bonds formed between bases of anticodon and codon (Crick, 1958). This seems to be in agreement with the results of X-rays analysis by Arnott *et al.* (1966). The authors suggest, moreover, that hydrogen bonds between OH groups of ribose and phosphate groups play an important role in the interaction of sRNA, mRNA and ribosomal RNA. The reduced binding of template to ribosomes in the presence of sRNA, observed in the present work, could be explained by the possibility that sRNA, when attached to template through anticodons and codons, may diminish the binding of mRNA to ribosomes. This appears to be probable in the case of *E. coli* template which is weakly bound to ribosomes. It would be interesting to study these processes also in systems which are known to possess a stable, tightly bound to ribosomes mRNA.

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WIĄZANIE NATURALNEJ MATRYCY Z RYBOSOMAMI *ESCHERICHIA COLI* W OBECNOŚCI ROZPUSZCZALNEGO RNA

Streszczenie

1. Rozpuszczalny RNA (sRNA) wyizolowany z *E. coli* i pozbawiony wielkocząsteczkowego RNA zmniejsza ilość wiązanego przez rybosomy szybko znaczonego [^{14}C]RNA.

2. sRNA wpływa również na utworzony kompleks matryca-rybosom.

3. Chlorotetracyklina, która blokuje wiązanie sRNA z rybosomami, nie zmienia jego hamującego wpływu na przyłączanie matrycy do rybosomów.

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SECONDARY STRUCTURE OF POLY-$N_{(4)}$,5-DIMETHYLCYTIDYLIC ACID AND ITS COPOLYMERS WITH CYTIDYLIC ACID

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1. $N_{(4)}$,5-Dimethylcytidine 5′-pyrophosphate has been synthesized and proved to be a substrate for polynucleotide phosphorylase. The resulting homopolymer does not enter a double stranded complex with poly-inosinic acid (poly-I). Copolymers containing cytidylic (C) and $N_{(4)}$,5-dimethylcytidylic (4,5-diMeC) residues form complexes with poly-I at a ratio of 1 : 1 indicating that 4,5-diMeC is able under proper conditions to form a pair with hypoxanthine. The resulting complex is much less stable than that formed by poly-C and poly-I. The complexing ability of methylamino derivatives of cytosine and adenine at the polymer level is discussed. 2. The $N_{(4)}$-methyl group does not appear to modify appreciably the structure of the polymer in neutral medium as compared to the single stranded configuration of poly-C and poly-5-methylcytidylic acid. In acid medium poly-4,5-diMeC does not attain any double helical structure in contrast to the two former polymers. The type of secondary structure existing in poly-4,5-diMeC at pH 4 resembles that of the neutral form in spite of the protonation of about 40% bases. 3. Poly-4,5-diMeC is degraded by pancreatic ribonuclease and snake venom phosphodiesterase. The rate of enzymic hydrolysis is in both cases considerably decreased as compared to the hydrolysis of poly-C.

A number of methylated polyribonucleotides have been synthesized in recent years with the help of polynucleotide phosphorylase and employed as models in studies on the structure and function of natural nucleic acid molecules. It has been shown, in particular, that substitution of a 5-methyl group in the pyrimidine ring of poly-U[1] and poly-C confers a remarkably stabilizing effect upon their 1 : 1 complexes with the corresponding complementary purine polyribonucleotides (Szer,

[1] Abbreviations used: poly-U, poly-uridylic acid; poly-C, poly-cytidylic acid; poly-5-MeC, poly-5-methylcytidylic acid; poly-4-MeC, poly-$N_{(4)}$-methylcytidylic acid; poly-4,5-diMeC, poly-$N_{(4)}$,5-dimethylcytidylic acid; poly-I, poly-inosinic acid; poly-(C+I) is the twin-stranded complex of poly-C+poly-I with similar connotations of the other complexes. Poly-(C, 4,5-diMeC) (70 : 30) represents a copolymer containing 70% of cytosine residues and 30% of $N_{(4)}$,5-dimethylcytosine residues. 4,5-diMeCDP is the 5′-pyrophosphate of $N_{(4)}$,5-dimethylcytidine. RNase, pancreatic ribonuclease. Other abbreviations are used according to the Tentative Rules of IUPAC-IUB, 1966, *J. Biol. Chem.* **241**, 527.

Świerkowski & Shugar, 1963; Szer, 1965; Szer & Shugar, 1966). On the other hand, methylation of an amino group or ring nitrogen involved in base-pair hydrogen bonding leads to a marked decrease in stability or prevents altogether the formation of complementary complexes (Szer & Shugar, 1961; Griffin, Haslam & Reese, 1964; Brimacombe & Reese, 1966). It subsequently appeared of interest to examine at the polymer level the simultaneous effect of methylation of the two relevant positions in a cytosine moiety. Other points of interest were the influence of an aminomethyl upon the structure of poly-C in acid medium, and the possible template properties of poly-4,5-diMeC and its copolymers with cytidylic acid. We also wished to find out, in line with our previous studies, the effect of methylation on nucleolytic enzymes. 4,5-diMeC was not detected among naturally occurring minor components but the presence of 4-methylcytosine in hydrolysates of ribosomal RNA from *E. coli* has been recently reported (Nichols & Lane, 1966).

A preliminary report of some of these results has been presented at the 3rd Meeting of the Biochemical Society of DDR in Suhl (Rabczenko & Szer, 1966).

MATERIALS AND METHODS

Poly-C and poly-I preparations employed were commercial samples purchased from Calbiochem (Los Angeles, Cal., U.S.A.); they were purified prior to use by deproteinization and exhaustive dialysis against 0.01 M-EDTA and 0.2 M salt solutions and finally against distilled water. Poly-4,5-diMeC and its copolymers containing various proportions of cytosine were prepared by the action of polynucleotide phosphorylase either from *Azotobacter vinelandii* or from *Micrococcus lysodeicticus* on 4,5-diMeC and its mixtures with CDP (Tables 1 and 2). The latter

Table 1

*Conditions of polymerization * of CDP and 4,5-diMeCDP*

Substrate, μmoles	Enzyme, source and exchange units**	Buffer (μmoles)	Time and temp.	Yield (%)
CDP, 60	*A. vinelandii*, 16	tris-HCl (150), Mg^{2+} (5), pH 8.2	3 hr., 30°	60
4,5-diMeCDP, 60	*A. vinelandii*, 16	tris-HCl (150), Mg^{2+} (5), pH 8.2	5 hr., 30°	trace
4,5-diMeCDP, 10	*A. vinelandii*, 16	tris-HCl (150), Mg^{2+} (5), pH 8.2	2.5 hr., 30°	50
CDP, 48	*M. lysodeicticus*, 16	tris-HCl (150), Mg^{2+} (10), pH 9.2	2 hr., 37°	45
4,5-diMeCDP, 48	*M. lysodeicticus*, 16	tris-HCl (150), Mg^{2+} (10), pH 9.2	5 hr., 37°	trace
4,5-diMeCDP, 10	*M. lysodeicticus*, 16	tris-HCl (150), Mg^{2+} (10), pH 9.2	2.5 hr., 37°	40

* Final volume 1 ml.

** See Basilio & Ochoa (1963).

Table 2

Copolymerization of CDP and 4,5-diMeCDP

Polymerization conditions (1 ml. final volume): total substrate, 10 μmoles; tris-HCl, 150 μmoles; Mg^{2+}, 10 μmoles; pH 9.2; 16 exchange units of *M. lysodeicticus* enzyme; incubation for about 2.5 hr. at 37°.

Relative proportion of substrates in incubation mixture (%)		Experimentally determined * proportion of bases in isolated polymer (%)	
CDP	4,5-diMeCDP	C	4,5-diMeC
40	60	45	55
55	45	70	30
80	20	83	17

* Exhaustive ribonuclease digestion (48 hr., 37°, 0.5 mg./ml. substrate, 0.05 mg./ml. enzyme) followed by paper chromatography (ethanol-1M-ammonium acetate, 7 : 3, v/v), elution of the corresponding spots and spectral estimation of components.

was a commercial product from Calbiochem. $N_{(4)}$,5-Dimethylcytidine was synthesized by thiation of 1-β-D(2′,3′,5′-tri-*O*-benzoyl)-ribofuranosylthymine and subsequent treatment of the thio derivative with a methanolic solution of methylamine (cf. Fox *et al.*, 1959, for $N_{(4)}$-methylcytidine). The resulting nucleoside was converted quantitatively into the 2′,3′-*O*-benzylidene derivative (Gulland & Smith, 1948) and phosphorylated with cyanoethylphosphate (Tener, 1961) to give $N_{(4)}$,5--dimethylcytidine-5′-phosphate. The latter was converted to the morpholine derivative and phosphorylated (Moffat & Khorana, 1961) to give 4,5-diMeCDP. Details of this procedure as well as spectral and other properties of the compounds involved will be described elsewhere (Rabczenko & Szer, in preparation).

4,5-diMeCDP did not undergo appreciable polymerization with any of the enzyme preparations employed under conditions considered standard for 5′-pyrophosphates of natural nucleosides (Basilio & Ochoa, 1963), differing in this respect from the behaviour of both 5-methylUDP and 5-methylCDP, which readily polymerize under standard conditions. A several-fold decrease in substrate concentration ensured reproducible homopolymer yields of 40 - 50% within 2 - 3 hr. (Table 1). The shortened incubation time had the advantage of giving an increased molecular weight product by avoiding extensive phosphorolysis and possible degradation by nucleases contaminating the enzyme preparation. The course of polymerization was followed by paper chromatography (ascending, sat. ammonium sulphate - 8.2% ammonium acetate - propan-2-ol, 48 : 10.8 : 1.2, by vol.), the polymers remaining at origin. Copolymers were prepared under conditions similar to those employed for the polymerization of 4,5-diMeCDP. The content of cytidine residues in a given copolymer was found to be usually higher than its relative proportion in the incubation mixture (Table 2) in accord with the greater affinity of the enzyme for CDP. The polymers were isolated as previously described (Szer & Ochoa, 1964).

Pancreatic ribonuclease (Reanal, Budapest, Hungary) five times recrystallized, and snake venom phosphodiesterase (Worthington, N. J., U.S.A.; batch VPH 125;

potency 0.3; c.f. Williams, Sung & Laskowski, 1961) were used in enzyme-catalysed hydrolysis of polymers. Substrates were prepared at a concentration of 5×10^{-5} M and the incubation was carried out at 37° directly in thermostated spectral cuvettes. The increase in extinction at λ_{max} was taken as a measure of enzymic hydrolysis of substrate. Buffers employed: 0.01 M-tris-HCl, pH 7.8 for ribonuclease digestion and 0.02 M-sodium borate - 0.01 M-magnesium chloride, pH 9.0, for snake venom phosphodiesterase.

Mixing experiments and UV-temperature profiles were run in an Unicam SP 500 as described in previous communications (e.g. Szer & Shugar, 1966).

RESULTS AND DISCUSSION

Poly-4,5-diMeC in neutral solution

Both poly-C and poly-5-MeC exhibit typical non-cooperative UV-temperature transitions in neutral medium. Fasman, Lindblow & Grossman (1964) have shown that optical rotatory dispersion properties of the neutral form of poly-C do not change upon formylation, but the Cotton effect of the major absorption band decreases gradually upon addition of ethylene glycol or rising temperature. They concluded that neutral poly-C, much like neutral poly-A (Leng & Felsenfeld, 1966), exists in a single stranded configuration with secondary structure maintained largely by base stacking without involvement of specific hydrogen bonding. No effect of the $N_{(4)}$-methyl substituent upon the behaviour of the neutral form poly-5-MeC can be detected (see Fig. 1), the thermal transition of the resulting poly-4,5-diMeC being almost identical to that of poly-5-MeC and poly-C. The shape of the temperature transition curve remained unchanged upon increasing sodium ion concentration or adding divalent cations, similarly to the behaviour of poly-C and poly-5-MeC. This is taken to imply that the kind of single stranded organization existing in poly-C and poly-5-MeC is maintained in poly-4,5-diMeC. Since the presence

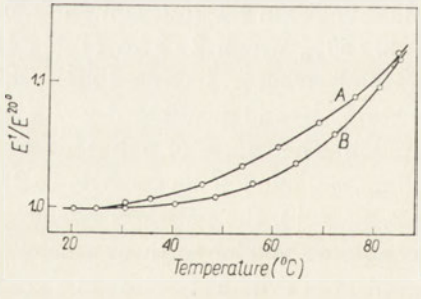

Fig. 1

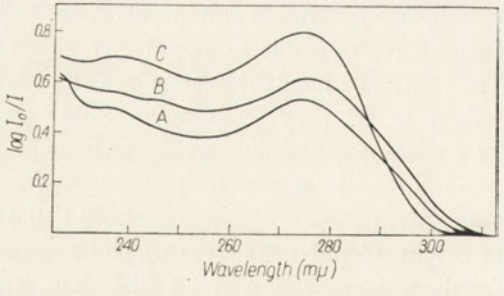

Fig. 2

Fig. 1. Temperature profiles in 0.1 M-NaCl - 0.05 M-tris, pH 7.5, of *A*, poly-5-MeC, and *B*, poly-4,5-diMeC. Changes in absorption were followed at 275 mμ for poly-5-MeC and at 278 mμ for poly-4,5-diMeC.

Fig. 2. Absorption spectrum of poly-4,5-diMeC: *A*, at 20°, *B*, at 85° and *C*, following hydrolysis to mononucleotides with ribonuclease. Buffer: 0.1 M-NaCl - 0.05 M-tris, pH 7.5.

of a $N_{(4)}$-methyl group interferes considerably with the formation of those structures in which specific hydrogen bonding does participate (see below; cf. also Brimacombe & Reese, 1966), the behaviour of neutral poly-4,5-diMeC may serve as additional evidence that hydrogen bonds are indeed not involved in the formation and maintaining of secondary structure in single stranded helices. Figure 2 presents the absorption spectrum of poly-4,5-diMeC at 20° and in the "melted" form at 85° along with the spectrum of its mononucleotide hydrolysis products. It will be seen that the hyperchromicity of the homopolymer on degradation to mononucleotides is 51%. This is to be compared with 38% and 52% hyperchromicity values for poly-C and poly-5-MeC, respectively. It follows that the hyperchromicity of poly-4,5-diMeC, while higher than that for poly-C, does not increase relative to poly-5-MeC. If the value of hyperchromicity is taken as a relative and rough measure of base stacking, then the $N_{(4)}$-methyl, in contrast to the 5-methyl group, apparently does not contribute to its enhancement. It is noted that all three polymers exhibit a similar percentage of temperature sensitive hyperchromicity in the range 20° - 85°, amounting to about 15%. It may be therefore concluded that the methylated polymers possess more "residual" hyperchromicity, i.e. temperature resistant interactions between chromophores, than poly-C. Copolymers of C and 4,5-diMeC exhibit melting curves similar to those shown in Fig. 1 and possess intermediate residual hyperchromicity values.

It is perhaps worthwhile to compare the effect of a 5-methyl substituent on poly-U and poly-C. The former is apparently intrinsically capable to form a U-U pair (Lipsett, 1960; cf. also Hamlin, Lord & Rich, 1965; Szer, 1966) and the 5-methyl group serves to increase the stability of the ordered state (Szer *et al.*, 1963). Poly-C and its $N_{(4)}$-methyl derivative are not able to form a double helical structure at neutral pH and the 5-methyl group presumably causes a higher degree of stacking between bases. In a way, the effect is analogous in both cases: it strengthens the preexisting structure.

It will be noted from Fig. 2 that poly-4,5-diMeC exhibits to the red of 292 mμ hyperchromicity with respect to its constituent mononucleotides which is even more pronounced for the melted form and extends to 287 mμ. The same observation has been made for poly-C and poly-5-MeC and discussed in an earlier paper (Szer & Shugar, 1966) in relation to the origin of hyperchromicity.

Poly-4,5-diMeC in acidic solution

Poly-C forms under acidic conditions an ordered state with an abrupt temperature transition and attains maximal thermal stability at pH 4, i.e. when half of the cytosine residues are protonated. The conclusion on the double helical configuration of this ordered state is based upon several lines of evidence including X-ray diffraction studies (Langridge & Rich, 1963), hydrodynamic, absorbance and optical rotatory dispersion properties (Akinrimisi, Sander & Ts'O, 1963; Fasman *et al.*, 1964; Hartman & Rich, 1965). It is assumed that two cytosine residues form

two hydrogen bonds across the helix center involving their respective 2-keto and 4-amino groups, and that the stability of the resulting helix is considerably enhanced by sharing a proton between the ring 3-nitrogens. The introduction of a 5-methyl substituent was shown to be without effect on the acid form of poly-C as may be inferred from melting profiles (Szer & Shugar, 1966) and optical rotatory dispersion measurements (Ulbricht & Szer, unpublished observations). Figure 3 reveals that poly-4,5-diMeC does not form any regular structure in acid as may be judged from the lack of a sharp temperature transition. The melting curve of the

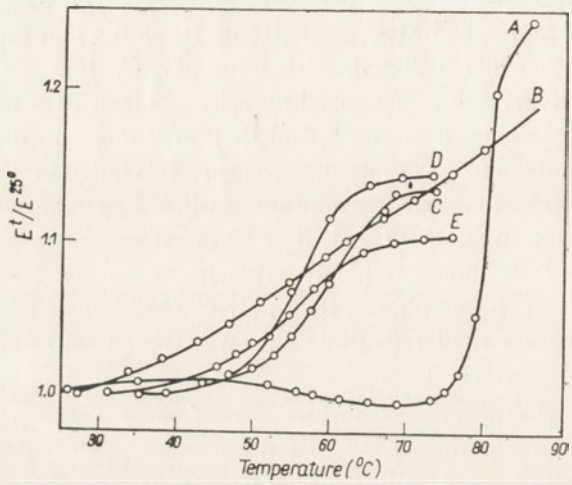

Fig. 3. Temperature profiles in 0.1 M-NaCl - 0.05 M-acetate buffer, pH 4.0, of *A*, poly-C; *B*, poly-4,5-diMeC; *C*, poly-(C, 4,5-diMeC) (83 : 17); *D*, poly-(C, 4,5-diMeC) (70 : 30); and *E*, poly-(C, 4,5-diMeC) (45 : 55). Changes in absorption were followed at λ_{max} for each preparation.

homopolymer resembles closely that of the neutral form, thus indicating that the presence of the $N_{(4)}$-methyl group interferes with the formation of the cooperatively melting ordered state. This is similar to the behaviour of poly-4-MeC (Brimacombe & Reese, 1966) and may be due to steric hindrance and charge distribution changes induced by the presence of the $N_{(4)}$-methyl group. Nonetheless, the polymer possesses thermal hyperchromicity between 25° and 85° (Fig. 3) and a relatively high percentage of residual hyperchromicity (Fig. 4). This indicates that base-base interactions still exist although at pH 4 about 40% of the bases are protonated. It is recalled that Warshaw & Tinoco (1965) have concluded, on the basis of optical rotatory dispersion measurements of dinucleoside phosphates at various pH values, that charges on both neighbouring bases cause unstacking but a charge on one base need not, and attraction forces between parallel aromatic rings still exist. It seems therefore reasonable to suggest that the single stranded configuration of the neutral form of poly-4,5-diMeC with stacked bases persists on acidification to pH 4 since the polymer is not capable of assuming the more stable and hence more favoured double helical configuration. On further acidification to about pH 3 most of the bases become protonated and the hyperchromicity vanishes as

indicated by melting curves and spectra of the polymer *versus* its constituent mononucleotides.

It will be seen from Fig. 3 that the shape of the acidic melting curves of copolymers containing a high proportion of cytosine residues becomes more asymmetrical and resembles, to some extent, that of poly-C. However, the hyperchromicities are diminished, the T_m values decrease and the width of the transition increases.

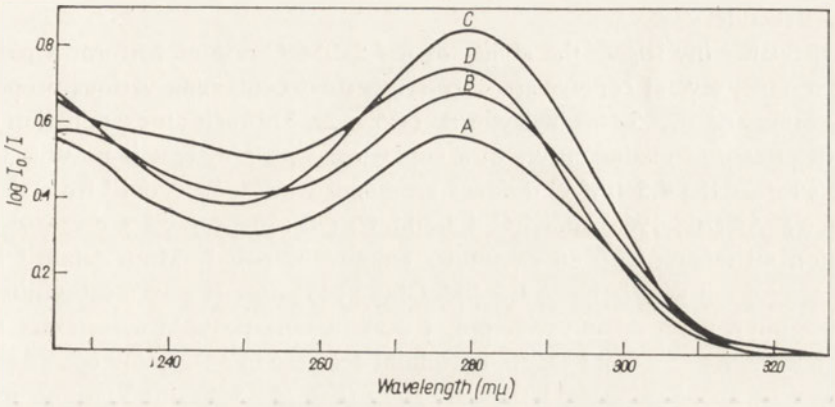

Fig. 4. Absorption spectra of poly-4,5-diMeC and its constituent mononucleotides at 25° and 85° in 0.1 M-NaCl - 0.05 M-acetate buffer, pH 4.0. *A*, Poly-4,5-diMeC at 25°; *B*, at 85; *C*, following hydrolysis with RNase at 25°; *D*, as above, at 85°.

All that is indicative of the melting of double stranded helices containing imperfections, e.g. looped out non-complementary residues (Fresco & Alberts, 1960). A copolymer composed of approximately 1 : 1 C and 4,5-diMeC residues does not give rise to a cooperatively melting structure (Fig. 3, curve *E*).

Complexing ability of poly-4,5-diMeC

It has been demonstrated previously (Szer & Shugar, 1966) that the replacement of cytosine by 5-methylcytosine in the twin stranded complex with poly-I results in a pronounced increase in the mid-point of the helix-coil transition, the difference amounting to about 18° within a Na^+ concentration range from 0.01 M to 0.2 M. Poly-4,5-diMeC appears to be *a priori* capable of forming a complex with poly-I. Indeed, poly-6-methyladenylic acid forms a complex with poly-U, although much weaker than that formed by poly-A with poly-U, the corresponding T_m values in 0.1 M-Na^+ being 15° and 55°, respectively (Griffin *et al.*, 1964). As inferred from the optical density of the mixture over the entire spectrum range, there is, however, no complex formation between poly-4,5-diMeC and poly-I even under most favourable conditions, i.e. on prolonged chilling in the presence of up to 1 mM-Mg^{2+}.

This is identical with the results of Brimacombe & Reese (1966) with poly-4--MeC and may be accounted for either by assuming that the temperature transition of such a complex would be very low, near 0°, or perhaps more likely, that the

ordered state of poly-I itself is more stable than the complex at the same ionic strength (cf. Doty, Boedtker, Fresco, Haselkorn & Litt, 1959), and therefore prevents its formation. At any event, since neither poly-4-MeC nor poly-4,5-diMeC is capable of entering the complex with poly-I, it is evident that the enhancing effect of the 5-methyl substituent does not manifest itself in this case. This supports the notion, likewise inferred from previous investigations (Szer & Shugar, 1966), that the 5--methyl substituent contributes to further stabilization of an already existing regular structure.

To further investigate the ability of a 4,5-diMeC residue to form a pair with an I residue, several copolymers were synthesized containing various proportions of cytosine and $N_{(4)}$,5-dimethylcytosine (Table 2). Through an examination of the stoicheiometry of mixing curves of a copolymer with poly-I, it is possible to evaluate whether the 4,5-diMeC residues are bonded or "looped out" from the helix (Fresco & Alberts, 1960). Poly-(C, 4,5-diMeC) (70 : 30) showed a decrease in absorption on mixing with an equimolar amount of poly-I. More detailed studies were carried out with poly-(C, 4,5-diMeC) (83 : 17) since it gave a substantial drop in absorption upon mixing with poly-I thus making possible more exact mixing experiments. As seen from Fig. 5, maximum decrease in absorption occurs at a copolymer to poly-I ratio of 1 : 1, indicating that $N_{(4)}$,5-dimethylcytosine residues do form pairs with hypoxanthine residues of poly-I. The resulting complex is much less stable than poly-(C+I) and the width of the melting profile is substantially increased. As seen from Fig. 6, Δt of the complex, i.e. the change in temperature

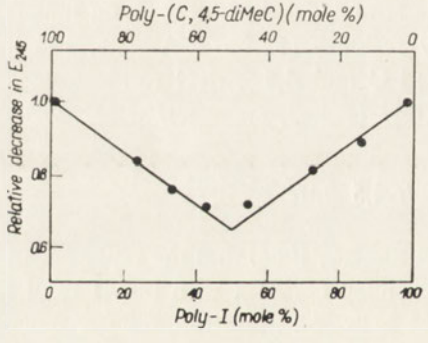

Fig. 5

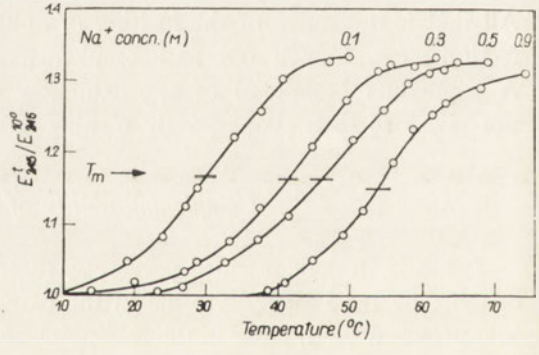

Fig. 6

Fig. 5. Mixing curve for poly-I and poly-(C, 4,5-diMeC) (83 : 17) in 0.01 M-phosphate buffer, pH 7.5, adjusted with NaCl to 0.1 M with respect to Na^+. Absorbancy of equimolar solutions of the two polymers at 245 mμ taken as 1.0.

Fig. 6. Transition profiles for the 1 : 1 complex of poly-I and poly-(C, 4,5-diMeC) (83 : 17) in 0.01 M-phosphate buffer, pH 7.5, in the presence of various concentrations of NaCl.

required to complete 2/3 of the optical density melting profile, amounts to 20°, while Δt for poly-(C+I) is about 4°. Thus, the cooperativeness of the transition is markedly affected. It follows that the introduction of approximately one 4,5--diMeC per five C residues exerts a considerable destabilizing effect on the helix.

Figure 7 reveals that the dependence of T_m of the complex on the logarithm

of salt concentration is linear as it is known for all the other ordered states. Furthermore, while the complex is much less stable than poly-(C+I), the slopes of the corresponding plots of T_m *versus* log of Na^+ molarity are fairly similar in both cases. It may be seen from Fig. 7 that the average difference in the T_m between the two complexes is 30° over a concentration range from 0.1 M-Na^+ to 1.0 M-Na^+.

The lack of complexing ability with poly-I, which, incidentally, may not apply to poly-G, exhibited by both homopolymers containing a $N_{(4)}$-methyl group, i.e. by poly-4-MeC and poly-4,5-diMeC, calls for some comments in connection with recent views on the role of specific hydrogen bonding and its contribution to helix stability. Provisional estimates of the free energy contribution are around 0.6 - - 1.0 kcal per hydrogen bond for various polynucleotide helices (Crothers & Zimm, 1964; Howard, Frazier & Miles, 1966) and, although the intrinsic strengths of various H-bonds may be different, it is widely accepted that they involve marginal energies of helix stabilization. Incidentally, the value of 0.6 kcal assigned to an additional hydrogen bond in the pair uracil+2-aminoadenine (estimated on the basis of the T_m value of the complex poly-U+poly-2-aminoadenylic acid, Howard *et al.*, 1966) is not much higher than the 0.45 kcal change in free energy due to a 5- -methyl substituent when comparing the pairs A+U relative to A+5-methylU and I+C relative to I+5-methylC (Szer *et al.*, 1963; Szer & Shugar, 1966); in

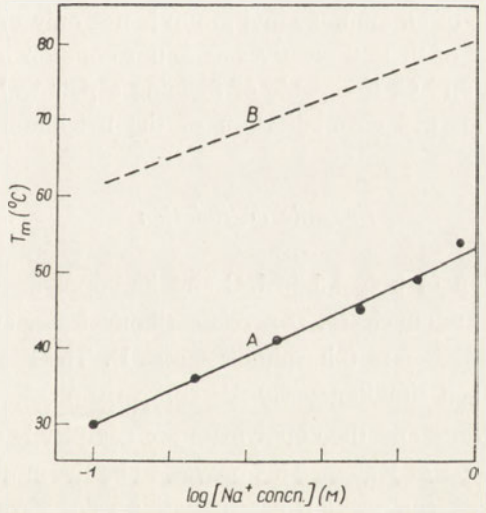

Fig. 7. Dependence of T_m on NaCl concentration, in 0.01 M-phosphate buffer, pH 7.5, for *A*, poly-I+poly-(C, 4,5-diMeC) (83 : 17) and *B*, poly-I+poly-C. The latter curve taken from Szer & & Shugar (1966).

this case any effect of the 5-methyl group on the hydrogen bonding capacities is to be excluded and enhanced stabilities are apparently due to increased stacking energies of the 5-methylated polymers. In spite of the low energy values involved in hydrogen bonding as compared to the free energy of formation of a base pair, it is generally assumed that H-bonds play the necessary discriminatory role in direc-

ting the highly selective base interactions at the polymer level. Yet, it seems that the behaviour of poly-4,5-diMeC cannot be satisfactorily explained in terms of H-bonding only. It has been shown that $N_{(3)}$-methyl derivatives of U and C as well as dimethylamino derivatives of A and C are rendered completely incapable of interacting with their complements (Szer & Shugar, 1961; Griffin *et al.*, 1964; Brimacombe & Reese, 1966). These results may be attributed chiefly to steric factors preventing hydrogen bonding and hence, close approach of bases and the operation of short range forces. The latter forces appear to be of real importance contrary to the somewhat oversimplified notion that a second H-bond is essential for base pairing. Obviously, close approach and H-bonding are interrelated but monomethylamino derivatives of C and 5-MeC which are *a priori* perfectly capable of forming such a second hydrogen bond, become complementary only in copolymers. The greatly reduced capacity of these derivatives to enter twin stranded helices is hard to explain in terms of only hydrogen bonding in view of the estimated strengths of H-bonds in aqueous media. The complexity of steric factors and stabilizing forces involved in various polynucleotide helices is further emphasized by the fact that the already mentioned homopolymer of $N_{(6)}$-methyladenylic acid will form a complex with poly-U (Griffin *et al.*, 1964), while a copolymer containing 70% $N_{(6)}$-methyladenine and 30% adenine fails to do so (Michelson & Pochon, 1966). This stands in sharp contrast with complexing properties of $N_{(4)}$-methyl derivatives of cytosine and 5-methylcytosine. Further clarification of the role of an aminomethyl group is not only of theoretical interest in view of the presence of these derivatives as minor components of some DNA's. Such derivatives of A and C, if capable of forming a pair with U (or T) and G (or I), would cause marked destabilization of the neighbouring helical region.

Enzymic degradation

Enzymic degradation of poly-4,5-diMeC and its copolymers with C has been investigated using an endonuclease, pancreatic ribonuclease, and an exonuclease, snake venom phosphodiesterase (phosphodiesterase I). The degradation of poly-C and poly-5-MeC was run simultaneously for comparison.

Both the homopolymer and the copolymers are completely degraded to mononucleotides with an excess of RNase. This proved to be the most accurate and convenient method for base composition determination of the copolymers (Table 2) and for measuring the extinction of constituent mononucleotides relative to the polymers (Fig. 2). At lower enzyme concentration, however, it became apparent that the rate and extent of degradation of poly-4,5-diMeC are significantly affected as compared to poly-C; e.g. at 0.05 μg./ml. enzyme poly-C and poly-5-MeC are 65% split within 6 min. and the reaction goes to completion within several hours, whereas poly-4,5-diMeC is degraded to an extent of 15% and the reaction stops presumably at the oligonucleotide level. Copolymers are degraded at intermediate rates. Figure 8 reveals that at still lower enzyme concentration poly-4,5-diMeC

is affected to a minimum extent as compared to poly-C and poly-5-MeC. It is also seen under these conditions that the hydrolysis of poly-5-MeC is slowed down relative to poly-C. Accordingly, it was found that the opening of 5-methylcytidine-2′,3′-cyclic phosphate proceeds at a rate fourfold smaller than that for cytidine-2′,3′-cyclic phosphate. It is not clear whether this reflects some changes in basicity of the 2-keto group and thus modifies the catalytic activity of the base (Witzel,

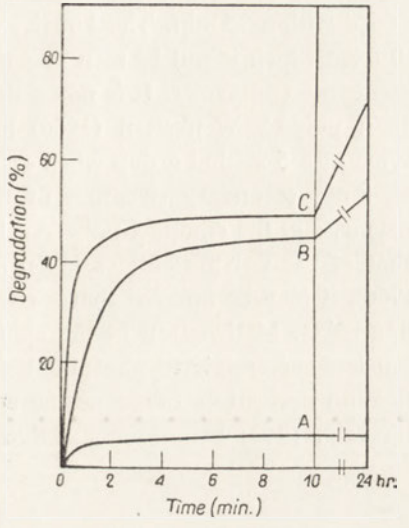

Fig. 8

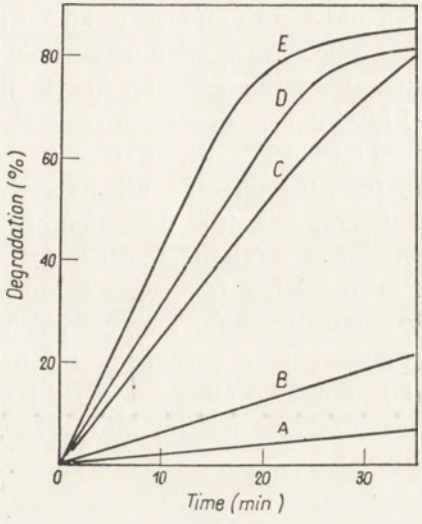

Fig. 9

Fig. 8. Hydrolysis by RNase of *A*, poly-4,5-diMeC; *B*, poly-5-MeC; and *C*, poly-C at 37° in 0.01 M-tris, pH 7.8. Substrate concentration 50 μM, enzyme concentration 0.005 μg./ml. Percent of degradation was calculated from $\frac{E - E_0}{E_t - E_0} \times 100$, where E_0 is initial absorbance, E_t final absorbance after hydrolysis to mononucleotides, E absorbance at time *t*.

Fig. 9. Hydrolysis by phosphodiesterase I of *A*, poly-4,5-diMeC; *B*, poly-5-MeC; *C*, poly-(C, 4,5-diMeC) (45 : 55); *D*, poly-(C, 4,5-diMeC) (70 : 30) and *E*, poly-C. Incubation at 37° in 0.02 M-borate buffer, 0.01 M-Mg^{2+}, pH 9.0. Substrate concentration 50 μM, enzyme concentration 20 μg./ml. For percent of degradation see legend to Fig. 8.

1963). The p*K* values for the nucleosides do not differ much: 4.15 and 4.07 for cytidine and 5-methylcytidine, respectively. A large decrease in p*K* as in poly-5-bromocytidylic acid (p*K* of 5-bromocytidine is 2.6) produces a sixfold decrease in the initial rate of hydrolysis relative to poly-C (Massoulié, Michelson & Pochon, 1966). Nonetheless, steric hindrance by the 5-methyl group does not appear to matter since, in a similar case, these authors report an increase in initial rate of hydrolysis of poly-5-methylU relative to poly-U. An increase in stacking interactions is also expected to enhance the rate (Witzel & Barnard, 1962). The limited susceptibility of poly-4,5-diMeC may be due to decreased polarizability of the base. A more detailed explanation is obviously called for and requires additional experimental evidence (e.g. rates of hydrolysis for the corresponding dinucleoside phosphates

and cyclic phosphates), especially in view of the unexpected finding by Brimacombe & Reese (1966) that $N_{(4)}$-methylcytidine-2′,3′-cyclic phosphate is not hydrolysed by RNase in contrast to the corresponding polymer.

The susceptibility of the methylated polymers toward degradation by phosphodiesterase I is also markedly decreased but at high enzyme concentration poly--4,5-diMeC can be completely hydrolysed. At 50 μg./ml. enzyme the degradation of poly-C is completed within 10 min., that of poly-5-MeC within 45 min., and poly-4,5-diMeC was affected to an extent of 66% within 75 min. The kinetics at lower enzyme concentration is shown in Fig. 9 from which it will be seen that the rate increases with an increase in cytidine content in a copolymer. It is noted that poly-5-MeC is degraded much more slowly than poly-C. Wenkstern (1966) has observed a similar inhibiting effect on the enzyme of a 5-methyl group when comparing ribosethymine and uridine derivatives. Since secondary structure of the substrate does not affect the action of this enzyme, e.g. the complex poly-(A+U) is degraded at a rate intermediate between that of the polynucleotides taken separately (Hadjiolov, Dolapchiev & Milchev, 1966), it may be inferred that a steric factor is responsible for the observed inhibiting effect. Methylation of the amino group renders the corresponding substrate even less susceptible to phosphodiesterase I as noted by other workers for methylamino derivatives of guanosine and cytidine (Baev, Wenkstern, Mirzabekov, Tatarskaia, 1963; Brimacombe & Reese, 1966).

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DRUGORZĘDOWA STRUKTURA KWASU POLI-$N_{(4)}$,5-DWUMETYLOCYTYDYLOWEGO I JEGO KOPOLIMERÓW Z KWASEM CYTYDYLOWYM

Streszczenie

1. Przeprowadzono syntezę 5′-pirofosforanu $N_{(4)}$,5-dwumetylocytydyny i stwierdzono, że jest on substratem fosforylazy polinukleotydowej. Otrzymany kwas poli-$N_{(4)}$,5-dwumetylocytydylowy (4,5-diMeC) nie tworzy podwójnołańcuchowego kompleksu z kwasem poli-inozynowym (poli-I). Kopolimery zawierające kwas cytydylowy (C) i 4,5-diMeC tworzą kompleks z poli-I w stosunku 1 : 1, co świadczy o tym, że $N_{(4)}$,5-dwumetylocytozyna może w określonych warunkach utworzyć parę z hipoksantyną. Kompleks ten ulega znacznie łatwiej dysocjacji termicznej niż kompleks utworzony przez homopolimery I i C. Omówiono własności kompleksotwórcze metyloaminowych pochodnych cytozyny i adeniny w polirybonukleotydach.

2. Grupa metylowa w pozycji $N_{(4)}$ nie zmienia w istotny sposób własnej struktury homopolimeru w środowisku obojętnym w stosunku do jednołańcuchowej konfiguracji występującej w poli-C i kwasie poli-5-metylocytydylowym. W środowisku kwaśnym, w odróżnieniu od obydwu wspomnianych modeli, poli-4,5-diMeC nie tworzy podwójnołańcuchowej struktury własnej. Występująca w tym wypadku w pH 4 struktura własna ma charakter podobny do formy obojętnej mimo protonacji około 40% zasad.

3. Poli-4,5-diMeC ulega degradacji pod wpływem fosfodwuesterazy jadu węża i rybonukleazy trzustkowej, jednak szybkość hydrolizy enzymatycznej jest w obu wypadkach znacznie mniejsza od szybkości hydrolizy poli-C.

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THE SEPARATION OF CHOLINEPHOSPHATE CYTIDYLYLTRANSFERASE FROM ETHANOLAMINEPHOSPHATE CYTIDYLYLTRANSFERASE OF RAT LIVER AND BRAIN BY GEL FILTRATION ON SEPHADEX G-200

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1. The cytidylyltransferases (EC 2.7.7.15 and EC 2.7.7.14) catalysing the formation of cytidine diphosphate choline and cytidine diphosphate ethanolamine were separated by gel filtration on Sephadex G-200. Molecular weights were estimated by gel filtration and found to be 130 000 and 40 000, respectively. 2. The Fiscus-Schneider (1966) phenomenon of cholinephosphate cytidylyltransferase activation by phospholipid was observed also in the crude enzyme preparation. The stimulation was most pronounced and consistent in preparations from rat brain.

Cytidine diphosphate choline (CMP-PC)[1] and cytidine diphosphate ethanolamine (CMP-PE), which are formed within the soluble fraction of the cell, are crucial intermediates in the biosynthesis of phospholipid diesters. On the basis of the observed differences in heat stability, Borkenhagen & Kennedy (1957) postulated the occurrence of two separate enzymic activities catalysing the formation of CMP-PC and CMP-PE, respectively. These enzymes have been later classified as CTP : cholinephosphate cytidylyltransferase (EC 2.7.7.15) and CTP : ethanolaminephosphate cytidylyltransferase (EC 2.7.7.14).

Another difference between the two activities has been reported by Fiscus & Schneider (1966) who were the first to observe in lipid-depleted supernatant fraction of rat liver, the activation of cholinephosphate cytidylyltransferase by the addition of phospholipids. This finding has been confirmed in this laboratory and direct proof for the presence of two separate enzymes was obtained by fractionation of soluble cytoplasmic fraction of rat liver and brain by gel filtration on Sephadex G-200.

[1] Abbreviations used: CMP-PC, cytidine diphosphate choline; CMP-PE, cytidine diphosphate ethanolamine; PC, phosphorylcholine; PE, phosphorylethanolamine.

MATERIALS AND METHODS

Chemicals. Phosphorylcholine (PC) and phosphorylethanolamine (PE) labelled with ^{32}P were prepared according to Riley (1944) and purified on ion exchange columns as described by Ansell & Chojnacki (1966). CTP was synthesized by the method of Moffat (1964) from CMP-morpholidate (CalBiochem., Los Angeles, Cal., U.S.A.) and pyrophosphate. The final step of CTP isolation from the reaction mixture was performed as described by Canellakis, Gottesman, Kammen & Irvin (1962). It included chromatography on Dowex-1-formate column, concentration of the CTP fraction on Dowex-1-chloride column and precipitation of barium salt with ethanol. The solution of sodium salt of CTP was obtained by precipitation of barium with sodium sulphate. Lecithin from spinal cord was a commercial preparation (Reinchemie, Berlin, Germany). Sephadex G-200 (particle size 40 - 120μ) was Pharmacia Ltd. (Uppsala, Sweden) product. Charcoal (Zakł. Elektr. Węgl., Racibórz, Poland) was prepared according to Threlfall (1957). Protein standards for molecular weight estimations were: ribonuclease A from bovine pancreas (mol. wt. 13 000) (Sigma, St. Louis, Mo., U.S.A.); human albumin (mol. wt. 69 000) and human γ-globulin (mol. wt. 150 000), products of Warsaw Serum and Vaccine Plant (Poland) kindly given by Prof. Dr. Kazimierz Zakrzewski.

Analytical. Phosphorus assays were carried out by the method of Fiske & Subbarow (1925) for amounts of 10 - 50 μg. P, and by the method of Ernster, Zetterström & Lindberg (1950) for those below 10 μg. P. The determination of ^{32}P was performed using a G. M. mica end window counter and conventional ancillary equipment. Protein was estimated spectrophotometrically by measuring the extinction at 280 mμ, and by the method of Lowry, Rosebrough, Farr & Randall (1951).

Homogenate and gel filtration. White Wistar rats, males and females, 6 weeks old, weighing 200 - 220 g., were used. They were stunned and decapitated, and livers and brains were homogenized in ice-cold 0.145 M-sodium chloride (1 g. of tissue in 4 ml. of 0.145 M-NaCl) in a Potter-Elvehjem homogenizer fitted with a teflon pestle. The homegenate was centrifuged for 1 hr. in the SW-30 rotor in a Spinco model L ultracentrifuge at 105 000 *g*. For gel filtration, the supernatant fraction (2 ml.) was applied to a Sephadex G-200 column (1.2×50 cm.) and eluted with 0.145 M-NaCl - 0.005 M-tris-HCl buffer, pH 7.5. Fractions of 1.8 - 2.0 ml. were collected with the aid of a fraction collector, and their exact volume measured afterwards.

Enzyme assay. Reaction mixture contained the following components in a total volume of 0.3 ml.: PC or PE labelled with ^{32}P (specific activity 3×10^5 to 1×10^6 counts/min./μmole), 0.05 μmole; CTP, 0.1 μmole; the mixture of tris and succinic acid, 5 μmoles of each, adjusted to pH 7.5 with NaOH; magnesium acetate, 3 μmoles; and 50 μl. of enzyme preparation. The tubes were incubated for 15 min. at 37°. The reaction was stopped by 0.3 ml. of 10% trichloroacetic acid and the precipitate removed by centrifugation. A 0.4 ml. sample of the supernatant was treated with 1 ml. of an aqueous suspension of charcoal (50 mg./ml.) and the labelled CMP-^{32}PC and CMP-^{32}PE were estimated by the procedure of Borkenhagen & Kennedy (1957).

RESULTS

Figure 1 represents a fractionation of rat liver supernatant on a column of Sephadex G-200. A clear separation of the two activities was obtained, and no cross-unspecificity of the separated enzymes was observed. The cholinephosphate cytidylyltransferase emerged first (peak *A*) in the effluent from the column and it was followed by the ethanolaminephosphate cytidylyltransferase (peak *B*).

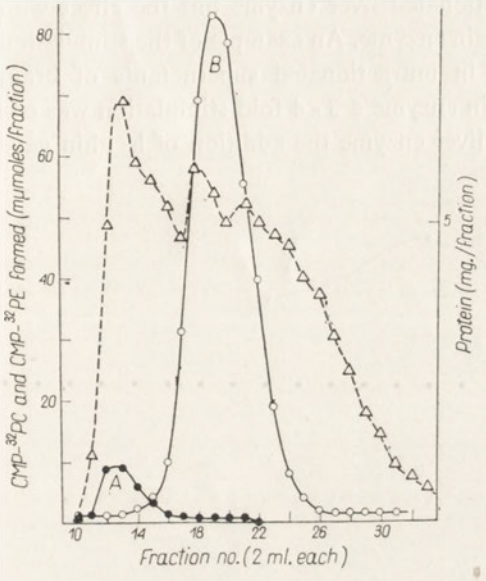

Fig. 1

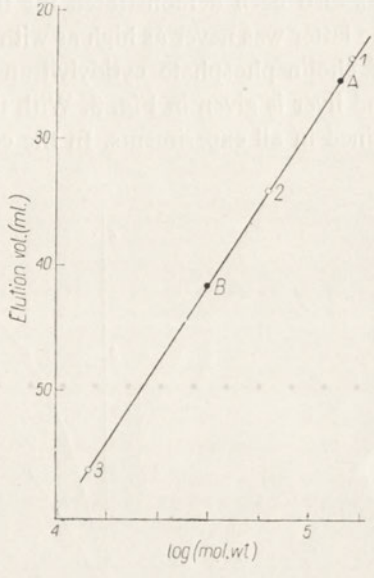

Fig. 2

Fig. 1. Fractionation of cholinephosphate- and ethanolaminephosphate cytidylyltransferases of rat liver by gel filtration on Sephadex G-200. 105 000 *g* supernatant of rat liver homogenate (2 ml.) was applied to a 1.2×50 cm. column of Sephadex G-200 and eluted with 0.145 M-NaCl - 0.005 M--tris-HCl, pH 7.5. Fractions of 2 ml. were collected. (●), Cholinephosphate cytidylyltransferase; (○), ethanolaminephosphate cytidylyltransferase; both activities expressed in mμmoles of synthetized CMP-PC and CMP-PE per fraction; (△), amount of protein (mg./fraction).

Fig. 2. Determination of molecular weights of cholinephosphate- and ethanolaminephosphate cytidylyltransferases of rat liver by Sephadex G-200 gel filtration. In the eluate from a 1.2×50 cm. column, the elution volumes of the enzymes studied were compared with those of proteins of known molecular weight as described by Andrews (1964). Standard proteins: *1*, human γ-globulin; *2*, human albumin; and *3*, RNase; *A*, cholinephosphate cytidylyltransferase; *B*, ethanolaminephosphate cytidylyltransferase.

For molecular weight determination, the Sephadex G-200 columns were standardized with pure proteins of known molecular weight (human γ-globulin and albumin, bovine•pancreas RNase). The values calculated for the two enzymes studied were 130 000 and 40 000 for cholinephosphate cytidylyltransferase and ethanolaminephosphate cytidylyltransferase, respectively (Fig. 2).

In contrast to a fairly high stability of rat liver enzymes in the crude 105 000 *g* supernatants or in effluents from Sephadex G-25 or G-50 columns containing total

protein, a considerable loss of activity was observed in fractions obtained by Sephadex G-200 gel filtration. Similar results were observed with rat brain preparations indicating that brain enzymes are identical with liver enzymes. The activities of the two cytidylyltransferases were, however, distinctly lower in brain than in liver, and in the former the cholinephosphate cytidylyltransferase activity could not be detected unless a trace of lecithin preparation was added to the reaction mixture.

The activation of cholinephosphate cytidylyltransferase by a lecithin preparation has also been demonstrated for the fractionated liver enzyme but the effect with the latter was never as high as with the brain enzyme. An example of the stimulation of cholinephosphate cytidylyltransferase in unfractionated supernatants of brain and liver is given in Fig. 3. With the brain enzyme a 3 - 4 fold stimulation was obtained in all experiments. In the case of liver enzyme the addition of lecithin gave

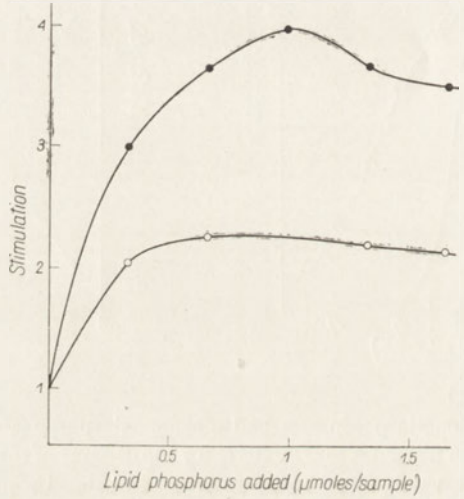

Fig. 3. Stimulation of cholinephosphate cytidylyltransferase of rat (○) liver and (●) brain by lecithin preparation. 0.05 ml. of 105 000 *g* supernatant of tissue homogenate (20%) in 0.145 M-NaCl was incubated in a medium containing 16.6 mM-tris-succinate, pH 7.5; 10 mM-magnesium acetate; 0.33 mM-CTP and 0.166 mM-[^{32}P]phosphocholine. Total volume, 0.3 ml. Procedure. Where indicated, different amounts of lecithin (as a chloroformic solution) were added to a series of tubes and the solvent evaporated in vacuum. The enzyme, tris-succinate and magnesium acetate were then added to the tubes and the residue of lecithin was emulsified for 30 sec. on a flask shaker (Griffin George, Ltd. England) using maximal speed. The reaction was started by adding ^{32}PC and CTP. Incubations were carried out for 15 min. at 37° and the amount of CMP-^{32}PC formed was estimated by charcoal adsorption. The amounts of CMP-^{32}PC obtained without adding lecithin taken as 1 were: 0.5 and 4.6 mμmoles for brain and liver enzyme, respectively.

usually about twofold stimulation of CMP-^{32}PC synthesis but the results were not consistent; often no effect or about 10% inhibition of the reaction was observed. The ethanolaminephosphate cytidylyltransferases of liver and brain were not affected by the addition of lecithin.

DISCUSSION

The presented data show that the two enzymes responsible for the formation of CMP-PC and CMP-PE in rat tissues belong to different molecular species. Their molecular weights were found to be about 130 000 and 40 000, respectively. Gel filtration on Sephadex G-200 gave clear-cut separation of the two activities. This strongly supports the observation of Chojnacki & Ansell (1967) that both enzymes are characterized by a high specificity when tested with phosphoric esters of unnatural bases.

The attempts of Schneider (1963) and Schneider, Fiscus & Lawler (1966) to fractionate the two cytidylyltransferases with ammonium sulphate, were unsuccessful as both activities were precipitated by 25 - 37.5% saturation. In our experiments similar results were obtained and a considerable loss of both activities was observed.

The existence of two separate cytidylyltransferases so far was assumed from indirect indications. The heat resistance was found by Borkenhagen & Kennedy (1957) to be specific for cholinephosphate cytidylyltransferase activity, and this observation was confirmed by Schneider (1963). Recently, Fiscus & Schneider (1966) demonstrated that in a lipid-depleted preparation only cholinephosphate cytidylyltransferase was stimulated by the addition of lecithin. This finding was confirmed in the present work; moreover, it appeared that even the native, not delipidated cholinephosphate cytidylyltransferase, both in the crude supernatant and after Sephadex G-200 gel filtration, was greatly stimulated by the addition of lecithin preparation. A high increase of the synthesis of CMP-PC was observed in preparations from brain. The results with liver enzyme were rather inconsistent which so far we are unable to explain. According to Fiscus & Schneider (1966) the stimulating effect depends on the presence of lysolecithin in the lecithin preparation. The preparation used in our experiments did also contain the lyso-compound as revealed by chromatography on silica impregnated paper (Marinetti, 1962). Our observation that the native, already active enzyme can also be stimulated indicates a regulatory effect of phospholipid in the synthesis of CMP-PC, as suggested by Fiscus & Schneider (1966). No data are so far available on the mechanism of this stimulation. Both the cholinephosphate- and ethanolaminephosphate cytidylyltransferase are cytoplasmic enzymes. However, the dependence of the cholinephosphate cytidylyltransferase activity on the presence of structural material of the cell (Fiscus & Schneider, 1966), and the occurrence of a small but distinct amount of this enzyme in endoplasmic reticulum (Wilgram & Kennedy, 1963; Schneider, 1963; Chojnacki, unpublished results), together with the activation by phospholipid indicate an interaction of the molecule of cholinephosphate cytidylyltransferase with lipid. This might have a similar nature as that described for β-hydroxybutyrate dehydrogenase (Jurtshuk, Sekuzu & Green, 1963).

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ROZDZIELANIE PRZY POMOCY SEFADEKSU G-200 CYTYDYLILOTRANSFERAZY CHOLINOFOSFORANOWEJ OD CYTYDYLILOTRANSFERAZY ETANOLOAMINOFOSFORANOWEJ ZAWARTYCH W WĄTROBIE I MÓZGU SZCZURA

Streszczenie

1. Filtracją na żelu Sefadeks G-200 oddzielono cytydylilotransferazę cholinofosforanową (EC 2.7.7.15) od cytydylilotransferazy etanoloaminofosforanowej (EC 2.7.7.14) występującej w supernatantach 105 000 *g* wątroby i mózgu szczura. Określono ich masy cząsteczkowe: 130 000 i 40 000.

2. Lecytyna zwiększa parokrotnie aktywność natywnej cytydylilotransferazy cholinofosforanowej występującej w rozpuszczalnej frakcji cytoplazmy mózgu, natomiast nie ma wpływu na enzym etanoloaminofosforanowy.

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Vo. XIV 1967 No. 3

RECENZJE KSIĄŻEK

PEPTIDES (L. Zervas, ed.) Pergamon Press, Oxford; 1966 str. XVI+386, cena 17.50 $

Recenzowana książka jest zbiorem referatów wygłoszonych na VI Europejskim Sympozjum Peptydowym, które odbyło się w Atenach we wrześniu 1963 r. W ateńskim Sympozjum brało udział 75 osób reprezentujących uczelnie, przemysł chemiczny i instytuty naukowe dwunastu krajów europejskich oraz Stanów Zjednoczonych i Izraela. Referaty te zostały w pełnym tekście wydane w omawianej książce (34 referaty w języku angielskim, 10 w niemieckim i 2 po francusku). Nie zamieszczono w książce tylko tych referatów, które wkrótce po Sympozjum opublikowane zostały w czasopismach. Każdy referat zawiera wykaz cytowanego piśmiennictwa. Zamieszczono również główne wypowiedzi dyskusyjne.

Każdy z siedmiu rozdziałów książki dotyczy innej grupy problemów. Rozdział I. poświęcony grupom osłaniającym oraz metodom sprzęgania aminokwasów zawiera 16 referatów. Pięć z nich dotyczy stosowania nowych grup ochronnych. Dziś można już powiedzieć, że karierę zrobiła opisana tam przez Zervasa grupa *o*-nitrofenylosulfenylowa, stosowana dla osłony funkcji α-aminowej. Cztery referaty opisują nowe lub ulepszone metody chemicznej syntezy wiązań peptydowych. Do najciekawszych w tym rozdziale należą opracowania wyjaśniające chemizm niektórych procesów od dawna stosowanych w praktyce: Rudinger wyjaśnił przebieg rozszczepiania grupy tozyloamidowej sodem w ciekłym amoniaku, Iselin opisał produkty przejściowe i uboczne redukcji reszty nitroargininowej, a Schnabel — uboczne skutki stosowania dwucykloheksylo-karbodwuimidu w syntezach peptydów. Te trzy prace wskazują na opóźnienie badań podstawowych w chemii peptydów, która goni za praktycznymi wynikami.

Rozdział II. poświęcony racemizacji zawiera cztery referaty. Główny z nich, przygotowany na życzenie organizatorów Sympozjum przez grupę specjalistów z Uniwersytetu w Oxfordzie z G. T. Youngiem na czele, kreśli obecny stan badań zjawiska racemizacji w syntezach peptydów. Zestawienie głównych metod badania zjawiska oraz ich wyników nie jest sporządzone krytycznie: autorzy cytują niekiedy obok siebie wyniki sprzeczne, jakich sporo jest w literaturze. Prox i Weygand przedstawili zasadę nowej metody badania racemizacji na drodze chromatografii gazowej oraz nakreślili szerokie możliwości tej metody.

Rozdział III. grupuje prace dotyczące degradacji łańcucha peptydowego. Ograniczona użyteczność enzymów w analizie peptydów (Zuber) uzasadnia poszukiwania nieenzymatycznych metod selektywnego rozszczepiania łańcuchów peptydowych. O metodach takich traktują referaty Witkopa i Patchornika. W tymże rozdziale przedstawiono możliwości modyfikowania bocznych łańcuchów tryptofanu i cysteiny w łańcuchu peptydowym, przydatne dla celów analitycznych.

Rozdział IV. poświęcony jest syntezom naturalnych polipeptydów i ich analogów. Aż pięć spośród dziesięciu prac zawiera próby uogólnień na temat zależności między strukturą a aktywnością biologiczną: w grupie oksytocyny i jej analogów (Rudinger), w grupie angiotensyn (Havinga), bradykinin (Schröder), eniantyn (Studer) i antybiotyków cyklodepsipeptydowych (Szemiakin). Wszystkie te podsumowania budzą pewien sceptycyzm z powodu nieznajomości konformacji zarówno rodzimych ciał czynnych, jak ich syntetycznych analogów.

W niewielkich rozdziałach V. i VI. poświęconych syntezie specjalnych i nietypowych peptydów zamieszczono ciekawe doniesienie o syntezie fragmentu łańcucha A insuliny, zawierającego

20-członowy pierścień dwusiarczkowy. Praca ta jest bardzo ciekawa, choć udana ostatnio synteza insuliny nie poszła drogą tam nakreśloną. Skomplikowane metody syntez niesymetrycznych peptydów kwasu mezodwuaminopimelinowego, jakie występują w mukopeptydach ścian komórek bakteryjnych, opisał Bricas. Dużą atrakcyjność opracowania Ivanowa o syntetycznych i naturalnych cyklodepsipeptydach stanowią zawarte tam rozważania konformacyjne, będące dotychczas wielką rzadkością w pracach peptydowych.

Cenną pozycją rozdziału VII. jest monograficzne opracowanie Weyganda o zastosowaniu chromatografii gazowej w chemii peptydów; zawiera ono zestawienie metod, jakimi można przeprowadzić aminokwasy i peptydy w lotne i trwałe pochodne, przydatne w badaniach chromatograficznych. Znaleźć tam można również przykłady zagadnień, jakie mogą być badane metodą chromatografii gazowej.

Omawiana książka przeznaczona jest w zasadzie dla chemików-organików, zajmujących się syntezą i analizą peptydów. Stanowi ona zwięzły przegląd wyników uzyskiwanych w najważniejszych działach chemii aminokwasów i peptydów. Dla osób nie zajmujących się praktycznie tym działem chemii może książka stanowić wprowadzenie do najbardziej aktualnej problematyki tej szybko rozwijającej się dziedziny wiedzy.

Teresa Sokołowska

RIBONUCLEIC ACID - STRUCTURE AND FUNCTION. (H. Tuppy, ed.) Pergamon Press, Oxford, London, Edinburgh, New York, Toronto, Sydney, Paris, Braunschweig 1966; str. 150; cena 30 s.

Treścią książki jest dziesięć programowych referatów wygłoszonych na sympozionie na temat kwasów rybonukleinowych, w ramach II Zjazdu Federacji Europejskich Towarzystw Biochemicznych w Wiedniu, 21 - 24 kwietnia 1965 r.

Pierwsze trzy referaty dotyczyły tRNA. W jednym z nich M. Staehelin z Zakładu Farmaceutycznego Ciby w Bazylei przedstawia próby wydzielenia, metodą rozdziału przeciwprądowego drożdżowego i wątrobowego tRNA, frakcji wiążących [^{14}C]serynę. Otrzymane frakcje, po ich strawieniu RNazą trzustkową, rozdziela na kolumnach z DEAE-celulozą a następnie metodą elektroforezy bibułowej i porównuje otrzymane wyniki.

Drugi referat dotyczy badań nad zagadnieniem roli metylowanych zasad we właściwościach tRNA. Uriel Z. Litauer i Ruth Milbauer z Instytutu Weizmanna w Rehovoth badali różnice w zachowaniu się tRNA wyizolowanego z komórek *E. coli*, hodowanych na pożywce pozbawionej metioniny („głodzone tRNA"), w porównaniu z „normalnym tRNA". Stwierdzili, że zubożenie tRNA w grupy metylowe nie wpływa na ich zdolność wiązania aminokwasów.

W trzecim referacie z tej serii H. G. Zachau i współpracownicy z Instytutu Genetyki w Kolonii przedstawiają wyniki pracy nad strukturą serylo-tRNA. Wyizolowali oni z drożdży trzy odmiany tego związku i omawiają budowę i własności fragmentów uzyskanych z częściowej hydrolizy. Uzyskane wyniki porównują z budową i własnościami alanylo-tRNA zbadanego przez Holley'a.

W trzech innych referatach omawiane są zagadnienia budowy i funkcji mRNA. W jednym z nich F. Gross i współpr. z Instytutu Biologii Fizyko-Chemicznej w Paryżu, przedstawiają prace na temat represorów syntezy mRNA. W szczególności omawiają wpływ rybosomów bądź rybosomowych białek, na mechanizm syntezy mRNA i dysocjację powstającego kompleksu mRNA-DNA.

W następnej pracy H. R. V. Arnstein z Narodowego Instytutu Badań Medycznych w Londynie przedstawia wyniki swoich badań nad mRNA retikulocytów żaby. Frakcjonowaniem w gradiencie sacharozy otrzymywał zarówno czysty mRNA o masie cząsteczkowej 150 000 - 300 000 jak i kompleksy mRNA-rRNA o większych cząsteczkach.

Inna z prac tej serii dotyczy wpływu azaguaniny na biosyntezę białka. Praca referowana była przez D. Grünbergera z Czechosłowackiej Akademii Nauk. Stwierdził on znaczne zahamowanie syntezy białka. Zdaniem autora azaguanina, wbudowująca się do łańcucha mRNA, utrudnia prze-

suwanie się rybosomów wzdłuż łańcucha; odpadają one przed zakończeniem biosyntezy łańcucha białkowego.

Dwie następne prace dotyczą pierwszo- i drugorzędowej struktury rybosomowego RNA. J. P. Ebel z Pracowni Chemii Biologicznej w Strasburgu przedstawia wyniki badań nad wpływem temperatury i jonów magnezu na niektóre frakcje drożdżowego rRNA. Podaje także procentowe zawartości zasad w poszczególnych frakcjach. R. A. Cox z Narodowego Instytutu Badań Medycznych w Londynie zwraca główną uwagę na wpływ pH, siły jonowej i temperatury na drugorzędową budowę rRNA. Stwierdza on, że 30s rRNA różnego pochodzenia jest bogatszy w guaninę i cytozynę i ma bardziej trwałą drugorzędową strukturę rRNA niż frakcje 16 - 19s.

Autorem przedostatniej pracy jest G. Koch z Instytutu Wirusologii i Immunologii w Hamburgu. Omawia on własności i działanie wirusowego RNA, jego mechanizm replikacji, a szczególnie możliwości transkrypcji genetycznych informacji z wirusowego RNA na DNA gospodarza.

Książka zakończona jest interesującym referatem na temat roli RNA w translacji aminokwasowego kodu. Autorem referatu jest S. Ochoa. Wielu polskim czytelnikom temat ten jest już znany, gdyż podobny referat, znacznie szerzej ujęty i uzupełniony nowymi osiągnięciami, wygłosił Ochoa w kwietniu 1966 r. w Warszawie na Czwartym Zjeździe Federacji Europejskich Towarzystw Biochemicznych.

Książka jest jak najbardziej godna polecenia, szczególnie biochemikom interesującym się budową i znaczeniem kwasów rybonukleinowych.

Bronisław Filipowicz

FLAVINS AND FLAVOPROTEINS (E. C. Slater, ed.) B.B.A. Library, Volume 8, Elsevier Publishing Company, Amsterdam, London, New York 1966; str. XII+549, cena Dfl 75.

Tom ten stanowi sprawozdanie z Sympozjum, które odbyło się w Amsterdamie od 10 do 15 czerwca 1965 roku. Było ono poświęcone flawinom i flawoproteidom. Z ramienia Międzynarodowej Unii Biochemicznej zorganizował to sympozjum Prof. E .C. Slater. Ilość uczestników wynosiła 56 osób, włączając w to 25 zaproszonych referentów. Zaproszeni referenci byli to badacze, którzy w ostatnich latach osiągnęli szczególnie interesujące wyniki w różnych dziedzinach chemii oraz biochemii związków flawinowych. Treść ich referatów oraz dyskusji, jaka się odbyła po każdym z nich, jest tematem recenzowanej książki.

Poszczególne rozdziały omawiają bardzo różne zagadnienia. Chociaż redaktor nie przeprowadza żadnego podziału można je jednak tematycznie podzielić na dwie grupy: 1) rozdziały poświęcone zagadnieniom ogólnym, 2) rozdziały poświęcone poszczególnym znanym flawoproteidom.

Do grupy pierwszej można zaliczyć referaty dotyczące roli kompleksów przenoszących ładunek („charge-transfer complexes") w chemii i biochemii flawin; intramolekularnych kompleksów flawin; badań rezonansu spinu elektronów oraz rezonansu paramagnetycznego elektronów; zastosowania izotopów wodoru do badań reakcji flawoproteinowych; współdziałania flawin z molibdenem i żelazem.

W grupie drugiej omówiono nowe wyniki badań dotyczących NADH peroksydazy, dehydrogenazy bursztynianowej, oksydazy D-aminokwasów i L-aminokwasów, oksydazy glukozy z *Aspergillus niger*, cytochromu b_2, NADPH-cytochrom oksydoreduktazy, NADH-cytochrom b_5 reduktazy, flawoproteidów przenoszących elektrony i ich współdziałania z łańcuchem oddechowym.

Książka ta z pewnością zainteresuje wszystkich, którzy zajmują się zagadnieniami związanymi z chemią flawoproteidów oraz ich udziałem w przemianach ustrojowych, przedstawiono bowiem w niej najistotniejsze zdobycze nauki w tej dziedzinie wiedzy, jakie osiągnięto w ostatnich latach.

Wiktor Rzeczycki

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Р. В. ШРАММ, Тереса МОССОР и Ч. ГЕРЧАК

ВЛИЯНИЕ СВЕТА НА СОДЕРЖАНИЕ ЦИТРАТА И МАЛАТА В ПРОРАСТАЮЩИХ СЕМЕНАХ И МОЛОДЫХ СЕЯНЦАХ БОБА *(VICIA FABA L. MINOR)*

Резюме

1. Свет влияет в значительно большей степени на изменения содержания цитрата и малата в оси сеянцев (побеги и корень) по сравнению с изменениями в котиледонах.

2. В периоде набухания семян цитрат потребляется зародышем.

3. В периоде образования проростка содержание цитрата в оси сеянца возрастает, а затем падает, тогда как содержание малата все время возрастает.

4. В раннем периоде прорастания малат частично переходит из котиледонов в ось сеянца. После приобретения сеянцами способности к фотосинтезу, количество малата в корне увеличивается за счет фотосинтетических процессов.

5. В котиледонах содержание цитрата и малата по отношению к общему сухому весу почти не изменяется.

А. Б. ЛЕГОЦКИ и Е. ПАВЕЛКЕВИЧ

ФЕРМЕНТЫ АКТИВИРУЮЩИЕ АМИНОКИСЛОТЫ В СЕМЕНАХ ЖЕЛТОГО ЛЮПИНА И ОЧИСТКА ЛЕЙЦИЛ-sRNA СИНТЕТАЗЫ

Резюме

1. В экстрактах из семян желтого люпина *L. luteus* L. констатировано присутствие ферментативной системы катализирующей зависящий от аминокислот обмен между неорганическим ^{32}P-пирофосфатом и АТР. Из белковых аминокислот наиболее интенсивно активировался лейцин, немного слабее метионин, валин, изолейцин и аланин другие аминокислоты почти не активировались. Наблюдалась также активация некоторых *N*-ацетил-производных аминокислот.

2. Лейцил-sRNA синтетаза очищена 270 раз. Исследованные свойства фермента сходны с описанными свойствами синтетазиного происхождения. Молекулярный вес синтетазы определенный методом гель-фильтрации составлял 170 000.

А. Б. ЛЕГОЦКИ, А. ШЫМКОВЯК, К. ПЭХ и Е. ПАВЕЛКЕВИЧ

ВЫДЕЛЕНИЕ И НЕКОТОРЫЕ СВОЙСТВА РАСТВОРИМОЙ РИБОНУКЛЕИНОВОЙ КИСЛОТЫ И СЕМЯН ЖЕЛТОГО ЛЮПИНА

Резюме

1. Из семян желтого люпина *(Lupinus luteus)* выделялась sRNA при помощи фенольной екстракции, осаждения цетилтриметил-аммоний бромидом и гель-фильтрации на колонке

Sephadex G-200. Полученные препараты sRNA обладали нормальной акцепторной активностью по отношению к аминокислотам.

2. Хроматография sRNA на колонке из DEAE-целлюлозы при температуре 72° дала острые максимумы акцепторной активности для лейцина, изолейцина и валина. Акцепторная активность для метионина была найдена в трех фракциях.

3. [^{14}C]-Аминоацил-sRNA фракционировали на протаминированной инфузорной земле. При этом была обнаружена гетерогенность лейцил-, изолейцил-, метионил- и валил-sRNA.

Т. БОРКОВСКИ, Ирена БОРКОВСКА, С. КУЛЕША и А. ПАПРОЦКИ

РИБОНУКЛЕНОВАЯ КИСЛОТА СОПРОВОЖДАЮЩАЯ ДЕЗОКСИРИБОНУКЛЕИНОВУЮ КИСЛОТУ ПРИ ЕЕ ЭКСТРАКЦИИ ИЗ МОЗГА

Резюме

1. Крысам вводили [^{32}P]интракраниально и через 1,5 часа и по истечении 24 часов из мозга экстрагировали DNA непосредственно по методу Кай'а, а затем фенольным методом.

2. Выделенные волокна DNA всегда содержали растворимую RNA и рибосомальную RNA. DNA очищенная на колонке из метилированного альбумина содержала небольшие количества RNA устойчивой к действию рибонуклеазы.

3. В белковой фракции обнаружено наличие быстро метящейся RNA с отношением G+C/A+U близким единицы.

Алиция БАРДОНЬ и Зофия ПАМУЛА

РИБОНУКЛЕАЗЫ ПЕЧЕНИ КРЫСЫ ВО ВРЕМЯ РАЗВИТИЯ

Резюме

1. Активность рибонуклеазы в ядерной и митохондриальной фракции из печени крысы является наиболее высокой на седьмой день жизни.

2. Оптимум pH основной рибонуклеазы в сульфосалициловокислых экстрактах ядерной и митохондриальной фракции печени взрослых и семидневных крыс было соответственно, pH 7,7 и 8,5.

3. 0,1 м-Mg^{2+} тормозил в 50% энзиматическую активность только в митохондриальных экстрактах взрослых крыс.

Мирослава ПЕХОВСКА и Д. ШУГАР

ПОВТОРИМАЯ ТРАНСФОРМАЦИЯ СТРЕПТОКОККОВ ГРУППЫ H С ВЫСОКИМ ВЫХОДОМ

Резюме

1. Приводится подробное описание трансформации двух гемолизирующих штаммов стрептококков из группы H: *Streptococcus Challis* и *Streptococcus Wicky*.

2. Получена повторимая трансформация с высоким выходом путем установления и выбора соответствующих условий эксперимента. Наиболее важными факторами оказались исходная концентрация культуры реципиента, присутствие в избытке фактора компетенции во время роста, отношение концентрации трансформирующего DNA к количеству клеток

реципиента, длительность контакта между ними, а также время посева трансформантов. Таким образом удалось получить 50% трансформантов в случае *S. Challis* и 60% в случае *S. Wicky*. Эти числа, выраженные числом колоний, являются суммой трансформаций двух независимых маркеров.

3. Показано, что в системе трансформации стрептококков избыток DNA, превышающий начальное „насыщение", может вызвать значительное уменьшение числа трансформантов.

4. В дискуссии обсуждается значение различных условий эксперимента, возможность увеличения выхода трансформации и возможность оценки числа трансформированных клеток.

С. ПЕЖЫНЬСКИ и П. ШАФРАНЬСКИ

СВЯЗЫВАНИЕ НАТУРАЛЬНОЙ МАТРИЦЫ С РИБОСОМАМИ *ESCHERICHIA COLI* В ПРИСУТСТВИИ РАСТВОРИМОЙ RNA

Резюме

1. Растворимая RNA (sRNA), выделенная из *E. coli* и лишенная примесей высокомолекулярной RNA уменьшает количество связываемой рибосомами быстрометящейся [^{14}C]RNA.

2. sRNA влияет также на образованный комплекс матрица - рибосомы.

3. Хлортетрациклин, который блокирует связывание sRNA с рибосомами не изменяет тормозящего влияния sRNA на связывание матрицы с рибосомами.

А. РАБЧЕНКО и В. ШЭР

ВТОРИЧНАЯ СТРУКТУРА ПОЛИ-$N_{(4)}$,5-ДИМЕТИЛ-ЦИТИДИЛОВОЙ КИСЛОТЫ И ЕЕ КОПОЛИМЕРОВ С ЦИТИДИЛОВОЙ КИСЛОТОЙ

Резюме

1. Проведен синтез 5′-пирофосфата $N_{(4)}$,5-диметилцитидина и установлено, что он является субстратом полинуклеотидфосфорилазы. Полученная поли-$N_{(4)}$,5-диметилцитидиловая кислота (4,5-diMeC) не образует двойной спирали с поли-инозиновой кислотой (поли-I). Кополимеры, содержащие цитидиловую кислоту (C) и 4,5-diMeC образуют комплекс с поли-I в отношении 1:1, что свидетельствует о том, что $N_{(4)}$,5-диметилцитозин может в определенных условиях образовывать пару с гипоксантином. Этот комплекс подвергается термической диссоциации значительно легче, чем комплекс образуемой гомополимерами I и C. Обсуждается способность к комплексообразованию метиламино-производных цитрозина и аденина в полирибонуклеотидах.

2. Метильная группа в позиции $N_{(4)}$ не изменяет существенным образом собственной структуры гомополимера в нейтральной среде по отношению к одноцепочечной конфигурации поли-C и поли-5-метилцитидиловой кислоты. В кислой среде поли-4,5-diMeC, в отличие от обеих вышеупомянутых моделей, не образует двойной цепи. В этом случае наблюдающаяся при pH 4 собственная структура имеет такой-же характер, как и при нейтральной среде, несмотря на протонацию приблизительно 40% оснований.

3. Поли-4,5-diMeC подвергается деградации под влиянием фосфодиэстеразы змеиного яда и панкреатической рибонуклеазы, однако, скорость энзиматического гидролиза в обоих случаях значительно меньше, чем скорость гидролиза поли-C.

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РАЗДЕЛЕНИЕ ПРИ ПОМОЩИ SEPHADEX G-200 ХОЛИНФОСФАТНОЙ ЦИТИДИЛИЛТРАНСФЕРАЗЫ И ЭТАНОЛАМИНФОСФАТНОЙ ЦИТИДИЛИЛТРАНСФЕРАЗЫ ИЗ ПЕЧЕНИ И МОЗГА КРЫСЫ

Резюме

1. При помощи гель-фильтрации на Sephadex G-200 удалось разделить холинфосфатную цитидилилтрансферазу (EC 2.7.7.15) и этаноламинфосфатную цитидилилтрансферазу (EC 2.7.7.14) находящихся в надосадочной жидкости после центрифугирования при 105 000 г гомогенатов печени и мозга крысы. Определены их молекулярные веса: 130 000 и 40 000.

2. Лецитин увеличивает в несколько раз активность нативной холинфосфатной цитидилилтрансферазы, находящейся в растворимой фракции цитоплазмы мозга, но не влияет на этаноламинфосфатный фермент.