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ACTA BIOCHIMICA POLONICA
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# INTERACTION OF ACETYLATED AND NON-ACETYLATED HISTONES WITH DNA*

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The ultraviolet difference spectra and melting profiles of histone-DNA complexes indicate that the complexes with acetylated histone fractions have lower stability than those with non-acetylated histones. Analysis of infrared spectra showed that acetylated histone *f3* has a more ordered structure than non-acetylated *f3*.

It has been demonstrated (Szopa, 1973) that the difference spectra at the wavelength range of 240 - 300 nm of the DNA complexes with total histone and histone fractions (except the DNA-histone *f1* complex) show minima at about 260 nm, and at higher wavelengths show maxima the positions of which vary with the kind of histone and the histone to DNA ratio. The minima were interpreted as pointing to stabilization of the double helical DNA, and maxima as corresponding to its destabilization.

The aim of the present work was to study the effect of histone acetylation on the structure and stability of histone-DNA complexes.

## MATERIALS AND METHODS

*Preparation of DNA.* Calf thymus DNA was isolated by the phenol method of Kirby in the modification of Maskos (1971). Denatured DNA was removed by hydroxyapatite column chromatography according to Bernardi (1969), hydroxyapatite being prepared as described by Tiselius *et al.* (1956). The obtained DNA preparation contained 0.36% of protein; its $E_{258}/E_{230}$ ratio was 2.20, and $E_{258}/E_{280}$, 1.75. After thermal denaturation it showed 35% hyperchromicity.

*Preparation of histones.* Histones were obtained in the same way as in the previous work (Szopa, 1973), fractionated according to method II of Johns (1964)

* This work was supported in part by the Committee of Biochemistry and Biophysics of the Polish Academy of Sciences.

and *f2a* was separated into *f2a1* and *f2a2* (Johns, 1967). The particular fractions were additionally purified on Bio-Gel P-30.

Acetylation of histones was carried out using acetic acid anhydride under the conditions described by Simpson (1971). To remove unstable acetyl groups, the preparations were incubated for 1 h at pH 11 and 25°C, then dialysed against 0.01 M-NaCl solution of pH 6.9 (Nohara *et al.*, 1968). The degree of acetylation was evaluated by free amino groups determination after Habeeb (1966); for fraction *f1* it was 20%, for *f2b* 58.5%, for *f2a1* 57.7%, for *f2a2* 60.5%, and for *f3* 54.5%.

The preparations of acetylated and non-acetylated histone fractions on polyacrylamide-gel electrophoresis (Panyim & Chalkley, 1969) were homogeneous. The histones after acetylation gave somewhat broader, less intensely stained and slower migrating single bands.

*Preparation of DNA-histone complexes.* To a solution of DNA in 0.01 M-NaCl, pH 6.9 (25 μg/ml) was added an equal volume of the histone solution of such a concentration as to obtain the required histone to DNA ratio. Each sample contained 12.5 μg of DNA/ml. The samples were filtered through a sintered glass filter G-4. The extinction at 340 and 400 nm of the nucleohistone solution did not exceed that of free DNA by more than 0.005.

*Difference ultraviolet spectra* were determined in 2 cm light-path vessels in a Unicam SP-800 spectrophotometer with an SP-825 photomultiplier.

*Infrared spectra* of the histones in solid state (1 mg of protein and 150 mg of KBr) were obtained in a Unicam SP-200 spectrophotometer, and as solution in $D_2O$ (Instytut Chemii Organicznej PAN, Warszawa) in a UR20 (U.S.S.R.) spectrophotometer. The spectra were interpreted according to Susi *et al.* (1967) and Timasheff *et al.* (1967).

*Other methods.* Protein was determined by the method of Lowry *et al.* (1951), DNA according to Spirin (1958) and phosphorus after Fiske & Subbarow.

## RESULTS

*Ultraviolet difference spectra.* Difference spectra of complexes DNA-*f1* and DNA-ac.*f1* at protein to DNA ratios of 0.1 and 0.3, are presented in Fig. 1. The increase in the content of the non-modified histone in the complex gave a blue shift of the whole spectrum, the minimum being displaced from 272 to 264 nm. On the other hand, the increase in the content of acetylated histone had no effect on the character of the spectrum, and the minimum was observed at about 300 nm. This red shift indicates that less energy is required for excitation and that the DNA-ac.*f1* complex has a less stable configuration.

Since the increase in the amount of histone *f1* stabilizes the complex, it could be expected that lowering of the histone *f1* to DNA ratio would result in a red shift and the minimum would eventually appear at about 300 nm, i.e. at a wavelength characteristic for the DNA-ac.*f1* complexes. This was, in fact, observed at the histone *f1* to DNA ratio of 0.066, the spectrum being identical with that of DNA-ac.*f1* complexes (Fig. 2).

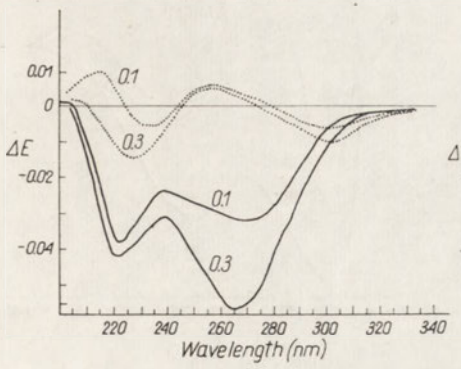


Fig. 1

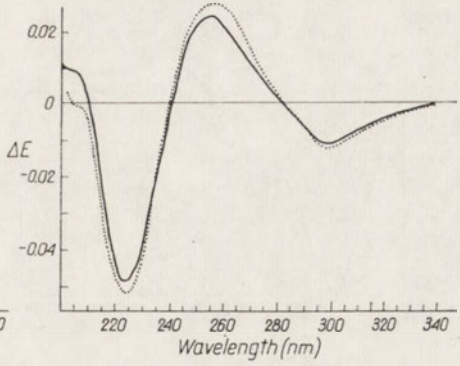


Fig. 2

Fig. 1. Ultraviolet difference spectra of DNA complexes with: —, non-acetylated histone *f1*, and ···, acetylated histone *f1*, at indicated histone to DNA ratios.

Fig. 2. Ultraviolet difference spectra of DNA complexes with: —, non-acetylated histone *f1*, and ···, acetylated histone *f1*, at histone to DNA ratio of 0.066.

The blue shift of DNA-*f1* complex observed at higher histone to DNA ratios may be explained by increased electrostatic interactions between basic amino acid residues and phosphate groups of DNA, enhancing the stability of the complex. The lack of such an effect of the amount of acetylated histone *f1* points, as suggested previously (Szopa, 1970, 1973), to the existence, in addition to electrostatic interactions, of another type of binding between histone *f1* and DNA.

The increase in the amount of non-acetylated histones *f2b* (Fig. 3a) and *f2a2* (Fig. 4a) in the complex gave a blue shift, similar to that observed with *f1* (Fig. 1). In contrast to DNA-*f1* complexes which exhibited only a minimum, the spectra of DNA-*f2b* and DNA-*f2a2* at histone to DNA ratios exceeding 0.1 gave maxima at longer wavelengths. In the case of DNA-*f2a1* complex (Fig. 5a) the increase in the amount of protein gave a red shift; in addition to a minimum, a maximum was also observed but, unlike that for the former two complexes, it appeared irrespective of the histone to DNA ratio.

The spectra of ac.*f2b*- and ac.*f2a2*-DNA complexes at increasing histone to DNA ratios showed a red shift (Figs. 3b and 4b). The shift was especially pronounced for the DNA-ac.*f2a2* complex: the maxima and minima were displaced toward longer wavelengths and the minimum at 300 nm disappeared completely at higher histone to DNA ratios.

Acetylation of *f2a1* resulted in irregular changes of the spectra of the complex (Fig. 5b). At 0.1 histone to DNA ratio, a red shift, and at 0.2, a blue shift, were observed. At the ratio of 0.3, the first maximum and the first minimum were slightly shifted toward shorter wavelengths, whereas the second maximum was displaced toward longer wavelengths, as compared with the spectra of DNA-*f2a1* complexes.

It seems possible to conclude that the increase in the content of the acetylated fractions *f2b*, *f2a2* and *f2a1* lowers the stability of their complexes with DNA; such an effect was not observed in the case of DNA-ac. *f1* complex.

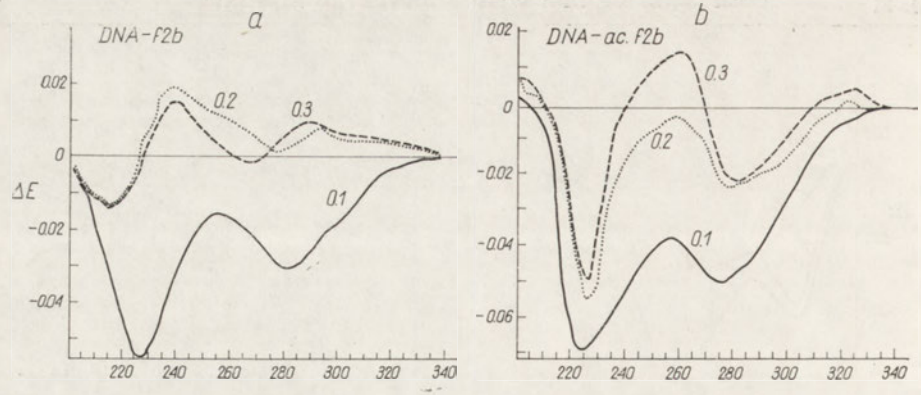


Fig. 3

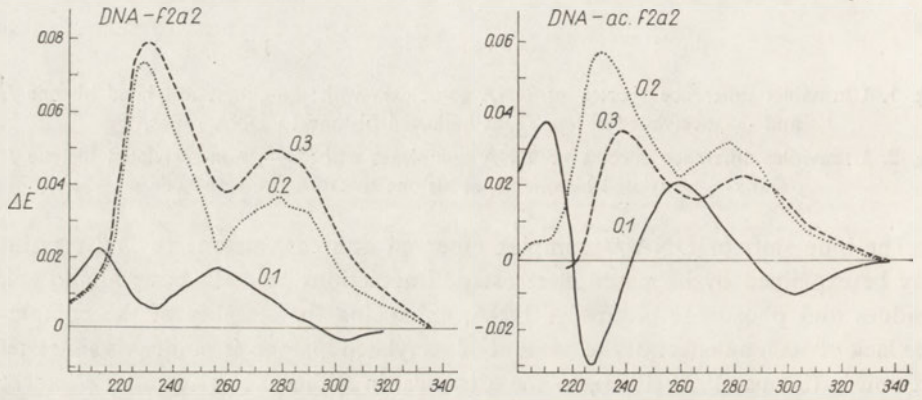


Fig. 4

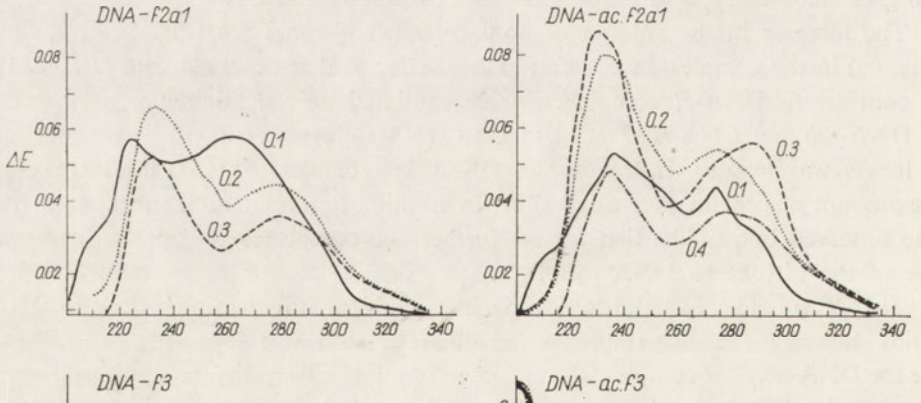


Fig. 5

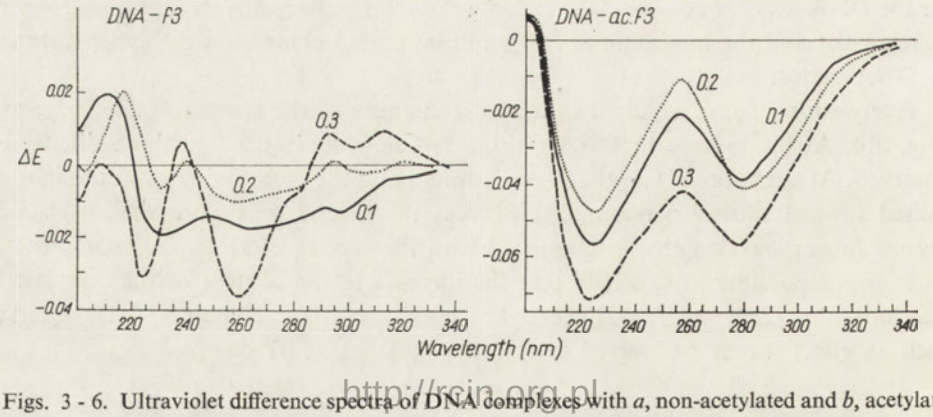


Fig. 6



Figs. 3 - 6. Ultraviolet difference spectra of DNA complexes with *a*, non-acetylated and *b*, acetylated histone fractions, at histone to DNA ratios indicated in the Figures.

The spectra of DNA-*f3* complexes are presented in Fig. 6a. At all histone to DNA ratios studied, a characteristic maximum appeared at about 294 nm, and a minimum at about 260 nm. The difference spectra of DNA-ac. *f3* complexes (Fig. 6b) show a considerable red shift as compared with those for DNA-*f3* complexes.

*Melting profiles.* The complexes of DNA with acetylated histones showing the red shift of the spectra should have a lower $T_m$ value. As expected, the melting temperatures for DNA-ac. *f3* complex were distinctly lower than the $T_m$ values for *DNA-f3* complex (Table 1). The $T_m$ values for DNA-ac. *f3* complex were, similarly as the u.v. spectra, independent of the histone to DNA ratio.

Table 1

*Effect of histone acetylation on the melting temperature of DNA-histone* f3 *complexes*

Each sample contained 12.5 μg of DNA/ml.

| Sample studied | Histone to DNA ratio | $T_m$ (°C) | |
|---|---|---|---|
| | | I | II |
| Free DNA | | 68.5 | |
| DNA-*f3* | 0.1 | 76.0 | |
| | 0.2 | 81.6 | |
| | 0.3 | 76.0 | 86.5 |
| DNA-ac.*f3* | 0.1 | 72.0 | |
| | 0.2 | 72.4 | |
| | 0.3 | 72.6 | |

The above results show that the determination of u.v. difference spectra may be used for evaluation of stability of the complexes.

*Infrared spectra.* The spectra of acetylated and non-acetylated histone *f3* in solid state and in solution in $D_2O$, are presented in Figs. 7a and 7b, respectively. In the amide I band of the non-acetylated *f3* in solid state the absorption peak at 1640 $cm^{-1}$ is indicative of the presence of β-structure and random conformation, and the peak at 1655 $cm^{-1}$, of α-helix (Timasheff *et al.*, 1967). The greater absorption at 1655 $cm^{-1}$ points to predominance of α-helix over the other conformations. This is confirmed by the amide II band at about 1540 $cm^{-1}$. The amide I band of histone *f3* in $D_2O$ at about 1650 $cm^{-1}$ also shows a predominance of α-helix.

The acetylated histone *f3* in solid state has the amide I band at 1633 $cm^{-1}$, indicating the prevalence of β-structure. The amide II band separated into three absorption peaks, of which the most pronounced was that at 1535 $cm^{-1}$; this also points to the prevalence of β-structure, similarly as the amide I band at 1630 $cm^{-1}$ observed for the solution of acetylated histone *f3* in $D_2O$.

The infrared spectrum of DNA-ac. *f3* complex exhibited an absorption peak at 1651 $cm^{-1}$, whereas for DNA-*f3* complex , at 1635 $cm^{-1}$ (Fig. 7c); this indicates

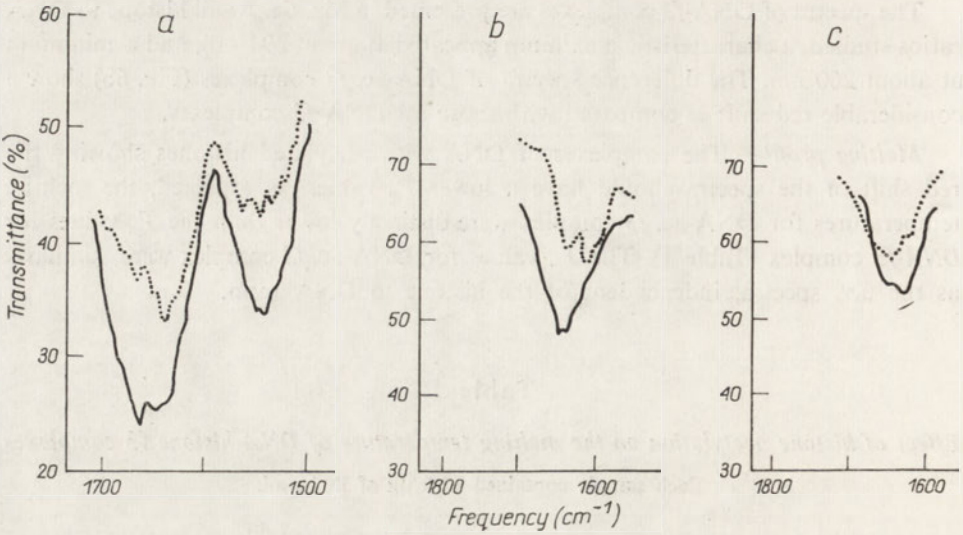


Fig. 7. Infrared spectrum of: ——, non-acetylated and · · ·, acetylated histone *f3*, (*a*), in solid state; (*b*), in $D_2O$ solution; (*c*), histone *f3* in complex with DNA in $D_2O$ solution, at histone to DNA ratio of 0.5.

that in the complex with DNA the acetylated histone *f3* is predominantly in *α*-helix structure, whereas the non-acetylated *f3*, mainly in *β*-structure. Thus, the acetylated histone *f3* in the complex has a more compact structure than the non-acetylated *f3*.

## DISCUSSION

The complexes of DNA with acetylated histones (except with histone *f2a1* at histone to DNA ratio higher than 0.1) are less stable than complexes with non-acetylated histones, as demonstrated by the red shift of the u.v. difference spectra and $T_m$ values. This lowered stability may be caused by decreased basicity of histones or by steric hindrance. The large acetyl residue may make difficult the interaction between the remaining free amino groups of histones and the phosphate groups of DNA. Moreover, the amide bonds formed on acetylation are involved in the intra- and inter-molecular secondary structure, causing a decreased reactivity of $-NH_2$ groups of basic amino acids.

As it appears from infrared spectra, histone *f3* after acetylation has an increased content of *β*-structure, which may be due to ordering of the random conformation or to transition $a \rightarrow \beta$ structure. On the other hand, in complex with DNA the acetylated histone *f3* is mainly in *α*-helix conformation.

From the behaviour of the u.v. and i.r. spectra as well as the $T_m$ values of the DNA-ac. *f3* complexes is may be concluded that the amide bond formed by acetylation of amino acid residues, is involved in formation of the ordered intra- and inter-molecular structure, as a result of which $-NH_2$ groups of amino acids are inaccessible to phosphate residues of DNA.

From the presented data it follows that complexes of DNA with acetylated histones have lower stability than the complexes with non-acetylated histones.

The author wishes to express his gratitude to Prof. Dr. Wanda Mejbaum-Katzenellenbogen for her interest in this work and helpful comments on the manuscript.

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## INTERAKCJA ACETYLOWANYCH I NIEACETYLOWANYCH HISTONÓW Z DNA

### Streszczenie

Z widm różnicowych w ultrafiolecie i oznaczania profilów topnienia wynika, że stabilność kompleksów z DNA frakcji histonów acetylowanych jest znacznie obniżona w porównaniu z kompleksami histonów nieacetylowanych.

Na podstawie widm w podczerwieni stwierdzono, że histony acetylowane w porównaniu do nieacetylowanych wykazują większe uporządkowanie.

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# EFFECT OF GROWTH SUBSTANCES ON RNA SYNTHESIS DURING MAIZE SEED GERMINATION*

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The rate of RNA synthesis in whole maize kernels treated with gibberellic acid ($GA_3$) and kinetin was approximately doubled after 24-h period of germination. This effect was, however, negligible in kernels germinated for shorter (12 h) or longer (48 h) periods. Stimulation of RNA synthesis was observed mainly in the embryo-less part of the seed. The isolated embryo responded to $GA_3$ treatment in very early stage, i.e. at 2 - 4 h of germination. 3-Indoleacetic acid had practically no stimulatory effect on RNA metabolism, except some enhancement of the synthesis of low-molecular--weight RNA after 24 h of germination.

It is well recognized that imbibition of water by mature plant seed triggers numerous biochemical processes, initiating its germination. Shortly afterwards differentiation starts as a result of sequential and specific transcription of the genome. It is generally believed that these processes are under precise hormonal control (Khan *et al.*, 1971). Therefore metabolic changes evoked by external application of the hormones, or their analogues, might to some extent reflect the natural control performed by these substances. The evidence accumulated during the last few years on the effect of phytohormones on RNA and protein metabolism in the whole plant or its parts was thoroughly reviewed (Trewavas, 1968; Key, 1969; Galston & Davies, 1969). Most of the authors (Chandra & Varner, 1965; Chandra & Duynstee, 1968; Jarvis *et al.*, 1968; Khan & Heit, 1969; Rejowski & Kulka, 1970; Barton *et al.*, 1971; Evins & Varner, 1971) were able to demonstrate only an overall enhancement of the synthesis of major RNA classes. In this respect the experiments of Zwar & Jacobsen (1972) with the barley aleurone layers were exceptional in proving the induction of a specific RNA fraction by $GA_3$.

* This work was supported by the Polish Academy of Sciences within the project 09.3.1 and in part by Grant No. FG-Po-262 from the United States Department of Agriculture, Agricultural Research Service.

The present work describes preliminary studies on the effect of gibberellic acid ($GA_3$), kinetin (K) and 3-indoleacetic acid (IAA) on the synthesis, during maize germination, of particular RNA species in whole kernels, isolated embryos and the embryo-less kernels.

## MATERIALS AND METHODS

*Chemicals.* Tris (hydroxymethylaminomethane), SDS (sodium dodecyl sulphate), EDTA (ethylenediaminetetraacetic acid, tetrasodium salt) and bentonite were obtained from Sigma (St. Louis, Mo., U.S.A.). Bentonite was purified by the method of Fraenkel-Conrat *et al.* (1961). *m*-Cresol was purchased from Schuchardt (München, G.F.R.). 2-Methoxyethanol and kinetin (6-furfurylaminopurine) were from Fluka A.G. (Buchs, Switzerland); IAA (3-indoleacetic acid) from Serva (Heidelberg, G.F.R.); CTAB (cetyl trimethyl ammonium bromide) from B.D.H. (Poole, Dorset, England); $GA_3$ (gibberellic acid) from Koch-Light (Colnbrook, Bucks., England); sucrose, special gradient grade, RNase free, from Schwarz-Mann (Orangeburg, N.Y., U.S.A.). [$^{32}$P]Orthophosphate (sodium salt) was purchased from the Institute of Nuclear Research (Świerk, Poland).

*Plant material.* Corn seeds: *Zea mays*, cultivar. Kb-260, Polish double-cross, were employed. For the experiment, 50 kernels (average weight 15 g), 100 embryos (av. wt. 8 g) or 15 g of embryo-less kernels, were used. All manipulations during imbibition and germination, as well as during incubation were performed under aseptic conditions. Whole kernels were sterilized by immersing for 15 min in 0.5% NaOCl, and the kernel parts, for 3 min in 0.25% NaOCl. After sterilization the samples were exhaustively rinsed with sterile water and transferred to Petri dishes containing 0.2 - 0.3 mCi of [$^{32}$P]orthophosphate (carrier-free) and plant hormones at concentrations of $10^{-5}$ - $10^{-7}$ M in the final volume of 7 ml. Petri dishes were placed in refrigerator at 2°C for 15 h imbibition. After this period the kernels were kept for 2 - 48 h at 22°C to germinate. At the end of the indicated germination time, the seeds were thoroughly washed with cold 0.1 M-Na-phosphate buffer, pH 7.0, and finally with water.

*Isolation of total RNA.* Total RNA was extracted using the phenol method of Kirby (1965) as modified by Cherry & Chroboczek (1966). For comparison, the methods of Brawerman *et al.* (1972) and of Ralph & Bellamy (1964) were used. The isolated RNA was centrifuged in continuous 5 - 20% buffered sucrose gradient at 4°C in 6 × 15 ml swing-out rotor of the MSE-65 ultracentrifuge at 24 500 rev/min for 16 h. The 20-drop fractions (about 0.45 ml) were collected and assayed spectrophotometrically for RNA content assuming that one $E_{260}$ unit equals 50 μg of RNA/ml. Radioactivity was measured using Tricarb Packard Liquid Scintillation Counter. Sedimentation values for two ribosomal RNA's were arbitrarily designated as 28S and 18S as referred to *E. coli* 23S and 16S rRNA. The third broad fraction consisted of RNA sedimenting below 13S and included well defined peak of tRNA.

## RESULTS AND DISCUSSION

During imbibition of aqueous solutions of nucleic acids and proteins by dry seeds at low temperature, no apparent initiation of germination could be observed (Marcus *et al.*, 1966; Rejman & Buchowicz, 1971). In our experiments, either, no incorporation of $^{32}P$ into RNA during 15-h imbibition at 2°C was detected.

On studying the effect of phytohormones on maize seed germination, the optimal concentration of $GA_3$, K and IAA was found to be $10^{-6}$M. Whole seeds were germinated at 22°C for 12, 24, or 48 h. After 12 h germination, $^{32}P$ was incorporated to a very small extent into the 28S and 18S rRNA fractions (about 50 - 80 c.p.m./$E_{260\,nm}$ unit), and to a higher degree into the <13S RNA fraction (500 - 650 c.p.m./$E_{260\,nm}$). The phytohormones applied had no effect on this incorporation. After 24 h of germination, as compared with 12 h, a remarkable stimulatory effect of $GA_3$ and K on RNA synthesis could be seen. In the presence of hormones, about twice as much of $^{32}P$ was incorporated both into two rRNA fractions and into the low-molecular-weight RNA fraction, in comparison with the control sample. IAA had no effect on rRNA synthesis, however, it stimulated significantly labelling of the <13S RNA fraction. Extension of the germination time up to 48 h resulted in a further several-fold increase in $^{32}P$ incorporation into all RNA fractions, but stimulation by phytohormones was negligible and limited to 28S rRNA only. Figure 1 represents the typical sucrose gradient sedimentation profiles of the RNA extracted from intact kernels germinated for 24 h in the presence or absence of phytohormones. The time-dependent response of germinating seeds to hormones is illustrated in Table 1.

Table 1

*Effect of growth substances on $^{32}P$ incorporation into RNA fractions during germination of maize kernels*

Phytohormones were applied in concentration of $10^{-6}$M. The results are expressed in c.p.m./$E_{260nm}$ unit

| Treatment | 28S rRNA | | | 18S rRNA | | | <13S RNA | | |
|---|---|---|---|---|---|---|---|---|---|
| | Germination (h) | | | | | | | | |
| | 12 | 24 | 48 | 12 | 24 | 48 | 12 | 24 | 48 |
| Control | 60 | 210 | 800 | 70 | 180 | 1800 | 650 | 640 | 3300 |
| $GA_3$ | 50 | 380 | 1020 | 55 | 380 | 1800 | 650 | 890 | 3300 |
| K | 50 | 400 | 1300 | 55 | 360 | 1750 | 500 | 1100 | 3500 |
| IAA | 60 | 200 | 700 | 80 | 200 | 1900 | 500 | 1200 | 2700 |

The lack of phytohormone effect after a longer germination period (48 h) might be due to the sufficient amount of growth substances synthesized endogenously by that time. On the other hand, the lack of response to the exogenous growth substances after 12 h of germination might have been caused by the insufficient —

at this time — synthesis of specific mediator-proteins, the presence of which is necessary for exertion of the effect of phytohormones (Matthysse & Philips, 1969; Matthysse & Abrams, 1970).

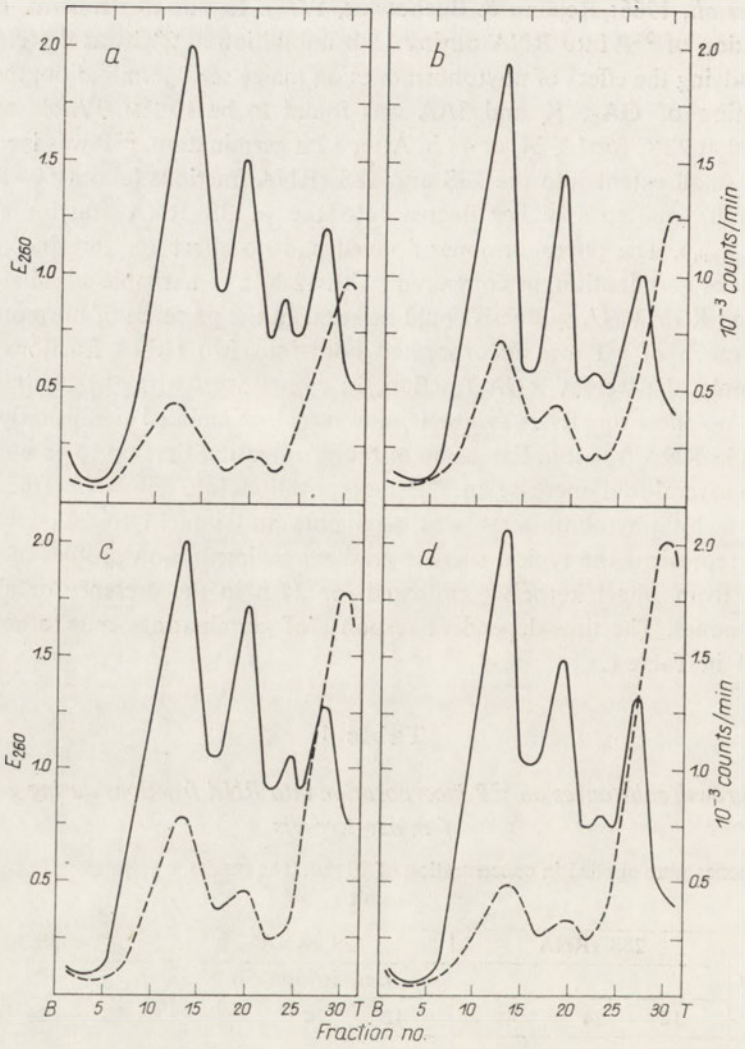


Fig. 1. Sucrose density gradient sedimentation pattern of total RNA extracted from maize kernels: control (*a*); and treated with $GA_3$ (*b*), K (*c*) and IAA (*d*). The seeds were germinated for 24 h at 22°C. $E_{260\ nm}$ (——); radioactivity (— — —); *T*, top; *B*, bottom.

According to our present understanding of the mode of rRNA synthesis, the rRNA fractions should be labelled in equimolar quantities. Such a labelling was observed, with but a slight deviation, after 12 and 24 h of germination. Therefore it is difficult to explain the disparity observed in the rate of labelling of 28S and 18S rRNA's in the seeds germinated for 48 h (see Table 1). A similar disparity in the

labelling of 26S and 17S rRNA's, and significant favouring of 17S rRNA labelling, was observed also by Van den Bos *et al.* (1971) upon a short u.v. irradiation of yeast cells. On the other hand, Cooper (1970) suggested that in resting lymphocytes approximately a half of the newly synthesized 18S rRNA molecules were selectively and rapidly degraded, probably without ever entering the cytoplasm.

In the next series of experiments, the embryo-less kernels and the isolated embryos were separately preimbibed with an aqueous solution of [$^{32}$P]orthophosphate and the phytohormone, and then incubated for 24 h at 22°C. The embryo-less seeds appeared to be very sensitive to the $GA_3$ and K treatment. As can be seen in Fig. 2,

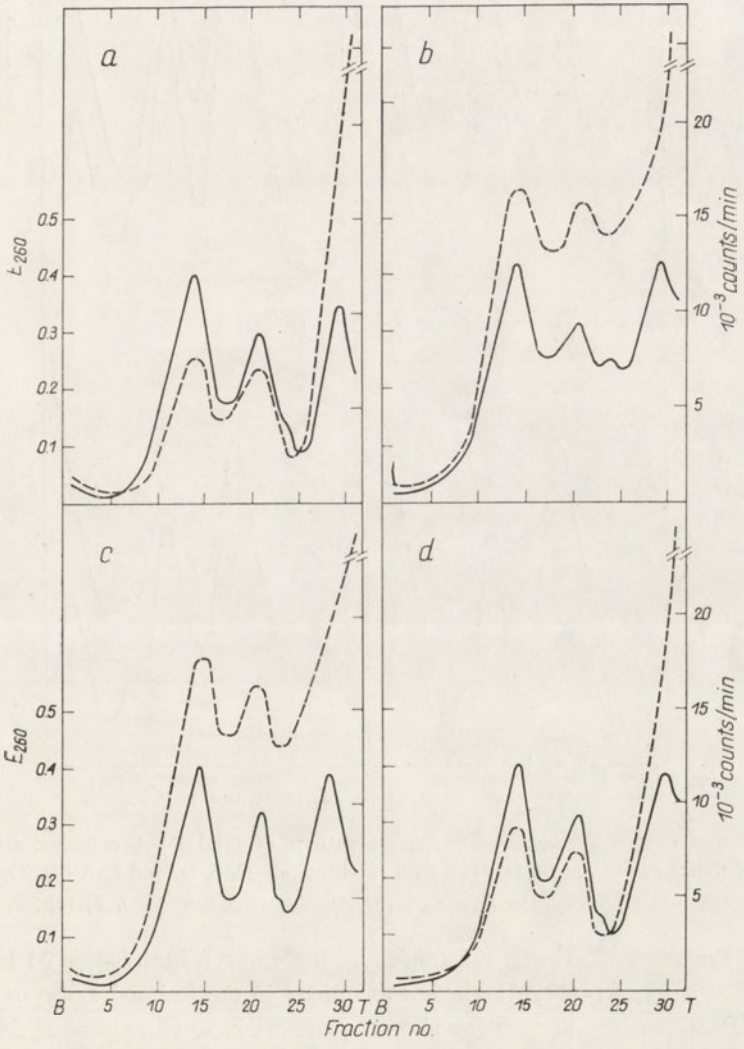


Fig. 2. Sucrose density gradient sedimentation pattern of total RNA extracted from embryo-less maize seeds: control (*a*); and treated with $GA_3$ (*b*), K (*c*) and IAA (*d*). The seeds were germinated for 24 h at 22°C. $E_{260\ nm}$ (—); radioactivity (— — —); *T*, top; *B*, bottom.

$GA_3$ and K stimulated about twofold (by 190% and 240%, respectively) the synthesis of both rRNA's. The isolated embryos did not show any significant response to phytohormones after 24 h of germination (Fig. 3). This might have been due to the

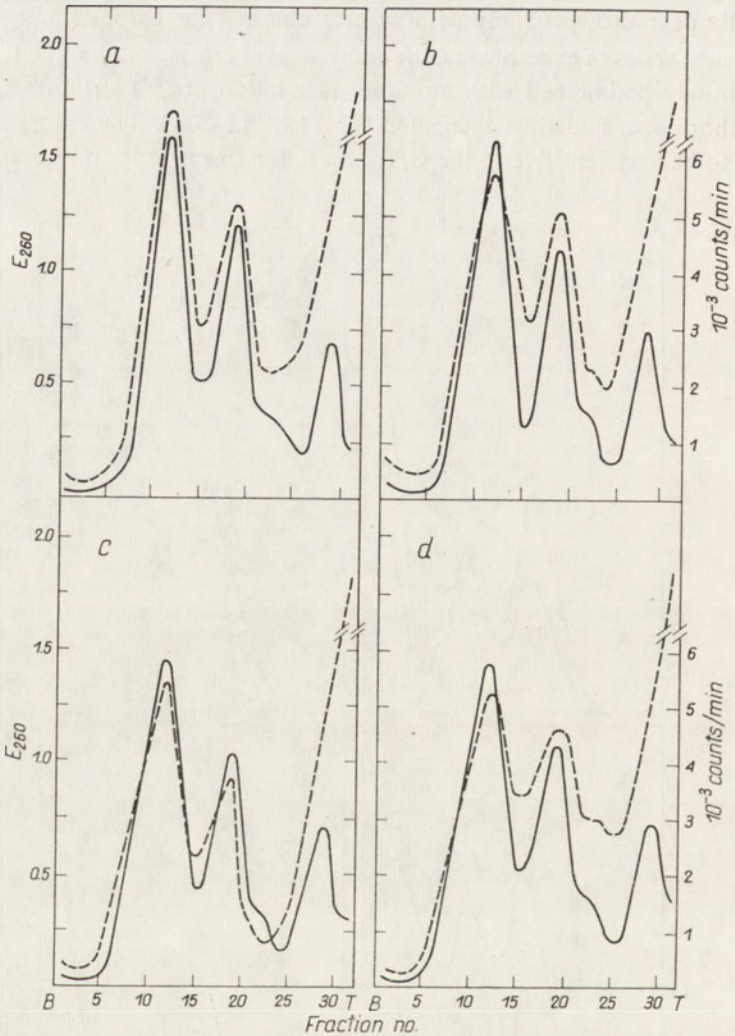


Fig. 3. Sucrose density gradient sedimentation pattern of total RNA extracted from isolated embryos of maize seeds: control (*a*); and treated with $GA_3$ (*b*), K (*c*) and IAA (*d*). The seeds were germinated for 24 h at 22°C. $E_{260\ nm}$ (—); radioactivity (— — —), *T*, top; *B*, bottom.

sufficient synthesis of growth substances by the embryo itself, after 24 h of germination. Therefore, RNA synthesis was examined in the isolated embryos incubated in the $^{32}P$-containing solution for shorter periods: 2, 4, 12 as well as 24 h, in the presence or absence of $GA_3$. The results presented in Table 2 support our suggestion and prove that stimulation of RNA synthesis by exogenous $GA_3$ takes place at the very early stages of germination.

Our data on the effect of phytohormones on the embryo-less part of the seed and the detached embryos are in agreement with the supposition (Chandra & Varner, 1965; Khan & Heit, 1969; Khan *et al.*, 1971) that the aleurone layer is the main target for phytohormones produced by the embryo.

Table 2

*Effect of gibberellic acid on the total RNA synthesis in isolated maize embryos*

Specific activities are expressed in relative values.

| Treatment | Germination (h) | | | |
|---|---|---|---|---|
| | 2 | 4 | 12 | 24 |
| Control ($H_2O$) | 100 | 100 | 100 | 100 |
| $GA_3$ ($10^{-6}$ M) | 120 | 140 | 98 | 103 |

It is of considerable interest that neither $GA_3$ nor K produced any qualitative changes in the RNA population. It seems that only IAA stimulated specifically the synthesis of the polydisperse low-molecular-weight RNA fraction (see Table 1). The values representing specific activity of this fraction are, however, elevated because of considerable contamination with $^{32}P$-labelled phosphate esters. The removal of these contaminants according to Ralph & Bellamy (1964) resulted in a significant diminution of the specific activity of the low-molecular-weight RNA fraction, but the relative values for the phytohormone-treated samples remained unchanged when compared with control.

The search for some RNA fraction specifically stimulated by $GA_3$ or K, undertaken with the procedure of Brawerman *et al.* (1972) was unsuccessful.

It is extremely difficult to demonstrate an induction of any particular class of heterogeneous RNA. Zwar & Jacobsen (1972), however, were able to demonstrate in barley aleurone layers, by the double-labelling technique, the synthesis of a specific, „$GA_3$-RNA" fraction despite the fact that its radioactivity was about 50 times lower than that of rRNA.

Further decisive experiments should be done to establish whether stimulation of RNA synthesis under phytohormone treatment represents an overall enhancement of the synthesis of the major RNA classes, or is restricted to a specific RNA fraction.

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## WPŁYW SUBSTANCJI WZROSTOWYCH NA SYNTEZĘ RNA PODCZAS KIEŁKOWANIA NASION KUKURYDZY

### Streszczenie

Wykazano około dwukrotną stymulację syntezy RNA pod wpływem kwasu giberelinowego i kinetyny w całym ziarnie kukurydzy po 24 godz. kiełkowania. Wpływ tych fitohormonów na syntezę RNA podczas krótszego (12 godz.) lub dłuższego (48 godz.) okresu kiełkowania był nieznaczny. Stwierdzono znaczne, ponad dwukrotne, zwiększenie syntezy RNA w izolowanych bezzarodkowych częściach ziarna (bielmo). Natomiast w izolowanych zarodkach wpływ fitohormonów był widoczny jedynie w bardzo wczesnym okresie kiełkowania (2 - 4 godz.). Kwas indolilooctowy praktycznie nie miał wpływu na metabolizm RNA w całym ziarnie lub jego częściach, z wyjątkiem niewielkiej stymulacji syntezy niskocząsteczkowej frakcji RNA.

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# PREPARATIVE SEPARATION OF SYNTHETIC OLIGONUCLEOTIDES BY THIN-LAYER CHROMATOGRAPHY ON DEAE-CELLULOSE*

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The use of thin-layer chromatography on DEAE-cellulose improved the separation of di- and trinucleoside phosphates: 2′-*O*-acetyl- and 2′-*O*-benzoyl-uridylyl(3′→5′)$N^4$,2′,3′-tribenzoylcytidine, and *N*,2′,5′-triacetyladenylyl((3′→5′)2′-*O*-acetyluridylyl (3′→5′)$N^4$,2′,3′-tribenzoylcytidine.

The yield of oligonucleotides obtained by the generally used procedures is often quite unsatisfactory because of formation of undesired side products and poor separation of the desired compounds from the reaction mixture. In separation of oligonucleotides, column chromatography on DEAE-cellulose is widely used (Lohrmann *et al.*, 1966; Griffin & Reese, 1969; Holy, 1970); however, the effectiveness of this procedure is rather poor, and the extent of intramolecular reactions leading to the side products is much higher when nucleoside 3′-phosphates rather than nucleoside 5′-phosphates are used as starting materials (Söll & Khorana, 1965).

The syntheses of protected dinucleoside phosphates $HOUOAcpC^{Bz}(OBz)_2$[1] and $HOUOBzpC^{Bz}(OBz)_2$ were performed after Lohrmann *et al.* (1966). The obtained dinucleotide $HOUOAcpC^{Bz}(OBz)_2$ was used as the substrate for further condensation with *N*,2′,5′-triacetyladenosine-3′-phosphate to yield trinucleotide $AcOA^{Ac}OAcpUOAcpC^{Bz}(OBz)_2$. The use of thin-layer chromatography with DEAE-cellulose improved substantially the yield of both di- and trinucleoside phosphates.

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[1] The following abbreviations are employed in this text: $HOUOAcpC^{Bz}(OBz)_2$ for 2′-*O*-acetyl-uridylyl(3′→5′)$N^4$,2′,3′-tribenzoylcytidine; $HOUOBzpC^{Bz}(OBz)_2$, for 2′-*O*-benzoyluridylyl(3′→5′)$N^4$,2′,3′-tribenzoylcytidine; $AcOA^{Ac}OAcpUOAcpC^{Bz}(OBz)_2$ for *N*,2′,5′-triacetyladenylyl(3′→5′)2′-*O*-acetyluridylyl(3′→5′)$N^4$,2′,3′-tribenzoylcytidine; U for uracil; $C^{Bz}$ for $N^4$-benzoylcytosine; DCC for dicyclohexylcarbodiimide; Bz for benzoyl; MMT for monomethoxytrityl.

## RESULTS AND DISCUSSION

The use of preparative thin-layer chromatography on DEAE-cellulose instead of column chromatography improved the yield of $HOUOAcpC^{Bz}(OBz)_2$ up to 60% as compared with 33% reported by Lohrmann *et al.* (1966). The properties of the obtained pure product on paper chromatography, paper electrophoresis, u.v. absorption spectra and enzymic degradation corresponded to the data reported for this compound.

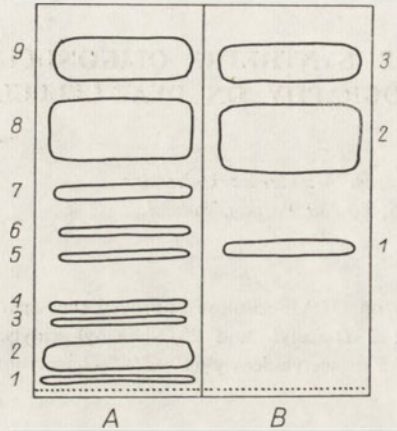


Fig. 1

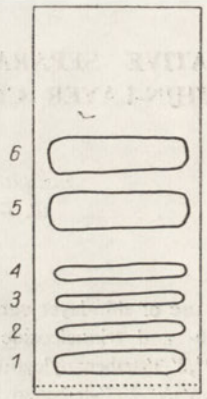


Fig. 2

Fig, 1. Thin-layer chromatography on DEAE-cellulose of the products formed on condensation of $HOC^{Bz}(OBz)_2$ with: *A*, 5′-MMT-U2′-*O*-Acp, and *B*, 5′-MMT-U2′-*O*-Bzp; solvent system *C*. For details see Experimental. Identification of spots: *A*, *1*, uridine 3′-phosphate and uridine 3′,5′-cyclic phosphate; *2*, 2′-*O*-acetyluridine 3′-phosphate and $P^1P^2$(2′-*O*-acetyluridine 3′)-pyrophosphate; *3*, *4*, *5*, *6*, unidentified products; *7*, *N*-(uridylyl)*N*,*N*′-dicyclohexylurea; *8*, 2′-*O*-acetyluridylyl(3′→5′)$N^4$,2′,3′-tribenzoylcytidine; *9*, $N^4$,2′,3′-tribenzoylcytidine. *B*, *1*, 2′-*O*-benzoyluridine 3′--phosphate; *2*, 2′-*O*-benzoyluridylyl(3′→5′)$N^4$,2′,3′-tribenzoylcytidine; *3*, $N^4$,2′,3′-tribenzoylcytidine.

Fig. 2. Thin-layer chromatography on DEAE-cellulose of the products formed on condensation of $HOUOAcpC^{Bz}(OBz)_2$ with $AcOA^{Ac}OAcp$; solvent system *C*. For details see Experimental. *1* and *2*, unidentified products; *3*, *N*,2′,5′-triacetyladenosine 3′-phosphate and 2′,5′-diacetyladenosine 3′-phosphate; *4*, *N*(adenylyl)*N*,*N*′-dicyclohexylurea; *5*, *N*,2′,5′-triacetyladenylyl(3′→5′)2′-*O*--acetyluridylyl(3′→5′)$N^4$,2′,3′-tribenzoylcytidine; *6*, 2′-*O*-acetyluridylyl(3′→5′)$N^4$,2′,3′-dibenzoylcytidine.

The thin-layer chromatography of the crude reaction mixture (Fig. 1A) revealed the presence of 6 additional minor components. Four of them were identified on the basis of $R_F$ values and electrophoretic mobilities as uridine 3′-phosphate and uridine 3′,5′-cyclic phosphate (spot *1*), *N*-(uridylyl)*N*,*N*′-dicyclohexylurea (spot *7*) and $P^1P^2$(2′-*O*-acetyluridine 3′)-pyrophosphate (spot *2*). A comparison of the obtained values with those reported by other authors is given in Table 1.

On hydrolysis with methanolic ammonium hydroxide all these minor side products yielded only uridine 3′-phosphate and uridine 2′,3′-cyclic phosphate; *N*-(uridylyl)*N*, *N*′-dicyclohexylurea did not hydrolyse completely under these conditions, which is in agreement with the results of Dekker & Khorana (1954) and Lapidot & Khorana (1963).

Examination of the products isolated by thin-layer chromatography showed that spots *1* and *2* on Fig. 1A consisted in fact each of two compounds, which were well separated by paper chromatography and electrophoresis. These limitations should be taken into account in application of t.l.c. for analytical and preparative purposes.

Table 1

$R_F$ *values and electrophoretic mobility of the reaction products of* $N^4$,2′,3′-*tribenzoylcytidine and* 5′-*monomethoxytrityl*-2′-O-*acetyluridine* 3′-*phosphate*

The electrophoretic mobilities were measured at pH 7.1 in relation to that for uridine 3′-phosphate taken as 1.00.

| Compound | $R_F$ | | | | Electrophoretic mobility | |
|---|---|---|---|---|---|---|
| | solvent *A* | | solvent *B* | | | |
| | found | lit. | found | lit. | found | lit. |
| Uridine 3′-phosphate | 0.08 | 0.09[a] | 0.27 | 0.30[a] | 1.00 | 1.00 |
| Uridine 3′,5′-cyclic phosphate | 0.32 | 0.26[a] | 0.52 | 0.58[a] | 0.68 | 0.63[a] |
| 2′-*O*-Acetyluridine 3′-phosphate | — | — | 0.45 | 0.46[a] | 0.84 | 0.87[a] |
| $P^1P^2$(2′-*O*-Acetyluridine 3′)-pyrophosphate | — | — | 0.72 | 0.78[b] | — | — |
| *N*-(Uridylyl)*N*,*N*′-dicyclohexylurea | 0.87 | 0.90[a] | 0.78 | 0.80[a] | 0.36 | 0.42[a] |
| Uridylyl(3′→5′)cytidine (after removal of protecting groups) | 0.17 | 0.18[c] | 0.38 | — | 0.38 | 0.35[d] |

[a] Söll & Khorana (1965).
[b] Lapidot & Khorana (1963).
[c] Holy (1970).
[d] Lohrmann *et al.* (1966).

In the synthesis of $HOUOBzpC^{Bz}(OBz)_2$ no side products were formed, even uridine 3′,5′-cyclic phosphate was not detected. $HOUOBzpC^{Bz}(OBz)_2$ was obtained in about 70% yield. The effect of 2′-*O*-protecting group in 5′-*O*-monomethoxytrityl uridine 3′-phosphate on formation of the side products deserves separate treatment.

Separation by t.l.c. of the reaction products of $HOUOAcpC^{Bz}(OBz)_2$ with $AcOA^{Ac}OAcp$ is presented in Fig. 2. The yield of $AcOA^{Ac}OAcpUOAcpC^{Bz}(OBz)_2$ was 75%. On examination of the isolated products by paper chromatography spot *3* was found to consist of two compounds.

Hydrolysis of spots *1 - 4* with methanolic ammonium hydroxide yielded adenosine 3′-phosphate and adenosine 2′,3′-cyclic phosphate with traces of unreacted *N*-(adenyl)*N*,*N*′-dicyclohexylurea (Dekker & Khorana, 1954). The $R_F$ values and electrophoretic mobilities of the side products formed in the synthesis of the trinucleoside, are given in Table 2.

## Table 2

$R_F$ *values and electrophoretic mobility of the reaction products of* 2′-O-*acetyluridylyl* (3′→5′)*N*⁴,2′,3′-*tribenzoylcytidine and N*,2′,5′-*triacetyladenosine* 3′-*phosphate*

The electrophoretic mobilities were measured at pH 7.1 in relation to that for adenosine 3′-phosphate, except for the trinucleotide.

| Compound | $R_F$ | | | | Electrophoretic mobility | |
|---|---|---|---|---|---|---|
| | solvent *A* | | solvent *B* | | | |
| | found | lit. | found | lit. | found | lit. |
| Adenosine 3′-phosphate | 0.12 | 0.10[a] | 0.18 | 0.21[a] | 1.00 | 1.00 |
| 2′,5′-Diacetyladenosine 3′--phosphate | — | — | 0.47 | 0.49[b] | — | — |
| *N*,2′,5′-Triacetyladenosine 3′--phosphate | — | — | 0.62 | 0.59[b] | 0.66 | 0.68[a] |
| *N*-(Adenylyl)*N*,*N*′-dicyclohexylurea | 0.83 | 0.80[a] | 0.87 | 0.85[a] | 0.20 | 0.28[a] |
| Adenylyl(3′→5′)uridylyl(3′→5′)cytidine (after removal of protecting groups)[d] | 0.62 | — | 0.54 | 0.56[c] | 0.56 | 0.57[c] |

[a] Söll & Khorana (1965).
[b] Lapidot & Khorana (1963).
[c] Lohrmann *et al.* (1966).
[d] $R_F$ values and electrophoretic mobility in relation to those for uridine 3′-phosphate taken as 1.00.

The presented results proved that t.l.c. may be successfully applied for isolation of the synthetic oligonucleotides.

## EXPERIMENTAL

*Preparation of protected nucleosides and nucleotides.* 5′-MMT-U2′-*O*-Acp and AcOA$^{Ac}$OAcp were prepared after Lapidot & Khorana (1963), 5′-MMT-U2′-*O*-Bzp according to Lohrmann *et al.* (1966), and HOC$^{Bz}$(OBz)$_2$ after Lohrmann & Khorana (1964).

In the preparation procedure, evaporations were carried out at a bath temperature not exceeding 35°C.

*Synthesis of* 2′-O-*acetyluridylyl*(3′→5′)N⁴,2′,3′-*tribenzoylcytidine*, 2′-O-*benzoyluridylyl*(3′→5′)N⁴,2′,3′-*tribenzoylcytidine and* N,2′,5′-*triacetyladenylyl*(3′→5′) 2′-O-

*-acetyluridylyl*(3′→5′)N$^4$,2′,3′-*tribenzoylcytidine*. The synthesis of HOUOAcpC$^{Bz}$(OBz)$_2$ from 5′-MMT-U2′-*O*-Acp (105 mg, 0.16 mmol) and $N^4$,2′,3′-tribenzoylcytidine (180 mg, 0.32 mmol); of HOUOBzpC$^{Bz}$(OBz)$_2$ from 5′-MMT-U2′-*O*-Bzp (100 mg, 0.13 mmol) and $N^4$,2′,3′-tribenzoylcytidine (140 mg, 0.28 mmol); and of AcOA$^{Ac}$OAcpUOAcpC$^{Bz}$(OBz)$_2$ from HOUOAcpC$^{Bz}$(OBz)$_2$ (80 mg, 0.08 mmol) and AcOA$^{Ac}$OAcp (144 mg, 0.28 mmol) was performed according to Lohrmann *et al.* (1966). All these compounds were pyridine-dried and were kept with 400 - 600 mg of DCC (Fluka AG, Buchs, Switzerland) in anhydrous pyridine (about 3 ml) at room temperature in the dark for 5 days.

*Separation on thin layers of DEAE-cellulose*. DEAE-TLC-cellulose (Serva, Heidelberg, G.F.R.), 15 g, was homogenized with 75 ml of water, applied to a glass plate (30×40 cm) and dried for 24 h at room temperature. The crude reaction mixtures were dissolved in methanol with a drop of pyridine, applied to plates of DEAE-cellulose and developed in 30% acetic acid - butan-1-ol (1 : 2, v/v) in a cold-room at an ambient temperature of 5°C. The bands corresponding to the synthesized compounds were eluted with pyridine, the solvent was removed under vacuum, and the residue repeatedly evaporated with dry pyridine. The final residue was dissolved in a small volume of dry pyridine and added dropwise to vigorously stirred ether. The precipitate was collected by centrifugation, washed with ether and dried over $P_2O_5$ in vacuum.

*Removal of the protecting groups*. The eluates of di- and trinucleotides from chromatographic plates were dried, the residue was dissolved in methanolic ammonium hydroxide (3 g of $NH_3$ in 10 ml of MeOH) and the mixture was kept at 0°C for 24 h. The solvent was evaporated, the residue dissolved in methanol and the products were identified as pure unblocked di- and trinucleotides by paper chromatography on Whatman no. 1 paper in solvent systems *A* and *B*, by thin-layer chromatography on DEAE-TLC-cellulose plates (10×20 cm) in solvent *C*, and by paper electrophoresis.

*Enzymic degradation*. After removal of the protecting group, the spot corresponding to uridylyl(3′→5′)cytidine was eluted with 0.05 M-Tris-HCl buffer, pH 6.9; 0.05 ml of pancreatic ribonuclease solution (5 mg of enzyme in 5 ml of buffer) was added and the mixture was incubated at 37°C for 1 h. Chromatography on Whatman no. 1 paper in solvent systems *A* and *B* showed complete degradation yielding uridine 3′-phosphate and cytidine. Quantitative analysis of these compounds was carried out by spectrophotometry after elution.

*Paper chromatography and electrophoresis*. Paper chromatography was performed on Whatman no. 1 paper using the ascending technique and the following solvent systems: *A*, propan-2-ol - conc. ammonia - water (7 : 1 : 2, by vol.); *B*, ethanol - 1 M-ammonium acetate, pH 7.5 (7 : 3, v/v); *C*, 30% acetic acid - butan-1-ol (1 : 2, v/v).

Paper electrophoresis was performed on Whatman no. 3 *MM* in 0.03 M-phosphate buffer, pH 7.1, at 2500 V using light petroleum (b.p. 50 - 60°C).

The separated nucleotides were detected under an u.v. lamp. In enzymic degradation of the synthetic products, pancreatic ribonuclease (Koch-Light, Colnbrook, Bucks, England) was used.

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## PREPARATYWNY ROZDZIAŁ SYNTETYCZNYCH OLIGONUKLEOTYDÓW NA DRODZE CHROMATOGRAFII CIENKOWARSTWOWEJ NA DEAE-CELULOZIE

Streszczenie

Zastosowanie chromatografii cienkowarstwowej na DEAE-celulozie do rozdziału dwu- i trójnukleozydofosforanów: 2′-*O*-acetylo- i 2′-*O*-benzoilourydylilo(3′→5′)$N^4$,2′,3′-trójbenzoilocytydyny oraz *N*,2′,5′-trójacetyloadenozylo(3′→5′)2′-*O*-acetylourydylilo(3′→5′)$N^4$,2′,3′-trójbenzoilocytydyny, w poważnym stopniu polepszyło wydajności produktów reakcji.

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# STUDIES ON HYDROGEN-DEUTERIUM EXCHANGE IN BASIC TRYPSIN INHIBITOR

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The H-D exchange in trypsin inhibitor was measured in the temperature range 20 - 75°C at pD 2.6, and the activation energy and the values of enthalpy and entropy of activation were calculated. The obtained thermodynamic parameters suggest the existence of two independent H-D exchange mechanisms in this protein, dominating at the temperature ranges of 20 - 60°C and 60 - 75°C, respectively. The results obtained in exchange experiments were compared with those of NMR spectroscopy.

Hydrogen isotope exchange between protein and water permits to obtain some information concerning the dynamic state of macromolecules in aqueous solution. On the basis of H-D exchange in α-chymotrypsin in the temperature range 20 - 40°C and the calculated activation parameters we have suggested (Kania & Siemion, 1973) that the rate of exchange of peptide protons in the α-chymotrypsin molecule is defined mainly by local conformational motions. In the present paper, the H-D exchange was studied over a broader temperature range (20 - 75°C) in the basic trypsin inhibitor, which shows an exceptional thermostability in this temperature range. The obtained thermodynamic parameters suggest the existence of two independent H-D exchange mechanisms in the investigated protein, one dominating between 20 and 60°C, and the other in the range of 60 - 75°C.

## MATERIALS AND METHODS

*Reagents.* The basic trypsin inhibitor was used in the form of hydrochloride; it was a gift from Zakłady Farmaceutyczne "Polfa" (Jelenia Góra, Poland). The preparation was pure as checked by zone electrophoresis on cellulose polyacetate

strips (Gelman Instrument Co., Ann Arbor, Mich., U.S.A.) and by stoichiometry of inhibition with trypsin. $D_2O$ was of 98.3% purity (Reachim, Moscow, U.S.S.R.).

*Infrared spectroscopy*. H-D exchange between peptide NH-groups and $D_2O$ was studied by the infrared spectroscopic method of Blout *et al.* (1961) and Hvidt & Nielsen (1966) as described previously (Kania & Siemion, 1973). The sample containing a 5% solution of the basic trypsin inhibitor hydrochloride in $D_2O$, and the reference cell containing $D_2O$ were inserted into thermostated cuvette holders and the spectra recorded over the temperature range of 20 - 75°C at intervals of 10 or 5°C. For each temperature 3 - 5 independent measurements were made and the mean value of rate constants of exchange calculated. The infrared spectra obtained at 70°C at different time intervals after solubilization of the inhibitor, are shown in Fig. 1.

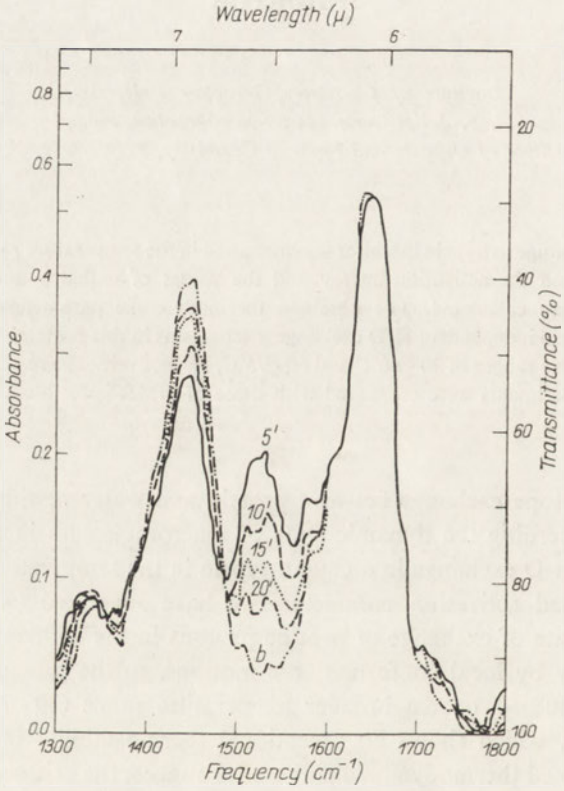


Fig. 1. Repeated scans of the infrared spectra of 5% basic trypsin inhibitor solution in $D_2O$ at pD 2.6 and temp. 70°C, started at the indicated time after dissolution. *b*, Background spectrum for exchange of basic trypsin inhibitor obtained by incubation for 2 hours at 75°C in $D_2O$.

The amide I band, mainly due to carbonyl stretching motion (Miyazawa *et al.*, 1958), has its maximum near 1645 $cm^{-1}$. Background absorption in the region of the amide I band was assumed to be insignificant, as judged from the negligible absorption just outside the region of about 1770 $cm^{-1}$. For the 5% inhibitor solution

at pD 2.6, the absorption of amide I band was 0.52. Considering an average molecular weight of an amino acid residue of 112 (Huber *et al.*, 1970), the apparent molar extinction coefficient $K_{C=O}$ of the amide I band was calculated to be 506 litre·mol$^{-1}$·cm$^{-1}$. The amide II band near 1535 cm$^{-1}$ originates from coupled C-N stretching and N-H bending motion (Miyazawa *et al.*, 1958). The rate of exchange was followed by absorption measurements of the amide II band. The first reading was made 5 min after protein solubilization. To determine absorption values of amide I and amide II bands, the tangent to the minimum absorption at 1770 cm$^{-1}$ was adopted as the base line. As the background of amide II band, the corresponding absorption value, measured after 2 h of protein incubation in $D_2O$ at 75°C was adopted. At this temperature the H-D exchange is completed within the first 30 min.

The kinetic evaluation of experimental data was performed as described previously (Kania & Siemion, 1973). The semilogarithmic plot of the obtained data permits to determine two exchange stages differing in their rate. The first stage comprises the initial 30 min of the reaction. For a given time, the percentage of unexchanged peptide hydrogen atoms (Remaining H %) was estimated from the equation:

$$\text{Remaining H } \% = (A_{1535}/A_{1645}) \cdot \frac{100}{0.43}$$

where $A_{1535}$ and $A_{1645}$ are absorption values corrected for the respective background absorption, and 0.43 is the absorption ratio of amide II to amide I for the investigated protein incubated in $D_2O$ at 20°C, determined by extrapolation to zero time in the semilogarithmic plot. At all temperatures studied, the percentage of exchange was calculated for the end of the first stage of exchange.

*Nuclear magnetic resonance measurements.* The NMR spectra of about 10% solutions (w/v) of the basic trypsin inhibitor were recorded in a Tesla BS 467-A 80 MHz spectrometer. Hexamethyldisiloxane was used as external reference.

*pD of medium.* pH of the basic trypsin inhibitor hydrochloride solution in $D_2O$ was measured with a Radiometer pH-meter fitted with microelectrodes (G 2201 and K 1301), and pD calculated using the conversion factor of Glasoe & Long (1960).

## RESULTS AND DISCUSSION

The exchange of the hydrogen atom of the *m*-th peptide group of a protein in water solution is described by the following reaction scheme (Hvidt & Nielsen, 1966):

$$N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} I \xrightarrow{k_3} \text{exchange} \qquad (1)$$

where N denotes protein molecules in which the *m*-th peptide group is buried within the protein matrix, thus unexposed to the solvent; I denotes protein molecules in which the *m*-th peptide group is freely exposed to the solvent; $k_1$ and $k_2$ are rate constants of conformational changes of the protein exposing and burying the *m*-th group; $k_3$ is the pseudo-first order rate constant of solvent-exposed peptide group.

Assuming the equilibrium to be undisturbed by the exchange reaction, an expression for the exchange of an arbitrary peptide hydrogen atom ($m$-th) in a protein can be given, according to the reaction scheme 1, by the following rate constant (Berger & Linderstrøm-Lang, 1957; Hvidt & Nielsen, 1966):

$$k_m = \frac{k_1 \cdot k_2}{k_1 + k_2 + k_3}$$

According to those authors, for the exchange process two extreme physical possibilities may be formulated:

$$\text{I. } k_m = k_1, \qquad \text{provided } k_2 \ll k_3$$

$$\text{II. } k_m = \frac{k_1}{k_2} \cdot k_3, \text{ provided } k_2 \gg k_3$$

In the first case the rate is determined by the opening reaction, and hydrogen isotope exchange measurement enables the evaluation of the unfolding reaction. This reaction has been termed the $EX_1$ exchange mechanism ("unimolecular" type of exchange). In the second case, termed the $EX_2$ mechanism ("bimolecular" type of exchange) the opening and closing reactions may proceed many times without exchange necessarily taking place. This case should provide the possibility of determining, by exchange experiments, the ratio of $k_1$ to $k_2$, i.e. the equilibrium constant for the transconformational reactions for the various peptide hydrogen atoms (Englander, 1967). On the other hand, Rosenberg & Enberg (1969) and Rosenberg & Woodward (1970) suggest that the exchange by the $EX_2$ mechanism is characterized by a high value of activation energy (the unfolding process and exchange). For a number of proteins the calculated activation energy has relatively low values which according to Woodward & Rosenberg (1971) indicate that the exchange takes place in the folded conformation (N states) and is determined by segmental non-cooperative changes of the surface of protein molecules. Hvidt & Wallevik (1972) regard this interpretation as not completely valid because in their opinion a low value of activation energy is not incompatible with the $EX_2$ mechanism.

The structure and unusual thermal stability of conformation of basic trypsin inhibitor is well known and has been investigated in detail (Acher & Chauvet, 1970; Huber *et al.*, 1970; Vincent *et al.*, 1971). The X-ray diffraction patterns of the trypsin inhibitor crystals obtained under various pH conditions (6.5 and 10.5) showed only small changes of the structure (Huber *et al.*, 1970). The protein has a molecular weight of 6500 and consists of 58 amino acids of known sequence (Kassell & Laskowski, 1965; Dlouhá *et al.*, 1966; Anderer & Hörnle, 1966; Chauvet & Acher, 1967). In this protein 25% $\alpha$-helix, 40% $\beta$-structure and 35% unordered conformation were found (Huber *et al.*, 1970). Basing on physico-chemical measurements Vincent *et al.* (1971) concluded that the folded conformation of the inhibitor remains unaltered at 77°C and pH 2.1 or in 6 M-guanidine·HCl at pH 7.5. No differences were observed in its viscosity at pH 2.1 over the temperature range 20 - 77°C. Changes in ORD spectra measured at 230 nm at pH 2.6 were observed only above 75°C. After chemical

modification of the inhibitor (carboxymethylation, carboxyamidomethylation, or aminoethylation) Vincent *et al.* (1971) obtained, however, a complete melting curve from which a melting temperature of about 60°C, at pH 2.6, may be calculated.

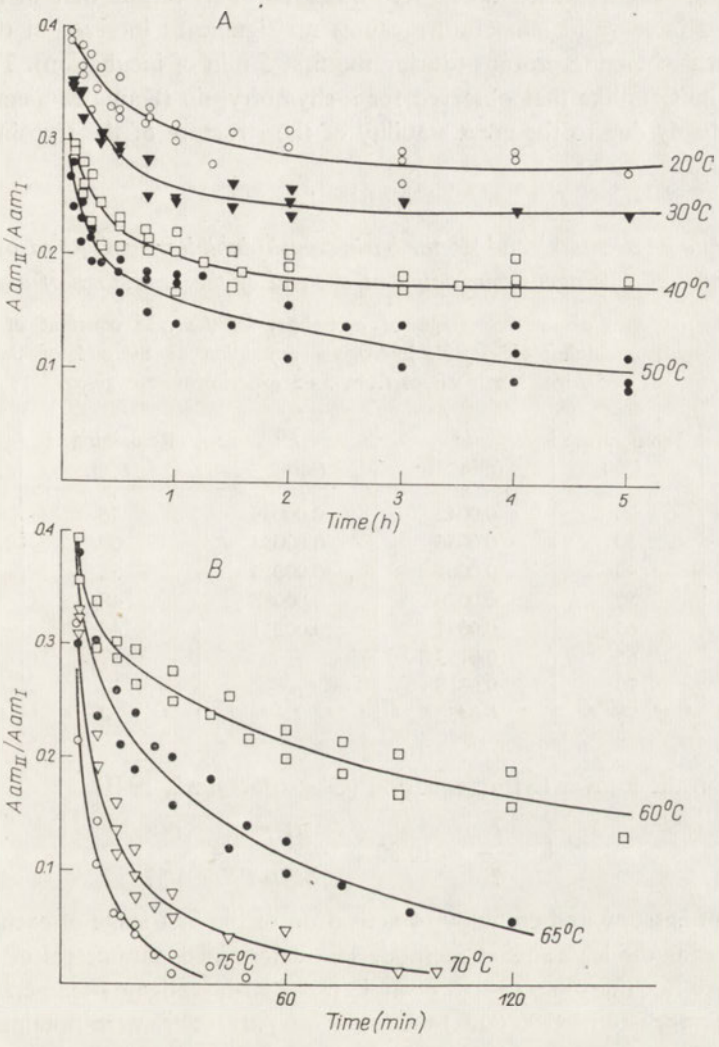


Fig. 2. The exchange of peptide hydrogens in the basic trypsin inhibitor in $D_2O$ at pD 2.6: *A*, over the temperature range 20 - 50°C; *B*, over the temperature range 60 - 75°C. The data from 2 - 3 independent experiments are plotted on the Figure.

In Figure 2A,B are shown the rates of H-D exchange for the basic trypsin inhibitor at various temperatures. The mean values for the graphically found rate constants presented in Table 1 show that all protons of the peptide groups are exchanged within 30 min only at 75°C; and at this temperature one stage of the exchange reaction is observed. At lower temperatures the H-D exchange can be approximately described as a two-stage process with different rate constants. The rate constants

for the second stage of exchange at 65 and 70°C are not included in Table 1 because the deviation from the mean values was higher than 15%. It should be pointed out that in the whole temperature range no significant differences for the $A_{amII}/A_{amI}$ values extrapolated to zero time, were observed. This means that in the trypsin inhibitor an increase in temperature causes no significant increase in the amount of very fast exchanging protons (during the first 5 min of incubation). This phenomenon is quite unlike that observed for α-chymotrypsin (Kania & Siemion, 1973) and is probably due to the great stability of the structure of the trypsin inhibitor.

Table 1

*Exchange rate constants* $k^I$ *and* $k^{II}$ *for basic trypsin inhibitor in* $D_2O$ *at* pD 2.6, *and percentage of hydrogens remaining at the end of the first stage of exchange*

$k^I$ is the rate constant of the first stage of exchange; $k^{II}$ the rate constant of the second stage of exchange; remaining H, peptide hydrogens remaining at the end of the first stage of exchange. Mean values from 3 - 5 experiments are given.

| Temperature (°C) | $k^I$ ($min^{-1}$) | $k^{II}$ ($min^{-1}$) | Remaining H (%) |
|---|---|---|---|
| 20 | 0.0042 | 0.00038 | 75 |
| 30 | 0.0056 | 0.00053 | 60 |
| 40 | 0.0061 | 0.00056 | 52 |
| 50 | 0.0076 | 0.00088 | 48 |
| 60 | 0.0097 | 0.00211 | 40 |
| 65 | 0.0153 | | 37 |
| 70 | 0.0318 | | 30 |
| 75 | 0.0458 | | 0 |

Basing on the known Eyring equation (Glasstone *et al.*, 1941)

$$\log \frac{k}{T} = 10.32 - \frac{\Delta H^*}{4.57 \cdot T} + \frac{\Delta S^*}{4.57}$$

the values of entropy and enthalpy of activation of the first stage of exchange were calculated using the least square method. The determined parameters of activation (Fig. 3) were not the same over the whole range of investigated temperatures. For the lower temperatures (20 - 60°C) the following parameters were obtained: $\Delta H^* = 3$ kcal/mol; $\Delta S^* = -65$ cal/mol·°K; $E^* = 3.6$ kcal/mol. A comparison of $\Delta H^*$ with $\Delta S^*$ shows that the value of free energy of activation of the exchange process is determined mainly by the entropy parameter. It is obvious that for the process of thermal transconformation a positive value of entropy of activation has to be expected. Therefore it may be assumed that at this stage the H-D exchange follows the mechanism of the segmental non-cooperative motion of the protein surface. For higher temperatures (60 - 75°C), the respective values were: $\Delta H^* = 20$ kcal/mol; $\Delta S^* = -15$ cal/mol·°K; and $E^* = 20.7$ kcal/mol. The activation energy and enthalpy of activation increased considerably with a simultaneous considerable decrease of the negative value of entropy of activation. This suggests that at 60 - 75°C there

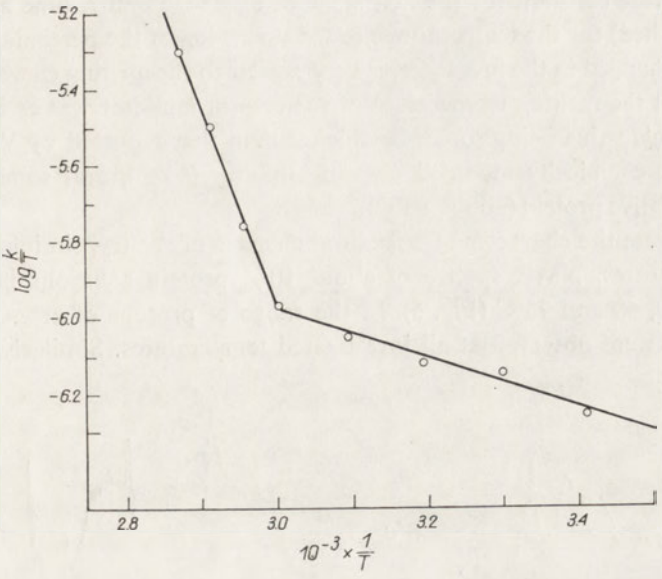


Fig. 3. The Eyring's plot of exchange rates showing two independent mechanisms of the process of exchange.

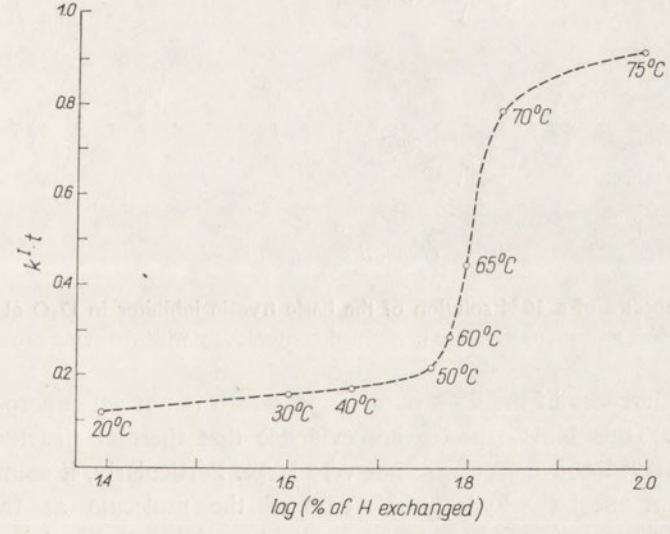


Fig. 4. The "melting curve" of the basic trypsin inhibitor obtained from spectroscopic i.r. data; $k^1$, rate constant for the first stage of exchange; $t$, time at which the first stage is finished; the percentage of exchanged hydrogen atoms refers to the exchange at the end of the first stage.

exists a second exchange mechanism which is superimposed over the first one. Thus it may be concluded that thermal transconformation of the trypsin inhibitor affects distinctly the rate of H-D exchange only at temperatures above 60°C (Fig. 3), its effect being parallel to the increase of temperature (Fig. 4). The plot of $k^1 \cdot t$ values

($k^I$ being the rate constant for the first stage of exchange and $t$, time at which this stage was finished) for the temperatures tested *versus* log of the percentage of hydrogen atoms exchanged in the first stage, is very similar to the melting curve of proteins. From this plot the melting temperature of the trypsin inhibitor was estimated to be about 65°C, this value being somewhat higher than that reported by Vincent *et al.* (1971) for the chemically modified trypsin inhibitor (60°C), and somewhat lower than that for native protein (about 80°C).

To investigate the character of structural changes of the trypsin inhibitor at elevated temperatures, NMR spectra of about 10% protein solutions in $D_2O$ were recorded at 20, 60 and 75°C (Fig. 5). In the range of protons of aromatic residues no differences were observed at all investigated temperatures. Similarly, there were

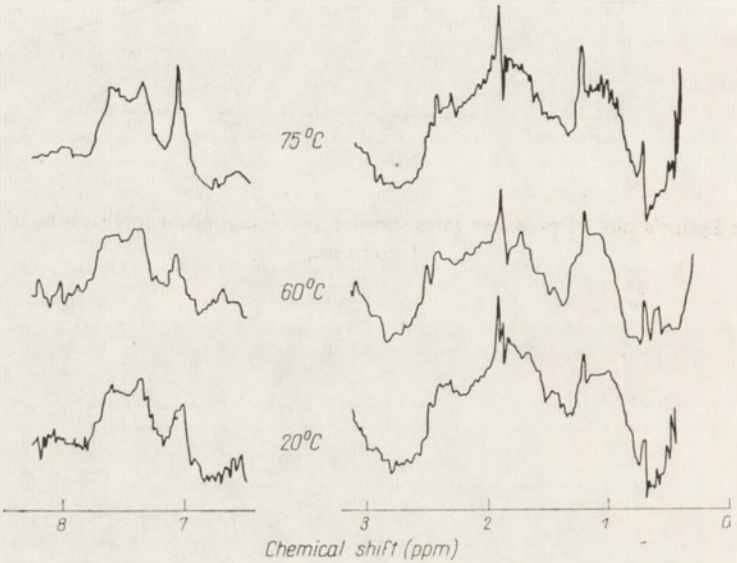


**Fig. 5. NMR spectra of a 10% solution of the basic trypsin inhibitor in $D_2O$ at the indicated temperatures. All measurements were performed continuously with the same protein solution.**

no distinct differences in the 0 - 3 ppm range, which is not yet superpositioned by HDO signals. Thus NMR spectra are evidence that thermal structural changes, which are observed above 60°C, are not very large. Particularly, it seems that these changes do not affect the hydrophobic core of the molecule as the magnetic environment of aromatic residues, which probably stabilize the protein structure, is essentially unaltered at elevated temperatures.

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## BADANIA NAD WYMIANĄ H-D W ZASADOWYM INHIBITORZE TRYPSYNY

### Streszczenie

Zbadano wymianę H-D w inhibitorze trypsyny w zakresie temperatur 20 - 75°C, przy pD 2.6, oraz wyliczono wartości energii aktywacji, entalpii i entropii aktywacji. Uzyskane parametry termodynamiczne sugerują istnienie dwóch niezależnych mechanizmów wymiany H-D w tym białku, dominujących odpowiednio w przedziale temperatur 20 - 60°C i 60 - 75°C. Dane doświadczalne z wymiany porównano z danymi uzyskanymi z analizy widm NMR inhibitora.

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# THE ATTACHMENT OF Vi-PHAGE III

## THE PRESENCE OF Vi-POLYSACCHARIDE DEACETYLASE**

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The activity of Vi-polysaccharide deacetylase in Vi-phage III and Vi-phage I has been demonstrated. The three morphologically distinct Vi-phages, I, II and III, have a common molecular mechanism of primary attachment consisting in complex formation of phage-bound enzyme with bacterial receptor-substrate.

Participation of phage-bound enzyme in the attachment to a substrate-receptor has been recognized in several phage-bacteria systems (Taylor & Taylor, 1963; Stirm, 1968).

The attachment of Vi-phage II corresponds to the formation of a complex involving the phage-bound deacetylase (Taylor, 1965) and microcapsular Vi-polysaccharide (*N*- and *O*-acetylated $\alpha$-1-4 linked 2-deoxy-2-amino-D-galacturonic acid) (Heyns & Kiessling, 1967). The deacetylase is probably situated in the spikes of Vi-phage II tail (Kwiatkowski, 1969). Thus Vi-phage II attachment results in Vi-polysaccharide deacetylation, which leads to the loss of the receptor activity of Vi-polysaccharide (Taylor, 1965) and consequently ghost release after DNA penetration (Taylor & Kwiatkowski, 1966).

A similar relationship was found by Stirm (1968) between K-phage 42 and *E. coli* A 295b (08:K42/A/:H$^-$). Stirm *et al.* (1971a,b) also described an endoglucosidase, localized in phage spikes, which depolymerized the capsular polysaccharide endowed with receptor activity.

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Other relevant experiments may also be those carried out by Lovett & Shockman (1970) on reversible attachment of phage N1 to *Micrococcus lysodeikticus*. Recently Takeda & Uetake (1973) and Kanegasaki & Wright (1973) showed independently that phage $\varepsilon^{15}$ destroys its receptor-bacterial lipopolysaccharide cleaving rhamnosyl-galactose linkages of polysaccharide chain.

This kind of interaction of phages with their receptors obviously does not cause inactivation of the phages but loss of the receptor activity of the corresponding polysaccharides.

The morphologically distinct Vi-phages I, II and III (Kwiatkowski, 1966; Ackerman *et al.*, 1970) belong to groups A, B and C, respectively, according to the classification of Bradley (1967). Common to all these phages is their receptor, Vi-polysaccharide of *Salmonella typhi*.

The molecular mechanism of the interaction of Vi-phages I and III with Vi-polysaccharide is unknown. Kwiatkowski & Taylor (1970) demonstrated that Vi-phage I is not inactivated by interaction with Vi-polysaccharide, which suggested that the receptor is also a substrate for Vi-phage I enzyme; however, it remained obscure whether a deacetylase (as in the case of Vi-phage II) or a glucosidase is involved.

So far, no studies have been performed on the attachment of Vi-phage III to determine whether it corresponds to any known enzymic mechanism or to irreversible binding reminiscent of the attachment of T3, T4 and T7 phages (Jesaitis & Goebel, 1952; Weidel, 1958; Beumer *et al.*, 1965). Therefore we have undertaken an investigation of the primary attachment of Vi-phage III in comparison with that of Vi-phages I and II.

## MATERIALS AND METHODS

*Bacteria and phages. Salmonella typhi* 21802 (Vi-phage type A) and phages Vi I, Vi II and Vi III were obtained from the National Reference Laboratory for Enteric Phage Typing (London, England).

All the Vi-phages were propagated on *S. typhi* 21802 in Stokes & Bayne (1958) basal medium supplemented with 1% of Difco lactalbumin hydrolysate (mSB). Phage titration was performed according to Adams (1959) with preincubation of phage with bacteria for 20 min at room temperature. Indicator bacteria for titration were prepared according to Kaiser (1955). The titers of phage stocks were about $10^{11}$ p.f.u./ml.

*Phage purification.* Vi-phage I was purified from the lysates by two cycles of differential centrifugation (12 000 *g*, 60 min and 3 000 *g*, 15 min), then sedimented again and resuspended finally at a concentration of $3 \times 10^{12}$ p.f.u./ml in the appropriate solution.

Vi-phage III was concentrated on ECTEOLA cellulose (Reanal, Budapest, Hungary) column (35 × 70 mm) equilibrated with 10 mM-phosphate buffer, pH 7. The phage (1 litre of lysate dialysed overnight against 20 volumes of water) was introduced into the column, washed with the equilibration buffer and eluted with a linear concentration gradient of NaCl (100 ml of 0.3 M-NaCl in 10 mM-phosphate

buffer, pH 7, and 100 ml of the same phosphate buffer). Finally, the phage suspension was centrifuged at 100 000 *g* for 60 min. The sedimented phages were resuspended in the appropriate solution at a concentration of $3 \times 10^{12}$ p.f.u./ml.

*The incubation solutions.* Three kinds of solutions were used: veronal-HCl buffer, 0.05 M, pH 7, containing 2 mM-$CaCl_2$ (veronal-$Ca^{2+}$ buffer), mSB, and mSB supplemented with 2 mM-$CaCl_2$ (mSB-$Ca^{2+}$). The physiological saline was buffered with 0.05 M-phosphate to pH 7.

*Vi-polysaccharide* was obtained from *E. coli* 5396/38 and purified by column chromatography on red blood cells stroma-celite 535 (Taylor & Taylor, 1965).

*Formolized sheep erythrocytes*, a 10% suspension, was prepared according to Weinbach (1958) and stored in 0.5% formaline solution at 4°C. The suspension was dialysed against tap water overnight, immediately before use. The erythrocytes were washed twice with water and once with physiological saline, then suspended in 9 parts of this solution for 1 part of sedimented cells.

*Membranes of human erythrocytes* coated with Vi-polysaccharide were prepared as previously described (Taylor & Kwiatkowski, 1963).

*Electron microscopy.* The specimens for the electron microscopy were prepared and chromium shadowed by the method of Kellenberger & Arber (1957) with the modification introduced by Kwiatkowski & Taylor (1970). The electron microscopes Zeiss-D 2 and Tesla BS 513 were used.

## RESULTS

As a first approach, Vi-phage III attachment on Vi-polysaccharide was examined. The Vi-polysaccharide was adsorbed on an inert surface of erythrocytes as in the experiments with Vi-phage II (Taylor & Kwiatkowski, 1963) and Vi-phage I (Kwiatkowski & Taylor, 1970). This procedure makes it possible to observe the attachment unobscured by subsequent steps of penetration.

Vi-polysaccharide, 2, 4, 6 and 8 μg in 1 ml of saline, were added to 0.5 ml portions of a 10% suspension of formolized erythrocytes and left overnight at 4°C. The Vi-polysaccharide-coated erythrocytes were washed once with cold saline by centrifugation. The sediment was treated with $2 \times 10^{11}$ p.f.u./ml of Vi-phage III in either 2 ml of cold mSB or veronal-$Ca^{2+}$ buffer. After 3 min incubation in ice-bath the erythrocytes were immediately centrifuged off and the unattached phage was titrated in the supernatant. In control experiments either Vi-phage I, instead of Vi-phage III, was added to the Vi-polysaccharide-coated erythrocytes, or non-coated erythrocytes were used.

The sediment of the erythrocytes with attached phages was resuspended in 2 ml of mSB or veronal-$Ca^{2+}$ buffer, respectively, and transferred to 37°C for 1 hour. Then the erythrocytes were centrifuged off and in supernatant the released phage was titrated (Fig. 1). The controls showed that there was no phage attachment on the non-coated erythrocytes, and that Vi-phage I, as in the previous experiments, was attached to Vi-polysaccharide at 0°C in both media and was released after the transfer to 37°C. It was surprising that, in contrast to this, no attachment

of Vi-phage III was observed in mSB medium. However, the attachment of Vi-phage III on Vi-polysaccharide in veronal-$Ca^{2+}$ buffer and release of infectious phage after elevation of temperature resembled closely the behaviour of Vi-phages I and II. The results observed in veronal-$Ca^{2+}$ buffer suggested by analogy that the receptor--destroying enzyme may be present also in Vi-phage III, as in the other Vi-phages.

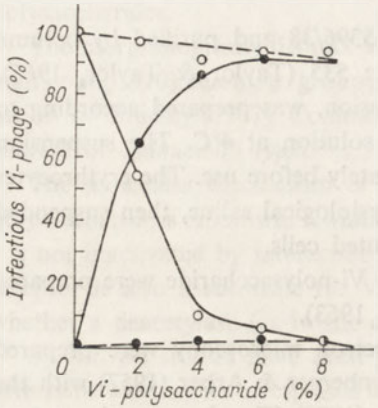


Fig. 1. Vi-phage III attachment on Vi-polysaccharide--coated erythrocytes in (——) veronal-$Ca^{2+}$ buffer, and (— — —) mSB; ○, phage unattached at 0°C; ●, attached phage released at 37°C. Non-coated erythrocytes were used as control (zero concentration of Vi-polysaccharide).

It seemed important to elucidate the behaviour of Vi-phage III in mSB, because this medium serves for phage propagation and provides the conditions for phage attachment in the natural infection. Our supposition was that in mSB the Vi-phage III attachment and receptor destruction proceed too quickly to be detected by our technique. This was based on an unpublished observation (B. Kwiatkowski and J. Lalko) that the adsorption constant for Vi-phage III attachment to the intact bacteria was considerably higher than for Vi-phages I or II.

The experiment with Vi-phage III attachment in mSB was repeated and after the removal of the free Vi-phage III, 2 ml of Vi-phage II ($2 \times 10^{11}$ p.f.u./ml) was added and allowed to attach at 0°C for 5 min. The control contained Vi-polysaccharide--coated erythrocytes not treated with Vi-phage III (Table 1). It was found that,

Table 1

*The attachment of Vi-phage II to Vi-polysaccharide-coated erythrocytes before and after treatment with Vi-phage III*

Details of the assay are described in the text.

| Expt. no. | Vi-phage II attachment | |
|---|---|---|
| | before treatment (%) | after Vi-phage III treatment (%) |
| 1 | 73 | 16 |
| 2 | 80 | 3 |
| 3 | 73 | 15 |
| 4 | 98 | 18 |

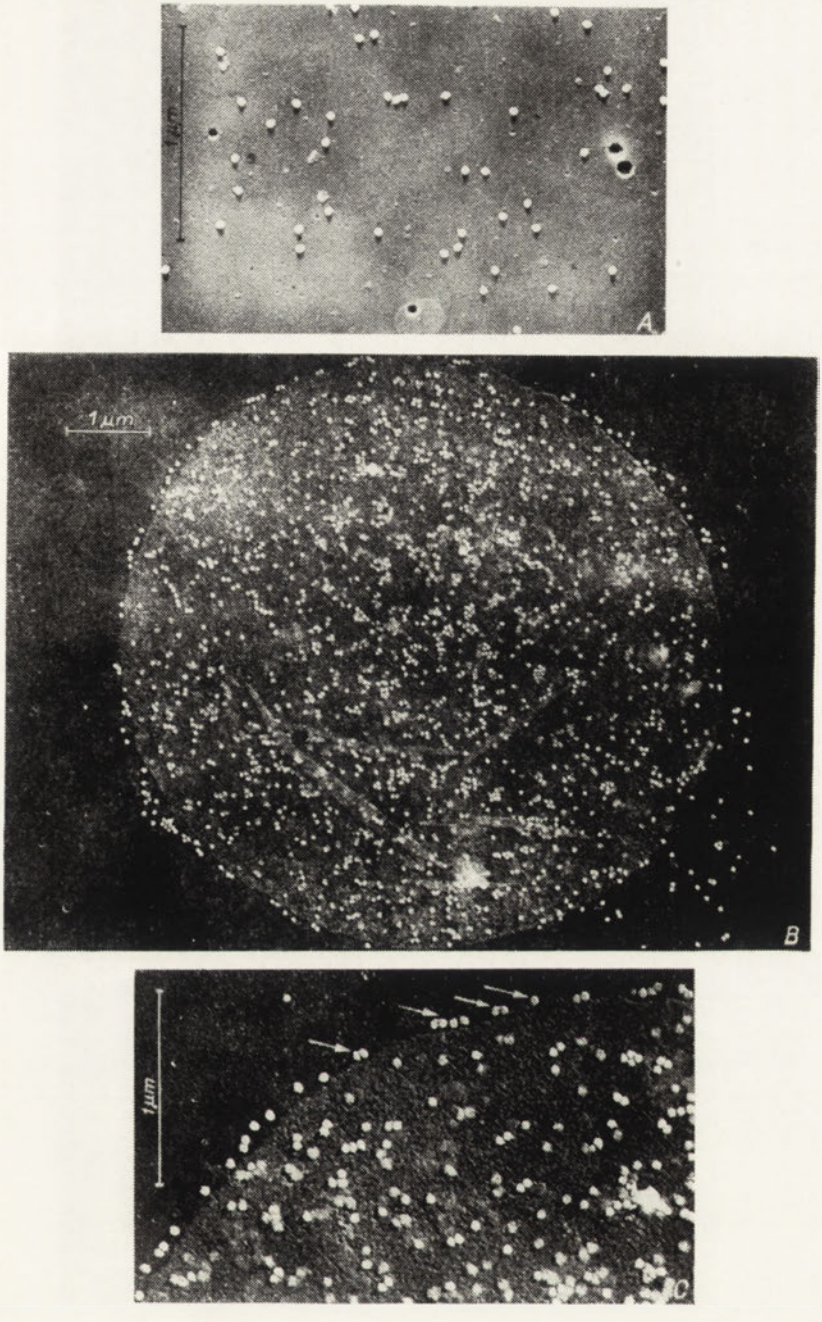


Fig. 2. Electron micrographs of chromium-shadowed preparations. *A*, purified Vi-phage III. *B*, Vi-phage III attachment to Vi-polysaccharide-coated erythrocyte membrane in veronal-$Ca^{2+}$ buffer. *C*, the enlargement of an edge of erythrocyte membrane from *B*; arrows point to Vi-phage III tails attached to the receptor.

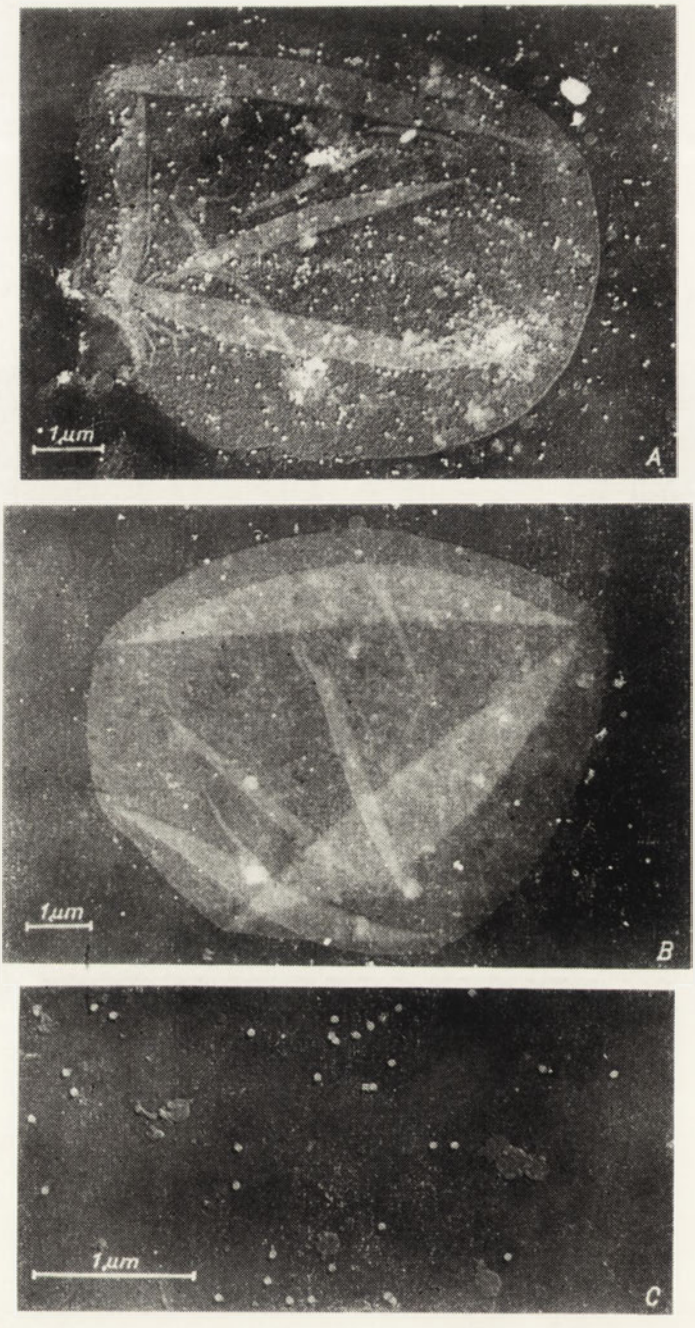


Fig. 3. Electron micrographs of chromium-shadowed preparations. *A*, Vi-phage III attachment to Vi-polysaccharide-coated erythrocyte membrane in mSB-$Ca^{2+}$; *B*, erythrocyte membrane after Vi-phage III release; *C*, the released Vi-phage III.

as anticipated, Vi-polysaccharide lost its receptor properties against Vi-phage II as a result of the treatment with Vi-phage III in mSB.

The mSB medium supplemented with 2 mM-$CaCl_2$ used in the same kind of experiment allowed for the attachment of about 30% of Vi-phage III added, but after the transfer to 37°C there was no release of infectious phage, as shown by titration.

The process was examined in the electron microscope, the method elaborated for Vi-phage I (Kwiatkowski & Taylor, 1970) and Vi-phage II (Taylor & Kwiatkowski, 1963) being employed. Vi-polysaccharide-coated erythrocyte membranes (2 ml) were mixed with 2 ml of Vi-phage III ($2 \times 10^{11}$ p.f.u./ml) either in veronal-$Ca^{2+}$ buffer or mSB and incubated for 2 min in ice-water. The membranes were centrifuged at 4°C, and carefully suspended in cold mSB. This medium does not cause phage detachment if the temperature is kept low. The membrane suspensions and the supernatants were examined in the electron microscope. Figure 2A presents the purified, chromium-shadowed preparation of Vi-phage III, and Fig. 2B the Vi-phage III attached to Vi-polysaccharide-coated erythrocyte membrane in veronal-$Ca^{2+}$ buffer. On the enlarged micrograph (Fig. 2C) it may be seen that Vi-phage III attached through its tail (arrows) which confirms the specificity of the attachment. No attachment could be observed in mSB but when mSB was supplemented with $CaCl_2$, some attachment of the phage appeared (Fig. 3A), its quantity being comparable to that found by titration. The erythrocyte membranes with attached phages from the experiment illustrated by Fig. 3A were transferred to 37°C for 60 min, centrifuged and again examined in the electron microscope. It appeared that the membranes were free of phages (Fig. 3B) and the phages themselves were found in the supernatant (Fig. 3C). Thus the release of phages from the receptor depends only on the temperature and it does not depend on the presence of $Ca^{2+}$. The released phages did not suffer any visible changes and contained their DNA. We do not know what is the basis of the loss of their infectivity.

The above experiments showed that Vi-phage III, like Vi-phage II, destroyed receptor properties of Vi-polysaccharide. It remained to check whether this was due to deacetylation or another reaction.

The suspension (4 ml) of phage III ($3 \times 10^{12}$ p.f.u./ml) in veronal-$Ca^{2+}$ buffer containing 0.1 mM-*p*-chloromercuribenzoate was incubated with 4 mg of Vi-polysaccharide at 37°C for 5 h. Phages were removed by centrifugation. The solution of the polysaccharide was dialysed against water, then against 10 mM-EDTA, pH 7, and bidistilled water, concentrated under reduced pressure and freeze-dried. The experiment with Vi-phage I was performed under the same conditions.

Samples of 800 μg of Vi-polysaccharide and phage-treated Vi-polysaccharide were pressed in KBr and their infrared spectra were taken in Perkin Infracord SP-200 spectrophotometer. Vi-polysaccharide showed absorption bands at frequencies of 1740 $cm^{-1}$ and 1240 $cm^{-1}$, corresponding to the presence of acetyl groups (Fig. 4A). These bands disappeared completely in Vi-polysaccharide preparations treated either with V-phage III (Fig. 4B) or Vi-phage I (Fig. 4C). This corresponds to deacetylation since Vi-polysaccharide does not contain any other ester group.

This result closely resembles the deacetylation of Vi-polysaccharide by Vi-phage II deacetylase demonstrated by Taylor (1965), which points to the presence of Vi-polysaccharide deacetylase in phages Vi III and Vi I.

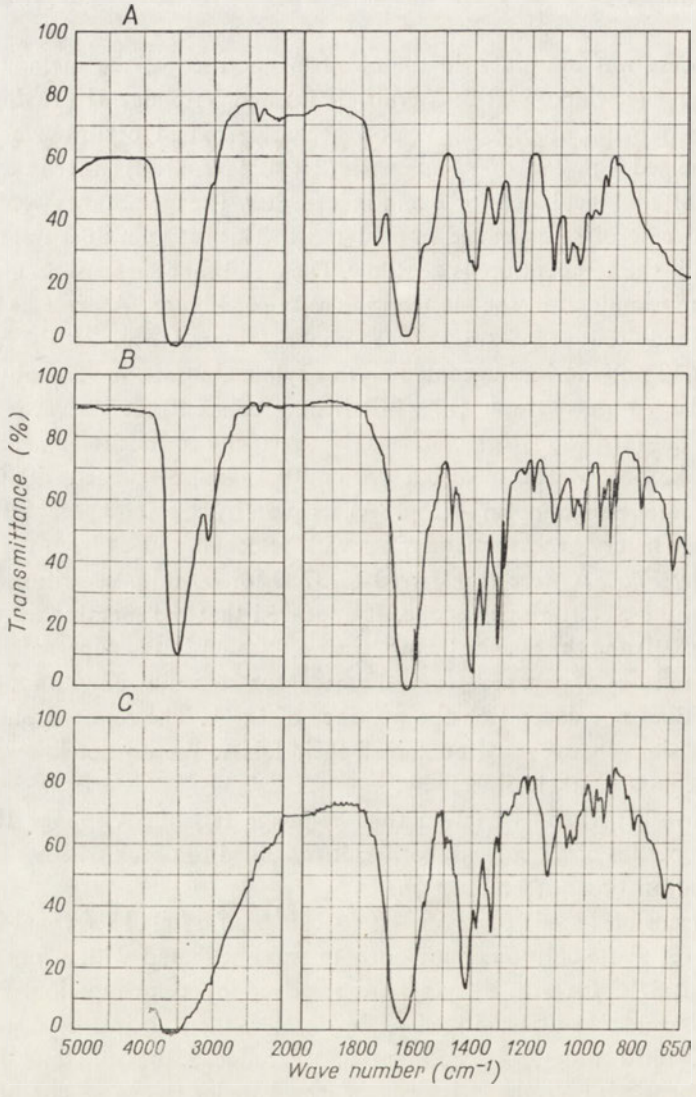


Fig. 4. The infrared absorption spectra of: *A*, initial Vi-polysaccharide, sodium salt; *B*, Vi-polysaccharide treated with Vi-phage III; *C*, Vi-polysaccharide treated with Vi-phage I. In *B* and *C* the absorption bands at frequencies of 1740 $cm^{-1}$ and 1240 $cm^{-1}$ are absent.

The above results indicate that the three morphologically different phages Vi I, II and III have the same molecular mechanism operating for the specific recognition of the sensitive bacteria. None of these three Vi-phages is bound by deacetylated

Vi-polysaccharide, and if the binding occurs, as in the case of Vi-phage I, it involves another component situated on bacterial cell wall.

## DISCUSSION

Two kinds of interaction of bacteriophages with their receptors are known. The first consists in a specific inactivation of phages due to the blocking of their organs of attachment. The molecular mechanism of the process remains obscure, though in numerous cases the specificity of the receptors could be correlated with the precise structure of the bacterial component (Coyette & Ghuysen, 1968; Lindberg & Holme, 1969; Lindberg *et al.*, 1970; Seltmann & Manson, 1970; Lindberg & Hellerquist, 1971; Tamaki *et al.*, 1971); also phage structure responsible for the attachment has been reported (Wilson *et al.*, 1970).

The other kind of attachment involves interaction of phage-bound enzyme with bacterial receptor-substrate (Taylor & Taylor, 1963; Taylor, 1965; Stirm, 1968; Takeda & Uetake, 1973; Kanegasaki & Wright, 1973). In such cases the receptor does not inactivate phages and therefore is harder to detect. It could be supposed that this type of interaction may be more common than indicated by the available evidence. For example, one can expect this is the case with T1 phage, for which inactivation experiments were unsuccessful (Weidel, 1958). The enzyme-substrate interaction may easily escape observation in cases analogical to Vi-phage I attachment, which proceeds in two steps occurring in rapid succession (Kwiatkowski & Taylor, 1970). In the first step, phage-bound deacetylase forms a complex with Vi-polysaccharide and removes acetyl groups. In the second, phage tail binds firmly with a still unknown component of bacterial cell wall, with a concomitant sheath contraction, but not DNA release. This step corresponds to Vi-phage I inactivation. For Vi-phage II the first step seems to be sufficient for phage DNA penetration with subsequent ghost release.

The enzyme-substrate type of attachment is not confined to a single morphological type of phage as it appears to operate for phages Vi I, Vi II and Vi III of different tail structures (morphological groups A, B, C; Bradley, 1967). As it follows from our studies on Vi-phages (Kwiatkowski & Taylor, 1970), the differences in the mechanism of attachment and penetration concern other steps of the process.

The physiological significance of Vi-polysaccharide deacetylation by an enzyme bound to Vi-phage spikes is not elucidated, and may only be the subject of speculation. Since both *O*- and *N*-acetyl groups are removed, the liberation of acetate may be preceded by *O*,*N*-transacetylation. It has been postulated by Taylor (1965) that due to the sequential removal of *O*- and *N*-acetyl groups the phage may be imagined to move along the linear Vi-polysaccharide towards the bacterial surface. If the polysaccharide is anchored at a specialized area like that described by Bayer (1968a,b) and Bayer & Starkey (1972), connected by a "duct" with the cytoplasmic membrane, the transacetylation may provide a means of reaching this particular spot, ensuring successful infection.

The authors thank Dr. J. Foks of the Institute of Technology in Gdańsk for making available the electron microscope Tesla BS 513. Thanks are due also to Dr. J. Szafranek of the University in Gdańsk for the infrared spectra. The excellent technical assistance of Mrs. A. Jastrzemska and Mrs. J. Żabina is gratefully acknowledged.

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## ADSORPCJA BAKTERIOFAGA Vi III

### OBECNOŚĆ DEACETYLAZY WIELOCUKRU Vi

Streszczenie

Wykazano obecność deacetylazy wielocukru Vi u fagów Vi III i Vi I. Trzy morfologicznie różne fagi Vi-, I, II i III, mają jednakowy mechanizm adsorpcji, który polega na tworzeniu się kompleksu fagowego enzymu z substratem-receptorem bakteryjnym.

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# THE ATTACHMENT OF VI-PHAGE III

## INTERACTION WITH CELL WALLS**

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The particular steps of Vi-phage III attachment to *Salmonella typhi* cell walls could be observed by the use of different media. In veronal-$Ca^{2+}$ buffer phage inactivation occurred due to phage binding to cell walls without DNA release. In nutrient medium mSB-$Ca^{2+}$ Vi-phage III attachment was followed by DNA release and ghost detachment.

The early step of phage penetration, the attachment, comprises several consecutive reactions. The difficulty in their examination consists in their very rapid succession during a natural infection. For example, Pryme *et al.* (1969) presented data that T2 phage penetration was accomplished in 35 seconds. Therefore a possibility of arresting this process at the particular steps could provide a useful experimental approach.

Attachment of T-even phages (morphological group A, Bradley 1967) has been described in detail on the basis of electron microscopy and of experiments on T4 phage mutants with defective attachment structure (Simon *et al.*, 1970; Eiserling *et al.*, 1967; Tamaki *et al.*, 1971). It has been demonstrated that the long tail fibers are responsible for the first contact of the phage with the bacterial cell. In the next moment the tail spikes bind to the bacterial surface and it is only then that contraction of the tail sheath follows and DNA release is provoked. The receptor which binds the long tail fibers is a lipopolysaccharide (Wilson *et al.*, 1970; Tamaki *et al.*, 1971). The activity resides in the core of the lipopolysaccharide which contains

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phosphate groups (Tamaki *et al.*, 1971). The molecular mechanism of the fiber binding remains obscure. Also the bacterial component which binds tail spikes and the mechanism of DNA release are unknown. The process of attachment of phages with non-contractile tails (morphological group B) and short-tailed phages (group C) is much less understood.

The mechanism of the attachment of Vi-phage I (morphological group A) was examined by Kwiatkowski & Taylor (1970), of Vi-page II (group B) by Taylor & Kwiatkowski (1966) and of Vi-phage III (group C) by Szczeklik *et al.* (1974). It has been shown that the first step of Vi-phage I, II and III attachment consists in the complex formation of phage-bound deacetylase with its receptor-substrate Vi-polysaccharide. Vi-phages I and II do not lose their infectivity after the first step if the process of penetration has been limited by the use of the isolated Vi--receptor adsorbed on the surface of erythrocytes. The deacetylated Vi-polysaccharide loses its receptor activity for Vi-phages I and II.

The next step has a different course with Vi-phage I and Vi-phage II. When the cell walls were used for the experiment, Vi-phage I was bound to them irreversibly with tail contraction and concomitant loss of infectivity, although there was no DNA release (Kwiatkowski & Taylor, 1970). It seems that in Vi-phage I attachment two receptor systems operate: the Vi-polysaccharide receptor responsible for the recognition of the sensitive bacteria and a second receptor, an unknown component of bacterial cell wall, which binds irreversibly Vi-phage I in the second step of attachment and probably causes its inactivation. As Vi-phage II is not bound by *S. typhi* cell walls, presumably the contact with the Vi-receptor on bacterial surface is sufficient for its penetration. After DNA injection the ghost of Vi-phage II detaches from the infected cell. The mechanism of DNA release is not known for either of the phages.

The aim of the present work was to examine whether penetration of Vi-phage III corresponds to the penetration scheme of either Vi-phage I or II, or is different from both.

## MATERIALS AND METHODS

The bacteriophages Vi I, Vi II and Vi III and bacteria *S. typhi* 21802 were the same as described in the accompanying paper (Szczeklik *et al.*, 1974), and the same methods of propagation, purification and titration, as well as veronal-$Ca^{2+}$ buffer, mSB, mSB-$Ca^{2+}$ and physiological saline were used.

The cell walls of *S. typhi* were prepared from 1 litre of the culture in mSB ($E_{575}$ = 1). The bacteria were centrifuged, suspended in 20 ml of buffered saline, pH 7, and frozen slowly to -20°C. Before use, the suspension was thawed in ice water, then mixed with cold Ballotini beads no. 16 and disintegrated in the apparatus described by Novotny (1964) at 3000 rev./min. Entensive washing was avoided not to remove entirely the loosely-bound capsular Vi-receptor. The beads were separated and washed once by decantation. The non-disintegrated bacteria were removed by centrifugation

and then the cell walls sedimented at 14 000 *g*. The cell wall pellet from 1 litre of the culture was suspended in 20 ml of mSB or veronal-$Ca^{2+}$ buffer and kept frozen at -20°C.

The specimens for electron microscopy were negatively stained with phosphotungstic acid according to Brenner & Horne (1959) as described by Kwiatkowski & Taylor (1970). The electron microscopes Zeiss D 2 and Tesla BS 513 were used.

## RESULTS

The aim of the presented experiments was to examine the interaction of Vi-phage III with *S. typhi* cell walls. The first question was whether Vi-phage III is firmly bound and inactivated by the cell walls like Vi-phage I, or is not inactivated like Vi-phage II.

The suspension of cell walls was diluted before use to $E_{620}=0.015$ with either mSB or veronal-$Ca^{2+}$ buffer. Then, to tubes containing 0.5 ml portions of Vi-phage III ($3\times10^6$ p.f.u./ml) in veronal-$Ca^{2+}$ buffer or mSB, increasing amounts of cell walls (0.1; 0.2 and 0.5 ml) in the corresponding solution were added. For comparison, Vi-phage I and Vi-phage II ($3\times10^6$ p.f.u./ml) were mixed with the cell walls under the same conditions. The mixtures were incubated at 37°C for 30 min. It has been found in preliminary experiments that the inactivation of Vi-phages by cell walls is completed within 30 min and prolongation of the time to 60 min has only a slight influence on the results. After incubation the mixture was chilled, diluted and titrated. For the control each of Vi-phages was incubated in parallel in appropriate medium for 30 min, and for calculation the titer of the controls was taken as 100%.

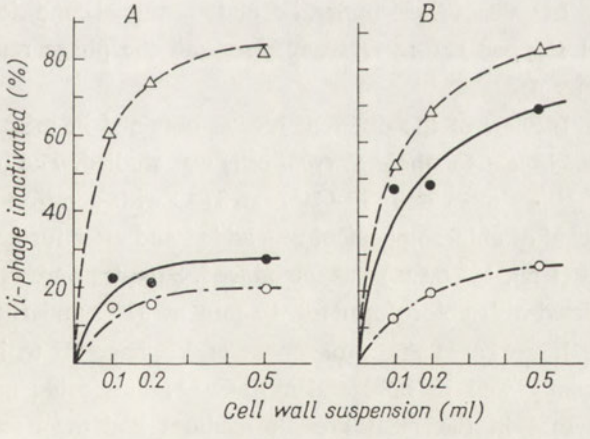


Fig. 1. Inactivation of Vi-phages by *Salmonella typhi* cell walls at 37°C: *A*, in mSB medium and *B*, in veronal-$Ca^{2+}$ medium. (△), Vi-phage I; (○), Vi-phage II; (●), Vi-phage III. The results are mean values from at least 5 experiments.

Figures 1A and B represent the inactivation curves in mSB and veronal-$Ca^{2+}$ media, respectively. In both media used, Vi-phage I was inactivated by the cell walls in about 80%. The inactivation of Vi-phage II did not exceed 27% in either medium. This figure may be considerably lower if fresh lysates are used. The results of in-

activation of Vi-phages I and II are consistent with the results of Taylor & Kwiatkowski (1966) and Kwiatkowski & Taylor (1970). Vi-phage III inactivation in mSB resembled that of Vi-phage II, reaching about 25%, and when fresh lysates were used, only a few percent of phage was inactivated. In mSB supplemented with 2 mM-$CaCl_2$ (mSB-$Ca^{2+}$) the inactivation of phage increased to 50%, whereas in veronal-$Ca^{2+}$ buffer 70% of phage was inactivated.

To examine the process of inactivation by electron microscopy, the undiluted suspension of the cell walls (0.2 ml) was incubated with 1.8 ml of Vi-phage III ($10^{11}$ p.f.u./ml) for 10 min at 37°C in either mSB, mSB-$Ca^{2+}$ or veronal-$Ca^{2+}$ buffer. After centrifugation, the sediment was resuspended gently in 0.1 M-ammonium acetate for negative staining. Figure 2A shows the electron micrograph of purified Vi-phage III, and Fig. 2B the cell walls treated with Vi-phage III in mSB. No phages were bound to cell walls and free, morphologically unchanged phages were found in the supernatant. This means that the inactivation of about 25% of the phage, observed in the previous experiment, was not caused by the binding to cell walls.

Vi-phages III after incubation with the cell walls in mSB-$Ca^{2+}$ medium were devoid of DNA and the majority of them detached from the cell walls (Fig. 3A). Figure 3B presents the enlargement of detached phage ghosts. It seems that after the interaction with the cell walls the structure of their tails was changed, but this would need further examination. This experiment demonstrated that Vi-phage III inactivation in mSB-$Ca^{2+}$ medium was due to DNA release. Thus the mSB-$Ca^{2+}$ - cell walls system contained all the components needed for provoking phage DNA release. The presence of numerous detached phage ghosts proves that *S. typhi* cell wall did not bind firmly Vi-phage III, similarly as Vi-phage II.

In veronal-$Ca^{2+}$ buffer, i.e. under artificial conditions, the penetration of Vi-phage III stopped before DNA release, and the phage remained bound to the cell wall (Fig. 4).

In view of the different results obtained in mSB and mSB-$Ca^{2+}$ media, phage attachment to whole *S. typhi* cells was studied. The bacteria were cultivated in 5 ml of either mSB or mSB-$Ca^{2+}$ at 37°C with aeration till $E_{575}$ reached 0.4, then 30 μg/ml of chloramphenicol was added and after further 15 min incubation, Vi-phage III (1 ml, $10^9$ p.f.u.) was introduced. Samples were taken at indicated time intervals, stirred with chloroform for 10 min at 37°C and titrated (Fig. 5).

It appeared that attachment of Vi-phage III to intact bacterial cells proceeded equally well in mSB and mSB-$Ca^{2+}$. This could imply that traces of $Ca^{2+}$ were present in the mSB growth medium and the bacteria were able to accumulate a sufficient amount of calcium on their surface. The analysis of Difco lactalbumin hydrolysate (batch no. 491206) showed the presence of about 50 μg of $Ca^{2+}$ per 1 g, which gives the final concentration of $Ca^{2+}$ in the mSB medium of the order of 10 μM.

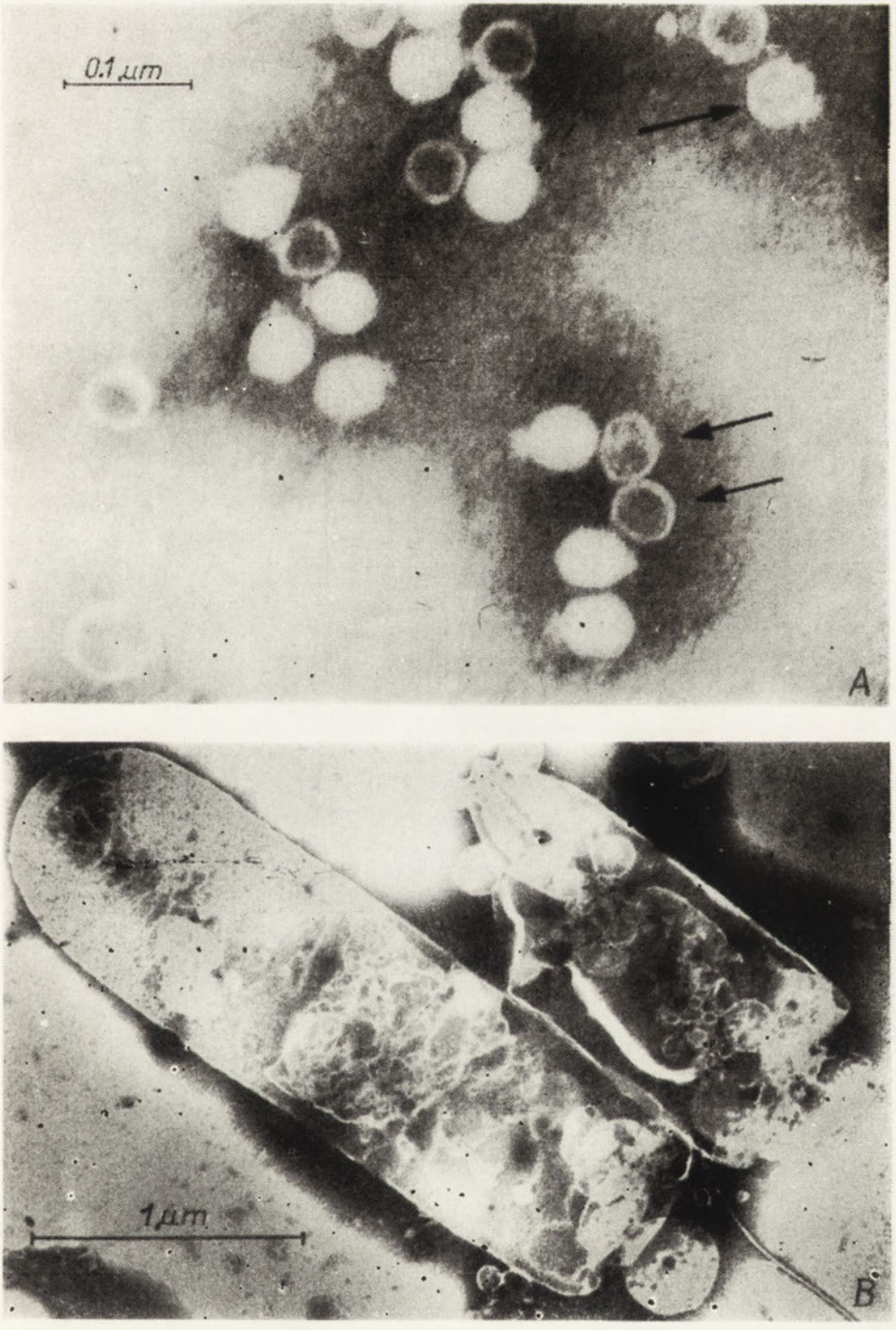


Fig. 2. Electron micrographs of negatively stained preparations. *A*, Purified Vi-phage III; the arrows point to phage ghosts. *B*, *Salmonella typhi* cell walls treated with Vi-phage III in mSB; no bound phages may be seen.

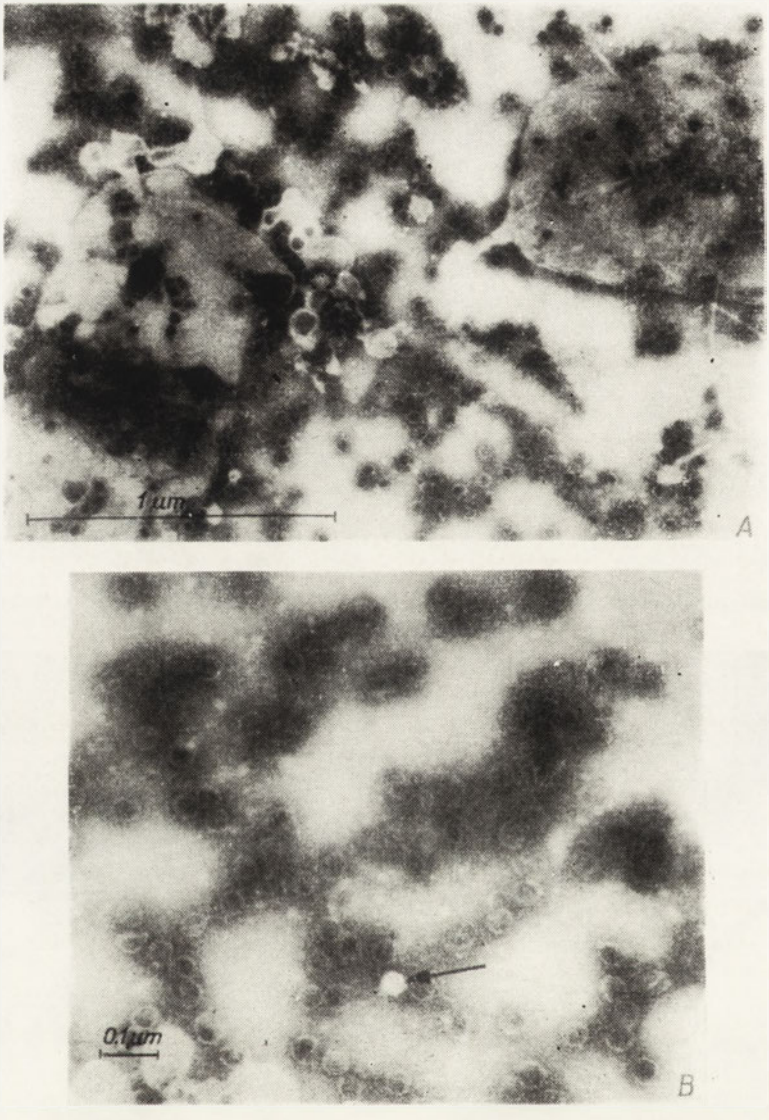


Fig. 3. *A*, Electron micrograph of the negatively stained preparation showing the interaction of Vi-phage III with cell walls in mSB-$Ca^{2+}$ medium; all phages are devoid of DNA and most of them are detached from the cells walls. *B*, The enlargement of detached Vi-phage III ghosts; the arrow points to intact phage.

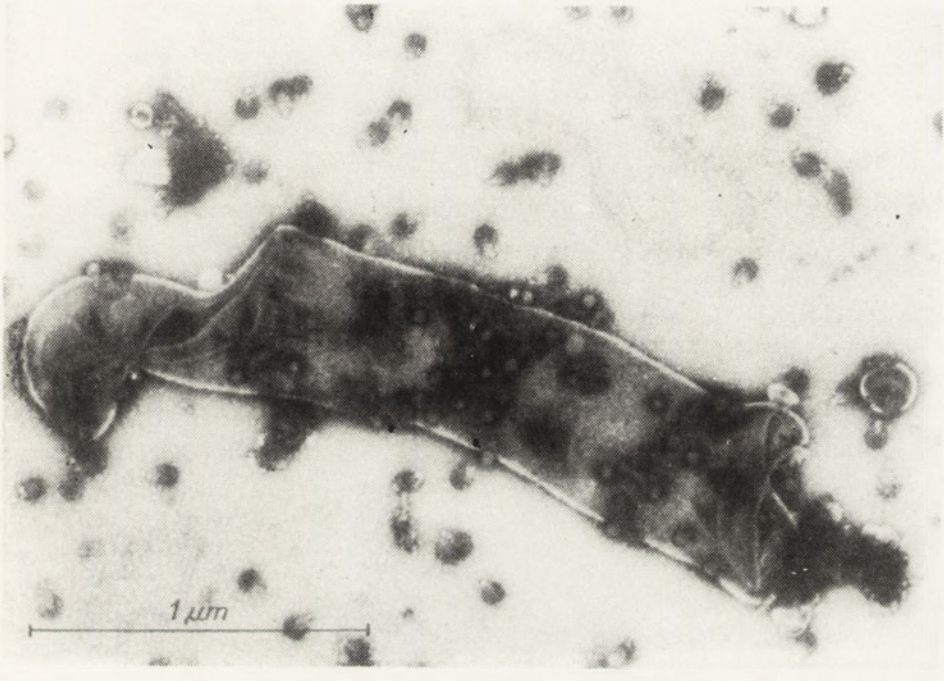


Fig. 4. Electron micrograph of the negatively stained preparation showing the Vi-phage III attachment to cell walls in veronal-$Ca^{2+}$ buffer; the majority of attached phages retain DNA.

## DISCUSSION

Vi-phage III attachment to *S. typhi* cells results in phage DNA injection and ghost release. The use of isolated Vi-receptor and cell walls in different media provides the possibility of arresting penetration of Vi-phage III at particular steps. The first specific contact of Vi-phage III with the bacterial cell is realized by the Vi-polysaccharide deacetylase (Szczeklik *et al.*, 1974) similarly as it is for Vi-phage I (Kwiatkowski & Taylor, 1970 ) and Vi-phage II (Taylor, 1965). This step of penetration may be best observed using the purified Vi-receptor adsorbed on the surface of red blood cells in veronal-$Ca^{2+}$ buffer at 0°C. After the elevation of temperature to 37°C, deacetylation of Vi-polysaccharide and release of infectious phage could be demonstrated. Under these conditions the behaviour of Vi-phage III differed from that of Vi-phage I and II, its attachment being influenced by $CaCl_2$ and components of mSB medium in a manner not clearly understood.

The next steps of attachment may be followed using cell walls. In veronal-$Ca^{2+}$ Vi-phage III was inactivated by cell walls, and electron micrographs showed that it was attached to them but did not release DNA. Therefore it seems that in the natural infection $Ca^{2+}$ may be needed for the step of attachment following Vi-polysaccharide deacetylation, which involves transitional phage binding with the cell, or capsid change promoting DNA release in a next step. It is known that in a variety of bacteriophages, divalent cations are required for the different steps of penetration, e.g. for attachment (Newbold & Sinsheimer, 1970), capsid rearrangement (Incardona *et al.*, 1972) or DNA penetration (Watanabe & Takesue, 1972).

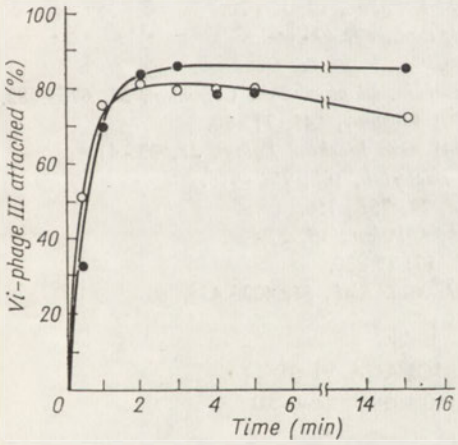


Fig. 5. Vi-phage III attachment to *S. typhi* cells in: (○), mSB, and (●), mSB-$Ca^{2+}$ media.

In mSB medium supplemented with $CaCl_2$ the attachment of Vi-phage III to cell walls was followed by DNA release and phage ghost detachment, whereas these processes did not occur in mSB not supplemented with $CaCl_2$. Apparently, the mSB-$Ca^{2+}$ medium contained some component(s) indispensable for phage DNA release. As the non-supplemented mSB medium proved adequate for propagation of Vi-phage III, it seems that this phage requires for growth only very small

amounts of calcium, which could be accumulated on the surface of growing bacteria from trace contaminations with $Ca^{2+}$, whereas larger amounts of calcium are required for the attachment and DNA release. If, as proposed by Costerton (1970), $Ca^{2+}$ is accumulated by acidic polysaccharide of gram-negative bacteria, it is to a large extent removed together with the polysaccharide during preparation of the cell walls. This would explain both the above observations and the finding that Vi-phage III attachment to intact bacteria proceeds equally well in mSB as in mSB-$Ca^{2+}$. It should be noted that mSB non-supplemented with $CaCl_2$ proved adequate for binding of Vi-phage I, which may indicate that the latter phage requires less calcium for its attachment than does Vi-phage III.

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## ADSORPCJA BAKTERIOFAGA Vi III
### REAKCJA FAGA ZE ŚCIANAMI KOMÓRKOWYMI

Streszczenie

Przy zastosowaniu różnych środowisk reakcji można obserwować poszczególne stadia wiązania się faga Vi III ze ścianą *Salmonella typhi*. W buforze weronalowym uzupełnionym jonami $Ca^{2+}$ inaktywacja faga polega na jego związaniu się ze ścianą, jednak bez utraty DNA. W pożywce mSB-$Ca^{2+}$ następuje wiązanie się faga Vi III ze ścianą komórkową, wypływ DNA i uwolnienie pustych cieni fagowych.

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# CHARACTERIZATION OF DNA POLYMERASE ACTIVITY IN ISOLATED PEA NUCLEI*

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TTP is incorporated into DNA by nuclei isolated from pea root tips. The reaction is stimulated by the presence of the other three deoxynucleoside-5′-triphosphates. ATP in concentration greater than 0.5 mM strongly inhibits the incorporation. Exogenous denatured DNA is an effective template for DNA synthesis. *In vivo* treatment of root tips with cycloheximide does not influence the DNA polymerase activity in isolated nuclei.

Nuclei isolated from various animal tissues can incorporate DNA precursors into acid-insoluble material (Lagunoff, 1969; Probst *et al.*, 1972). Recently Hershey *et al.* (1973) have shown that DNA synthesis observed in isolated HeLa nuclei could be a continuation of the *in vivo* process, however, for the majority of other systems studied it is not clear whether the *in vitro* process is a direct continuation of native replication or is a sum of repair and terminal addition processes. On the other hand, Chang *et al.* (1973) reported that a considerable part of DNA polymerase activity was found in the cytoplasmic fraction, and that the cytoplasmic DNA polymerase differed in molecular weight (6 - 8S) and other properties from nuclear 3.4S polymerase.

The aim of the present work was to investigate some properties of the nuclear DNA-synthesizing system obtained from pea root meristem.

## MATERIALS AND METHODS

*Root tips.* From 4-day-old pea seedlings (*P. sativum*, var. Kujawski), root tips (0.3 - 0.5 cm) were cut and immediately used. In the experiments with cycloheximide, the root tips before processing were preincubated for 2.5 h with cycloheximide (250 μg/ml).

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*Isolation of nuclei.* Nuclei were prepared by the method of Sadgopal & Bonner (1970). In the experiments with cycloheximide the homogenization buffer and 2.0 M-sucrose solution contained additionally 250 μg/ml of cycloheximide.

*Enzyme assays.* The activity of DNA polymerase was measured in 10 mm × 80 mm glass tubes, at 34°C. The standard reaction mixture in a final volume of 200 or 750 μl contained: 25 mM-Tris-HCl buffer (pH 8.0), 5 mM-EDTA, 25 mM-KCl, 4 mM-magnesium acetate, 5 mM-2-mercaptoethanol, 15% glycerol, dATP, dCTP and dGTP, 0.045 mM each, 0.002 mM-[$^3$H]TTP (spec. radioact. 28 Ci/mmol), and the nuclear preparation containing about 30 μg of DNA. After an appropriate period of time, 150 μl samples were taken and spotted on filter paper discs (Whatman 3 *MM*). The discs were washed sequentially at 0°C with: 10% trichloroacetic acid (TCA) in 0.1 M-sodium pyrophosphate (60 min); 5% TCA in 0.1 M-sodium pyrophosphate (30 min); 5% TCA in water (30 min); ethanol-ethyl ether mixture, 1:1, v/v (10 min); ethanol - ethyl ether, 1:3, v/v (10 min); ethyl ether (10 min). Liquid scintillation counting was performed in Nuclear Chicago Isocap 300 Scintillation Counter, as described previously (Jerzmanowski & Toczko, 1973)

*Determination of DNA.* DNA concentration was measured by the method of Burton (1956) using calf thymus DNA as standard.

*Reagents.* Unlabelled deoxyribonucleoside triphosphates were from Sigma (St. Louis, Mo., U.S.A.), ATP was obtained from Serva (Heidelberg, West Germany), calf thymus DNA was from Worthington Corp. (Freehold, N.J., U.S.A.); it was denatured by heating a sample containing 1000 μg DNA/ml of water in a boiling-water bath for 15 min, followed by rapid cooling. [$^3$H]TTP (spec.radioactivity 28 Ci/mmol) was from the Radiochemical Centre (Amersham, England) and [$^3$H]thymidine (spec. radioactivity 18 Ci/mmol) from UVVVR (Prague, Czechoslovakia). Other reagents were analytical grade products supplied by Polskie Odczynniki Chemiczne (Gliwice, Poland).

## RESULTS

The purity of nuclei obtained from pea root tips was checked both by light microscopy and by determination of the DNA/protein ratio. The preparation contained no intact cells and the DNA /protein ratio was 1:3, which is typical for nuclei.

The ability of the nuclear preparation to synthesize DNA was measured in the standard incubation mixture by the incorporation of [$^3$H]TTP into the acid-insoluble material (Fig. 1). In the range of concentrations studied, the incorporation was linear with the amount of nuclei (up to 120 μg of nuclear DNA). When dATP, dCTP and dGTP were omitted from the reaction mixture, the incorporation of [$^3$H]TTP was smaller and the time-course was linear only for 30 min instead of at least 50 min as in the standard incubation mixture (Fig. 2). On replacement of [$^3$H]TTP by the same amount of [$^3$H]thymidine, the incorporation of radioactivity was lower by a half, the time-course in both cases being similar. The incorporation of [$^3$H]thymidine into DNA observed in the preparation of isolated nuclei points to the pre

sence of active thymidine kinase. This activity was also observed in animal nuclear systems (Lagunoff, 1969).

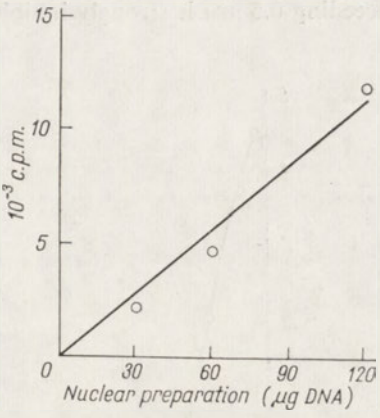


Fig. 1. Effect of the amount of the nuclear preparation from pea root tips on DNA synthesis, measured by [$^3$H]TTP incorporation. The incubation was carried out for 35 min in the standard reaction mixture; final volume 200 μl. For details see Methods.

To check whether the preparation of pea nuclei contained any DNA polymerase activity undetected with endogenous DNA, denatured calf thymus DNA was used as an additional primer. In the standard reaction mixture with the added exogenous DNA, the incorporation of radioactivity was twice as high as in the presence only of the endogenous primer (Fig. 3).

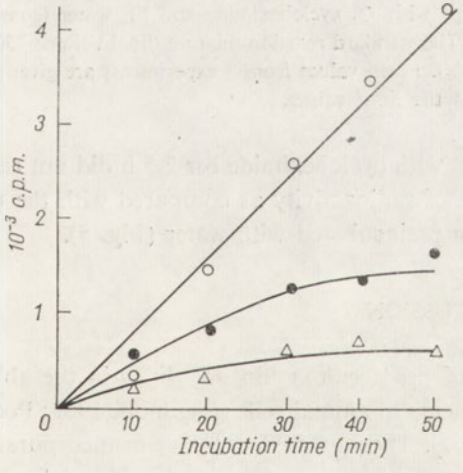


Fig. 2

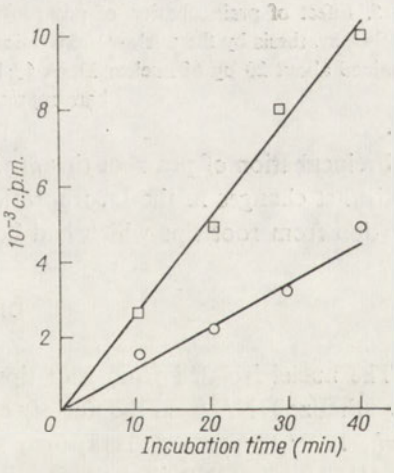


Fig. 3

Fig. 2. Rate of [$^3$H]thymidine and [$^3$H]TTP incorporation into DNA by the nuclear preparation from pea root tips. ○, Standard reaction mixture; ●, [$^3$H]TTP without dATP, dCTP and dGTP; △, [$^3$H]thymidine alone (0.002 mM, spec. act. 18 Ci/mmol), without the four deoxynucleoside triphosphates. Final volume of the reaction mixture was 750 μl.

Fig. 3. Effect of exogenous denatured DNA on DNA synthesis by the nuclear preparation from pea root tips. ○, Standard reaction mixture; □, with the addition of 20 μg of denatured calf thymus DNA. Final volume of the reaction mixture was 750 μl.

Although the purified DNA polymerases are known not to require ATP, the majority of nuclear systems from animal cells are ATP-dependent. For the activity of the preparation from pea nuclei ATP was not required, and at concentrations exceeding 0.5 mM it strongly inhibited DNA synthesis (Fig. 4).

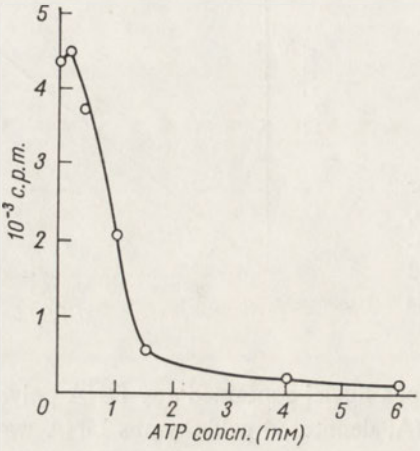


Fig. 4

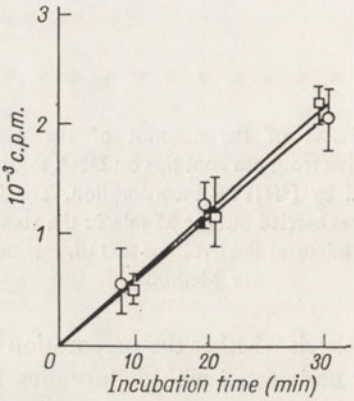


Fig. 5

Fig. 4. Effect of ATP on DNA synthesis by the nuclear preparation from pea root tips. The standard reaction mixture (final volume 200 µl) was incubated for 35 min with the indicated amounts of ATP.

Fig. 5. Effect of preincubation of pea root tips with ○, cycloheximide and □, water (control), on DNA synthesis by the nuclear preparation. The standard reaction mixture (final volume 750 µl) contained about 20 µg of nuclear DNA (±10%). Mean values from 3 experiments are given; the bars represent the limit values.

Preincubation of pea root tips *in vivo* with cycloheximide for 2.5 h did not result in distinct changes in the incorporation of radioactivity as compared with the preparation from root tips which had been preincubated with water (Fig. 5).

## DISCUSSION

The nuclei isolated from root tips of pea seedlings do not differ in the ability to synthesize DNA from the nuclear systems of animal cells (Lagunoff, 1969; Probst *et al.*, 1972; Shimada & Terayama, 1972). The observed decrease in incorporation of [$^3$H]TTP into DNA in the absence of the other three deoxynucleoside triphosphates indicates that replication rather than terminal addition of deoxynucleotides is involved. The fact that in the absence of dATP, dCTP and dGTP the rate of DNA synthesis was linear for a shorter time as compared with the synthesis in their presence, suggests that the observed decrease in [$^3$H]TTP incorporation was due mainly to exhaustion of endogenous precursors. The stimulation of DNA synthesis on addition of the exogenous DNA primer to the complete system indicates that the amount of DNA polymerase was not a limiting factor. Probst *et al.* (1972) demon-

strated that in rat liver nuclei there are some minor differences between products primed by endogenous and exogenous DNA. As concerns pea nuclei, we cannot say at the present time whether the DNA synthesized with the exogenous primer differs from that synthesized with the endogenous one.

The strong inhibitory effect of ATP on [$^3$H]TTP incorporation is difficult to explain. It could be due either to the specificity of plant nuclear system or to the observed incorporation being the ATP-independent repair process. The latter supposition is supported by the observation that the pea nuclear system is insensitive to preincubation of root tips with cycloheximide, which is known to inhibit the native DNA synthesis by about 50% (Jerzmanowski & Toczko, 1973).

Chang *et al.* (1973) have demonstrated that of the two different forms of eucaryotic DNA polymerase only the high-molecular-weight cytoplasmic polymerase undergoes the activity changes parallel to the *in vivo* changes of mitotic activity of the cell. The data presented in this work concerning the properties of nuclear polymerase, especially those showing the insensitivity of the polymerase activity to the *in vivo* treatment with cycloheximide and the inhibitory effect of ATP, confirm the suggestion that the polymerase present in nuclear preparations is not alone responsible for native replication.

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## CHARAKTERYSTYKA AKTYWNOŚCI POLIMERAZY DNA W IZOLOWANYCH JĄDRACH KOMÓRKOWYCH GROCHU

Streszczenie

Izolowane jądra komórkowe grochu włączają tymidyno-5′-trójfosforan do DNA. Reakcja jest stymulowana obecnością pozostałych trzech dezoksynukleozydo-5′-trójfosforanów. ATP w stężeniach większych niż 0.5 mM silnie hamuje reakcję. Egzogenny, zdenaturowany DNA jest efektywną matrycą w syntezie DNA. Preinkubacja tkanki grochu *in vivo* z cykloheksymidem nie wpływa na aktywność polimerazy DNA w izolowanych jądrach komórkowych.

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# THE INVOLVEMENT OF NADH-CYTOCHROME $b_5$ REDUCTASE AND CYTOCHROME $b_5$ COMPLEX IN MICROSOMAL NADH-CYTOCHROME $c$ REDUCTASE ACTIVITY

## RESOLUTION OF THE COMPLEX BY TRITON X-100*

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Triton X-100 added to microsomal suspension causes a breakdown and gradual solubilization of cytochrome $b_5$ and NADH-cytochrome $b_5$ reductase. The sensitivity to 4,4,4-trifluoro-1-(2-thienyl)-1,3-butadione (TFTB) of NADH oxidation by the solubilized system measured with cytochrome $c$ as an acceptor, is similar to the sensitivity of NADH oxidation by the unchanged system measured with exogenous cytochrome $b_5$. It is suggested that differences in the sensitivity to inhibitors of NADH-exogenous cytochrome $b_5$ reductase and NADH-cytochrome $c$ reductase are due to differences between reduction of membrane-bound and solubilized cytochrome $b_5$.

Microsomal NADH-cytochrome $b_5$ reductase and cytochrome $b_5$ exhibit the activity of NADH-cytochrome $c$ reductase (Strittmatter, 1968). However, Schulze *et al.* (1970) have found that the activity of NADH-exogenous cytochrome $b_5$ reductase and that of NADH-cytochrome $c$ reductase differed in the sensitivity to pCMB[1] and TFTB. This indicates the existence of two pathways of electron transport from NADH to cytochrome $c$ with only one pathway involving cytochrome $b_5$. Our previous work (Staroń & Kaniuga, 1972) has shown that Triton X-100 inhibited only partially NADH-cytochrome $c$ reductase activity. The activity unaffected by this detergent responded to pCMB and TFTB similarly as NADH-exogenous cytochrome $b_5$ reductase in intact microsomes.

* This work was supported in part by a grant from the Committee of Biochemistry and Biophysics of the Polish Academy of Sciences.

[1] Abbreviations: pCMB, *p*-chloromercuribenzoate; TFTB, 4,4,4-trifluoro-1-(2-thienyl)-1,3-butadione.

The results of the present study show similarity in the properties of NADH--exogenous cytochrome $b_5$ reductase and NADH-cytochrome *c* reductase in Triton X-100 treated microsomes.

## MATERIAL AND METHODS

*Animals.* Albino male Wistar rats (100 - 200 g) starved for 24 hours were killed by decapitation. Livers were removed, washed with ice-cold 0.25 M-sucrose, dried with filter paper and immediately used for preparation of microsomes.

*Preparation of microsomes.* Microsomes were obtained according to the method described previously (Staroń & Kaniuga, 1971) including chromatography on Sepharose 2B.

*Centrifugation of detergent-treated microsomes.* Samples of microsomes suspended in 0.1 M-sodium phosphate buffer, pH 7.4 (9 ml) were treated with 1 ml aqueous solution of Triton X-100 (1 ml, final concentration from 0.05 to 0.5%); 1 ml of water was added to the control sample. Final concentration of protein was about 1.5 mg/ml. Samples were centrifuged in a VAC-60 (H. Janetzki, Leipzig, G.D.R.) centrifuge for 90 min at 105 000 *g*. The pellet was suspended in 5 ml of 0.1 M-sodium phosphate buffer, pH 7.4.

*Chromatography of detergent-treated microsomes.* Samples of microsomes suspended in 0.1 M-sodium phosphate buffer, pH 7.4 (11.7 ml) were treated with aqueous solution of Triton X-100 (1.3 ml, final concentration from 0.15 to 0.75%); 1.3 ml of water was added to the control sample. Samples were applied on the Sepharose 2B column (4 × 20 cm) equilibrated with 0.1 M-sodium phosphate buffer, pH 7.4. Subsequent elution was performed with the same buffer. Samples of about 4.5 ml were collected.

*Determination of enzymic activities.* NADH-cytochrome *c* reductase and NADH--ferricyanide reductase activities were determined at 25°C according to Takesue & Omura (1970). TFTB in methanol was added directly to the cuvette and incubated at 25°C for 5 min.

*Determination of cytochrome* $b_5$. Cytochrome $b_5$ was determined from the absorption difference between reduced and oxidized form at 425 and 410 nm under conditions described by Nebert (1970), using 0.1 M-sodium phosphate buffer, pH 7.4. The samples were reduced with NADH or with $Na_2S_2O_4$.

*Protein* was estimated by the method of Lowry *et al.* (1951).

*Reagents.* The reagents used were as follows: NADH, Sigma (St. Louis, Mo., U.S.A.); Triton X-100, Serva (Heidelberg, G.F.R.); Sepharose 2B, Pharmacia (Uppsala, Sweden); TFTB (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) was a kind gift from Prof. Dr. J. F. Howland. Cytochrome *c* was obtained from bovine heart according to the method of Keilin & Hartree (1945) modified by Margoliash (1954).

## RESULTS

*Release of cytochrome* $b_5$ *and NADH-cytochrome* $b_5$ *reductase by Triton X-100.* Increasing concentrations of Triton X-100 result in the increased solubilization of microsomal proteins and their release into 105 000 *g* supernatant (Fig. 1). When $Na_2S_2O_4$ is used for measurement, the appearance of characteristic absorption ($\Delta A_{425-410\ nm}$, typical for cytochrome $b_5$) in the soluble fraction can be observed even with the lowest concentration of Triton X-100 used. When NADH is used for reduction instead of $Na_2S_2O_4$, $\Delta A_{425-410\ nm}$ absorption is observed with 0.2% or higher concentration of the detergent. Concentration of cytochrome $b_5$ was measured in the same time interval (1 min) after the addition of the reducing agent. The obtained results indicate that the complex of NADH-cytochrome $b_5$ reductase and cytochrome $b_5$ is broken down by Triton X-100 with a gradual release of cytochrome $b_5$ followed by the release of reductase.

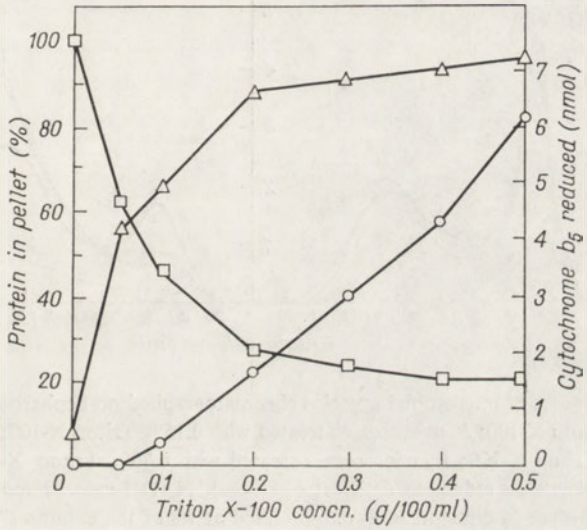


Fig. 1. The release by Triton X-100 of microsomal protein and $Na_2S_2O_4$- or NADH-reducible cytochrome $b_5$. (□), protein in pellet; (△), $Na_2S_2O_4$-reduced cytochrome $b_5$ in supernatant; (○), NADH-reduced cytochrome $b_5$ in supernatant.

The same order of release of the enzymes is observed when the samples of Triton X-100 treated microsomes are chromatographed on Sepharose 2B (Fig. 2). If the sample of microsomes treated with 0.15% Triton X-100 is applied to the column, cytochrome $b_5$ is found in the fraction of soluble proteins (Staroń & Kaniuga, 1971) (Fig. 2B, fraction *b*), while NADH-cytochrome $b_5$ reductase (measured with ferricyanide as an electron acceptor) is found in this fraction when microsomes treated with 0.30% Triton X-100 are analysed (Fig. 2C, fraction *b*). If 0.75% Triton X-100 is used, most of cytochrome $b_5$ and NADH-ferricyanide reductase activity is present in the soluble fraction (Fig. 2D, fraction *b*).

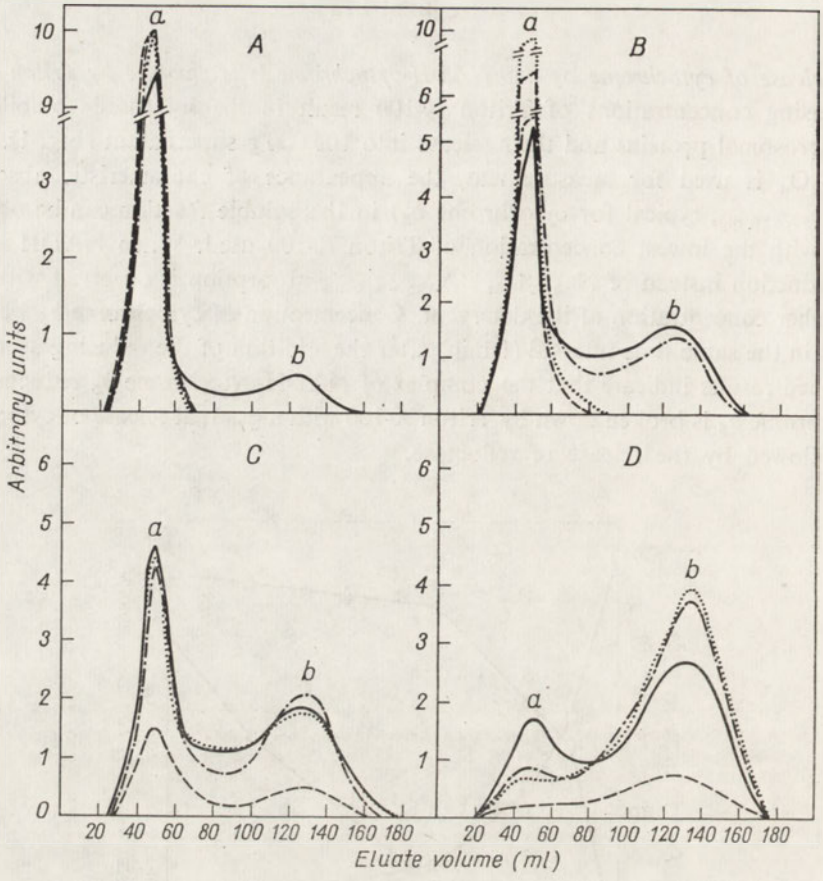


Fig. 2. Elution profiles of microsomal proteins chromatographed on Sepharose 2B: *A*, microsomes untreated with Triton X-100; *B*, microsomes treated with 0.15% Triton X-100; *C*, microsomes treated with 0.30% Triton X-100; *D*, microsomes treated with 0.75% Triton X-100. (—), protein; (···), NADH-ferricyanide reductase activity; (— — —), NADH-cytochrome *c* reductase activity; (—·—·—), cytochrome $b_5$ content. *a*, Fraction eluted at $V_0$ of the column ("insoluble" fraction); *b*, fraction eluted at $V_t$ of the column ("soluble" fraction).

NADH-cytochrome *c* reductase activity appears in the soluble fraction together with NADH-ferricyanide reductase activity (Fig. 2C, 2D).

*Effect of TFTB on NADH-cytochrome* c *reductase activity in the soluble fraction.* TFTB stimulates microsomal NADH-cytochrome *c* reductase activity (Schulze *et al.*, 1970) and inhibits partially the activity unaffected by Triton X-100 in high concentrations (0.5 - 1.0%) (Staroń & Kaniuga, 1972). In the preparations treated with 0.3% Triton X-100, only a small activation by TFTB is observed (Table 1). However, the NADH-cytochrome *c* reductase activity found in the soluble fraction isolated from microsomes treated with 0.3% Triton X-100 (see Fig. 2C, fraction *b*), is inhibited by TFTB (Table 1). The effect of TFTB on this enzymic activity in the

soluble fraction obtained from microsomes treated with 0.3% Triton X-100 is identical with that observed in microsomes treated with higher concentrations of Triton X-100.

Table 1

*Effect of TFTB on NADH-cytochrome* c *reductase activity in intact and solubilized microsomes*

The activity was measured with 2.5 mM-TFTB and expressed in relation to the activity of NADH-cytochrome *c* reductase activity in the absence of TFTB, taken as 100.

| Sample | Specific activity |
|---|---|
| A. Microsomes untreated with Triton X-100 | 143 |
| B. Microsomes treated with 0.30% Triton X-100 | 106 |
| "Insoluble" fraction (fraction *a*, Fig. 2) | 114 |
| "Soluble" fraction (fraction *b*, Fig. 2) | 85 |
| C. Microsomes treated with 0.75% Triton X-100 | 87 |

## DISCUSSION

The results of our previous work (Staroń & Kaniuga, 1972) indicated that the part of microsomal NADH-cytochrome *c* reductase activity which was unaffected by Triton X-100 in high concentration (0.5 - 1.0%) showed similar sensitivity to pCMB and TFTB as NADH-exogenous cytochrome $b_5$ reductase activity (Schulze *et al.*, 1970). The present study shows that Triton X-100 causes a breakdown of the complex of cytochrome $b_5$ and NADH-cytochrome $b_5$ reductase and a differential solubilization of these two enzymes. An inhibitory effect of TFTB on NADH-cytochrome *c* reductase activity is observed always in the soluble fractions, i.e. in the completely solubilized preparation, and in the soluble fraction obtained from partially solubilized preparation. On the other hand, inhibition of the reduction of cytochrome $b_5$ in intact microsomes (measured with NADH) by mersalyl, an inhibitor of sulfhydryl groups, is correlated with the inhibition of NADH-cytochrome *c* reductase activity (Franklin & Estabrook, 1971). The similar close correlation between the inhibition of the reduction of cytochrome $b_5$ and NADH-cytochrome *c* reductase activity was found in our laboratory with pCMB as the inhibitor of sulfhydryl groups (unpublished result).

The data presented in this work show that there is an essential similarity between the NADH-oxidizing system which is composed of intact microsomes and exogenous cytochrome $b_5$ as an electron acceptor, and the system containing Triton X-100 treated microsomes and cytochrome *c* as the acceptor. In both cases cytochrome $b_5$ is not attached to the membrane in contrast to the NADH-oxidizing system in the intact microsomes measured with cytochrome *c* as the electron acceptor. It is

suggested that the observed difference in the sensitivity to TFTB and pCMB of NADH--exogenous cytochrome $b_5$ reductase and NADH-cytochrome *c* reductase activities (Schultze *et al.*, 1970) is a result of different relation of cytochrome $b_5$ to NADH--cytochrome $b_5$ reductase in intact microsomes.

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## KOMPLEKS REDUKTAZY NADH-CYTOCHROM $b_5$ I CYTOCHROMU $b_5$ A AKTYWNOŚĆ MIKROSOMALNEJ REDUKTAZY NADH-CYTOCHROM *c*

### ROZBICIE KOMPLEKSU PRZEZ TRITON X-100

### Streszczenie

Triton X-100 powoduje rozbicie kompleksu cytochromu $b_5$ i reduktazy NADH-cytochrom $b_5$ i kolejne upłynnienie obu enzymów. Wrażliwość na TFTB utleniania NADH przez rozbity kompleks, mierzonego stopniem redukcji cytochromu *c*, jest podobna do wrażliwości utleniania NADH przez nienaruszony kompleks, mierzonego w obecności egzogennego cytochromu $b_5$ jako akceptora elektronów. Różnice pomiędzy redukcją związanego w błonie i upłynnionego cytochromu $b_5$ są najprawdopodobniej przyczyną różnic we wrażliwości na inhibitory reduktazy NADH-egzogenny cytochrom $b_5$ i reduktazy NADH-cytochrom *c*.

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# THE INVOLVEMENT OF NADH-CYTOCHROME $b_5$ REDUCTASE AND CYTOCHROME $b_5$ COMPLEX IN MICROSOMAL NADH-CYTOCHROME *c* REDUCTASE ACTIVITY

## CHANGES IN NADH-CYTOCHROME *c* REDUCTASE ACTIVITY FOLLOWING PHENOBARBITAL TREATMENT*

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Phenobarbital administered to rats decreases the total microsomal NADH-cytochrome *c* reductase activity in the system in which NADH-cytochrome $b_5$ reductase and cytochrome $b_5$ are attached to the membrane. The part of NADH-cytochrome *c* reductase activity found in the solubilized system is constant and correlated with NADH-ferricyanide reductase activity and cytochrome $b_5$ content. The phenobarbital-induced changes in the sensitivity of NADH-cytochrome *c* reductase to trypsin permit to assume changes in the vicinity of membrane-bound cytochrome $b_5$. These changes seem to be responsible for the lack of correlation between NADH-ferricyanide reductase activity, cytochrome $b_5$ content and total NADH-cytochrome *c* reductase activity.

Phenobarbital administered to rats results in a decrease of NADH-cytochrome *c* reductase activity whereas little or no changes are observed in the content of the enzymes responsible for this activity, i.e. cytochrome $b_5$ (Orrenius *et al.*, 1965) and NADH-cytochrome $b_5$ reductase (Estabrook & Cohen, 1969). On the other hand, if actinomycin D or puromycin are administered together with phenobarbital, the changes induced by phenobarbital in microsomes fail to develop (Orrenius *et al.*, 1965).

NADH-cytochrome *c* reductase activity is partially inhibited by Triton X-100 (Staroń & Kaniuga, 1972). The activity unaffected by the detergent represents the activity of solubilized NADH-cytochrome $b_5$ reductase and cytochrome $b_5$ (Staroń & Kaniuga, 1974). On the other hand, if both enzymes are attached to the mem-

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brane, the NADH-cytochrome *c* reductase activity is stimulated by trypsin digestion (Orrenius *et al.*, 1969).

These two properties of NADH-cytochrome *c* reductase activity were taken into consideration in the present work in an attempt to explain the lack of correlation between NADH-cytochrome *c* reductase activity and both NADH-cytochrome $b_5$ reductase and cytochrome $b_5$.

Preliminary reports of the results have been presented (Kaniuga & Staroń, 1972; Staroń *et al.*, 1972).

## MATERIAL AND METHODS

*Animals.* Albino male Wistar rats (100 - 200 g) starved for 24 hours were used for the experiments. When indicated, the animals were pretreated with two doses of phenobarbital (100 mg/kg/day) or with two corresponding doses of 0.9% NaCl. The agents were administered intraperitoneally in a volume of 1 ml/100 g. Animals were killed by decapitation. Livers were removed, washed with ice-cold 0.25 M-sucrose, dried with filter paper and immediately used for preparation of microsomes.

*Preparation of microsomes.* Microsomes were obtained according to the method described previously (Staroń & Kaniuga, 1971) including chromatography on Sepharose 2B. In the experiments with trypsin the livers were homogenized in 0.25 M-sucrose in 16 mM-Tris-HCl buffer, pH 7.4, containing 25 mM-KCl instead of the originally used 0.25 M-sucrose in 0.1 M-sodium phosphate buffer, pH 7.4. In this case the Tris-KCl buffer was also used for Sepharose 2B chromatography.

*Trypsin digestion.* Samples containing trypsin (specific activity 2 Kunitz units per mg) and microsomes (final concentration 1.5 mg protein per ml) in Tris-KCl buffer were incubated at 25°C. After 15 min a twofold excess of soyabean trypsin inhibitor was added. Two kinds of samples were used as control: without trypsin, and with trypsin inhibitor added before addition of trypsin.

*Determination of enzymic activities.* NADH-cytochrome *c* reductase and NADH-ferricyanide reductase activities were determined at 25°C according to Takesue & Omura (1970). NADH-cytochrome *c* reductase activity unaffected by Triton X-100 was determined in the microsomal suspension after the addition of 7.5% Triton X-100 to final concentration of 0.75%.

*Determination of cytochrome* $b_5$. Cytochrome $b_5$ was determined from the difference in absorption of reduced and oxidized form at 425 and 410 nm under conditions described by Nebert (1970), using 0.1 M-sodium phosphate buffer, pH 7.4. The samples were reduced by NADH.

*Protein* was estimated by the method Lowry *et al.* (1951).

*Reagents.* The reagents used were as follows: NADH, Sigma (St. Louis, Mo., U.S.A.); Triton X-100 and trypsin, Serva (Heidelberg, G.F.R.); soyabean trypsin inhibitor, Calbiochem (Los Angeles, Calif., U.S.A.); Sepharose 2B, Pharmacia (Uppsala, Sweden); phenobarbital, Polfa (Tarchomin, Poland). Cytochrome *c* was obtained from bovine heart according to the method of Keilin & Hartree (1945) modified by Margoliash (1954).

## RESULTS

*Relationship between NADH-cytochrome* c *reductase activity, cytochrome* $b_5$ *content and NADH-ferricyanide reductase activity.* Phenobarbital administered to rats causes a decrease in total NADH-cytochrome *c* reductase activity expressed in moles of reduced cytochrome *c*/min/mol of cytochrome $b_5$ as compared with control or saline-treated rats (Table 1). Only the activity responding to Triton X-100 treatment accounts for this fall; the activity insensitive to the detergent is not affected by phenobarbital.

Table 1

*Effect of phenobarbital on microsomal NADH-cytochrome* c *reductase activity*

The values are average of 8 experiments.

| Treatment | NADH-cytochrome *c* reductase activity (mol reduced cyt. *c*/min/mol cyt. $b_5$) | | | The activity ratio: Triton X-100 sensitive/Triton X-100 insensitive |
|---|---|---|---|---|
| | Total | Triton X-100 | | |
| | | sensitive | insensitive | |
| None | 1570 | 1210 | 360 | 3.35 |
| Saline | 1620 | 1240 | 380 | 3.25 |
| Phenobarbital | 1150 | 770 | 380 | 2.00 |

A similar relationship is observed when NADH-cytochrome $b_5$ reductase activity, measured with ferricyanide as an electron acceptor, is compared with NADH-cytochrome *c* reductase activity. NADH-ferricyanide reductase activity is not changed upon phenobarbital treatment. In consequence, the ratio of NADH-ferricyanide reductase activity to the Triton X-100 inhibited NADH-cytochrome *c* reductase activity increases after phenobarbital treatment (Table 2). On the other hand, the activity ratio of NADH-ferricyanide reductase to the Triton X-100 insensitive NADH-cytochrome *c* reductase is independent of phenobarbital treatment.

Table 2

*Effect of phenobarbital on the NADH-ferricyanide reductase/NADH-cytochrome* c *reductase activity ratio*

The values are the average of 4 experiments.

| Treatment | Ratio of the reductase activities: NADH-ferricyanide/NADH-cyt. *c* | |
|---|---|---|
| | Triton X-100 | |
| | sensitive | insensitive |
| Saline | 5.1 | 18 |
| Phenobarbital | 8.1 | 17 |

*Effect of trypsin on NADH-cytochrome* c *reductase.* Figure 1 illustrates the effect of trypsin on NADH-cytochrome *c* reductase activity. Under the conditions described in Material and Methods, a slight stimulation of the total activity of NADH-cytochrome *c* reductase is observed at the concentration of 10 μg of trypsin per 1 ml (curve *A*), but only the NADH-cytochrome *c* reductase activity sensitive to Triton X-100 is responsible for this stimulation (curve *B*). The activity not responding to the detergent is even somewhat decreased (curve *C*).

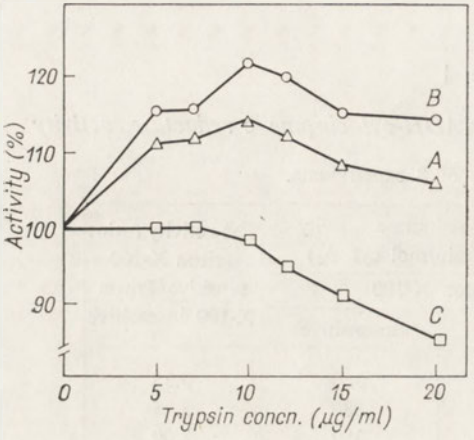


Fig. 1

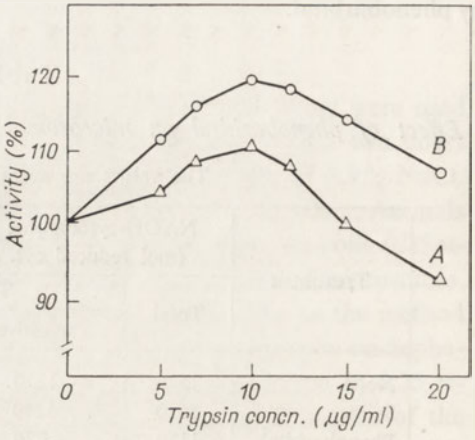


Fig. 2

Fig. 1. Effect of trypsin on NADH-cytochrome *c* reductase activity. *A*, Total activity; *B*, Triton X-100 sensitive activity; *C*, Triton X-100 insensitive activity. The activity is expressed in % of specific activity in the control sample.

Fig. 2. Effect of phenobarbital on the sensitivity of NADH-cytochrome *c* reductase activity to trypsin. *A*, Control; *B*, phenobarbital-treated. The activity is expressed in % of specific activity in the control sample.

Phenobarbital treatment decreases the ratio of the Triton X-100 sensitive to the Triton X-100 insensitive NADH-cytochrome *c* reductase activity (Table 1). However, this decrease is accompanied by the increased stimulation of NADH-cytochrome *c* reductase activity by trypsin (Fig. 2) from 11% for control animals to 20% for the phenobarbital-treated ones. (The values of six control experiments ranged from 8% to 13%; the values of four phenobarbital experiments ranged from 18% to 25%).

## DISCUSSION

Changes induced by phenobarbital in the activities of microsomal enzymes result from changes in the population of particular proteins (Orrenius *et al.*, 1965; Dehlinger & Schimke, 1972). It has been shown (Orrenius *et al.*, 1965) that the increase in the activity of the oxidative demethylation of aminopyrine caused by phenobarbital is closely correlated with the increase of NADPH-cytochrome *c* reductase activity

and the content of cytochrome P-450, which are responsible for the oxidative demethylation of aminopyrine in microsomes. A similar correlation was found in this work when NADH-cytochrome $b_5$ reductase (measured as NADH-ferricyanide reductase), cytochrome $b_5$ content and the Triton X-100 insensitive NADH-cytochrome *c* reductase activity were compared. The Triton X-100 sensitive NADH--cytochrome *c* reductase activity was not correlated either with NADH-ferricyanide reductase activity or with cytochrome $b_5$ content. This part of the reductase activity, however, does not seem to be connected with the additional pathway of electron transport from NADH to cytochrome *c*, as suggested by the following facts: a) phenobarbital treatment results in the increase of trypsin-dependent stimulation of total NADH-cytochrome *c* reductase activity; b) phenobarbital treatment decreases the reductase activity responding to trypsin stimulation. Therefore it may be supposed that the phenobarbital-induced changes in total NADH-cytochrome *c* reductase activity are the result of changes in the vicinity of cytochrome $b_5$ due to modification of the interaction between cytochrome $b_5$ and cytochrome *c*. The modification may be caused by some protein factor. In this case the stimulating effect of trypsin on total NADH-cytochrome *c* reductase activity could be due to solubilization of this modifying factor. Thus, an increase in the amount of this factor by phenobarbital treatment might result in a decrease of total NADH-cytochrome *c* reductase activity on one hand and, on the other, in an increase of stimulation caused by trypsin. It is possible that NADPH-oxidizing enzymes, i.e. NADPH-cytochrome *c* reductase and cytochrome P-450, could be identical with the modifying factor. Both of them are localized in the vicinity of cytochrome $b_5$ (Orrenius *et al.*, 1969; Hildebrandt & Estabrook, 1971) and both are induced upon phenobarbital treatment (Orrenius *et al.*, 1965; Dehlinger & Schimke, 1972). NADPH-cytochrome *c* reductase should be especially taken into consideration since its solubilization by trypsin precedes solubilization of cytochrome $b_5$ as well as inhibition of NADH-cytochrome $b_5$ reductase (Orrenius *et al.*, 1969).

The data presented in this and preceding paper (Staroń & Kaniuga, 1974) show that microsomal NADH-cytochrome *c* reductase activity is connected with one enzymic system composed of NADH-cytochrome $b_5$ reductase and cytochrome $b_5$. The properties of the membrane-bound system differ from those of the solubilized system. First, the solubilization causes partial inhibition of NADH-cytochrome *c* reductase activity, probably resulting from the change in the rate of cytochrome $b_5$ reduction by NADH-cytochrome $b_5$ reductase (Hara & Minakami, 1971). Secondly, the solubilized system differs from the membrane-bound one in the sensitivity to *p*-chloromercuribenzoate and 4,4,4-trifluoro-1-(2-thienyl)-1,3-butadione. Thirdly, the reduction of cytochrome *c* by the membrane-bound system is dependent on the phenobarbital-inducible factor.

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## KOMPLEKS REDUKTAZY NADH-CYTOCHROM $b_5$ I CYTOCHROMU $b_5$ W MIKROSOMALNEJ AKTYWNOŚCI REDUKTAZY NADH-CYTOCHROM *c*

### ZMIANY AKTYWNOŚCI REDUKTAZY NADH-CYTOCHROM *c* PO PODANIU LUMINALU

### Streszczenie

Podanie szczurom 5-etylo-5-fenylobarbituranu (luminalu) powoduje spadek całkowitej aktywności mikrosomalnej reduktazy NADH-cytochrom *c* w układzie, w którym reduktaza NADH-cytochrom $b_5$ i cytochrom $b_5$ są związane z błonami. Część aktywności odpowiadająca upłynnionemu układowi reduktazy NADH-cytochrom $b_5$ i cytochromu $b_5$ nie ulega zmianie i jest skorelowana z aktywnością reduktazy NADH-żelazicyjanek i z zawartością cytochromu $b_5$. Powodowane przez luminal zmiany wrażliwości reduktazy NADH-cytochrom *c* na trypsynę pozwalają przypuszczać, że za brak korelacji pomiędzy aktywnością reduktazy NADH-żelazicyjanek i zawartością cytochromu $b_5$ a całkowitą aktywnością reduktazy NADH-cytochrom *c* odpowiedzialne są zmiany zachodzące w błonie w otoczeniu cytochromu $b_5$.

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# EFFECT OF UREA ON THE THERMAL DENATURATION OF PARTIALLY DEHISTONIZED CHROMATIN*

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Derivatives of melting profiles of calf thymus chromatin depleted of histone *f1* or all lysine-rich histones, were determined at pH 7.0 in 0.25 mM-EDTA and in EDTA supplemented with 3 M- or 5 M-urea. For either preparation, in the absence of urea two melting bands, and in its presence three bands were obtained. These results seem to indicate that hydrophobic interactions between DNA and arginine-rich histones (*f3*, *f2a1*) predominate over those between DNA and lysine-rich histones (*f1*, *f2a2*, *f2b*).

The spatial arrangement of histones in chromatin and the character of forces maintaining the complexes of nucleic acid and histones is studied in several laboratories. One of the major tools used for these studies is examination of the thermal stability of DNA double-helix. It has been shown by several authors (Li & Bonner, 1971; Ansevin *et al.*, 1971; Subirana, 1973) that derivatives of melting profiles of chromatin are distinctly different from that for free DNA. The histone to DNA ratio appears to be the most important parameter to define the denaturation properties of a chromatin preparation (Subirana, 1973). The shape of melting curves also depends on what kinds or how much histone has been removed from chromatin (Li & Bonner, 1971). Apart from histones, also various other factors (pH, ionic strength, urea etc.) influence the behaviour of chromatin denaturation (Li & Bonner, 1971; Ansevin *et al.*, 1971; Subirana, 1973).

In this paper the effect of urea concentration on thermal denaturation of chromatin preparations was studied in an attempt to determine the contribution of hydropho-

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bic interactions between DNA and particular histones. For these investigations the calf thymus chromatin depleted of histone *f1* and of all lysine-rich histones (*f1*, *f2a2*, *f2b*) was used.

## MATERIALS AND METHODS

*Chromatin.* Calf thymus chromatin was prepared as described by Marushige & Bonner (1966) except that ultracentrifugation through 1.7 M-sucrose was omitted.

*Chromatin depleted of histone* f1. This was obtained according to Ohlenbush *et al.* (1967) using chromatography on Sepharose 4B instead of ultracentrifugation to separate the dissociated histone *f1*. Chromatin preparation equivalent to 300 extinction units at 260 nm was suspended in 10 ml of deionized water, sheared in the Unipan type 203 homogenizer for 90 sec at 120 V and centrifuged at 12 000 *g* for 30 min. To the supernatant (10 ml) was added 40 ml of 0.75 M-NaCl (final concentration 0.6 M-NaCl), stirred for 2 h at room temperature, then 30 ml of the solution was applied to Sepharose 4B column (35 × 3.5 cm, $V_t$ 150 ml). The elution was performed with 0.6 M-NaCl at a rate of 35 ml/h. Fractions of 4 - 5 ml were collected and the extinction at 260 and 230 nm was measured. The chromatin fractions (eluted in the range 0.3 - 0.6 $V_e/V_t$) were combined, the nucleoprotein precipitated with two volumes of cold ethanol and stored at 0°C.

*Chromatin depleted of all lysine-rich histones (f1, f2a2, f2b).* Chromatin preparation equivalent to 300 $E_{260}$ units was suspended in 50 ml of 0.8 M-sodium phosphate - 2 M -urea buffer of pH 5.6, sheared as above and after 12 h at 18 - 20°C centrifuged at 12 000 *g* for 30 min. The dissociated histones were separated from chromatin by chromatography on Sepharose 4B column. For equilibration of the column and elution 0.8 M-sodium phosphate - 2 M-urea, pH 5.6. was used. The pooled chromatin fractions were dialysed against 100 vol. of 0.14 M-NaCl, then the nucleoprotein was precipitated with two volumes of cold ethanol and stored at 0°C.

*Derivatives of melting profiles.* The samples of depleted chromatin or DNA were dissolved in 0.25 mM-EDTA supplemented with 3 M- or 5 M-urea, pH 7.0, and dialysed for 16 h at 0°C against the same solution immediately before thermal denaturation. The rate of temperature increase was 1°C/min. Hyperchromicity ($h_{260}$) was calculated by dividing absorbance at a given temperature by the value observed at room temperature. Derivatives of melting profiles $\left(\frac{dh_{260}}{dT} \textit{ vs.} T\right)$ were found from melting curves ($h_{260}$ *vs.*T) according to the equation of Li & Bonner (1971):

$$\frac{dh_{260}}{dT} = \frac{h_{260}(T+1) - h_{260}(T-1)}{2}$$

where $h_{260}(T)$ is the hyperchromicity at 260 nm and at temperature T.

*Determination of DNA and histones.* DNA was determined according to Munro & Fleck (1966) assuming that 1 $E_{260}$ unit is equivalent to 44.3 μg DNA. Histones were

twice extracted from chromatin with 0.3 N-HCl for 30 min at 0°C and determined by the procedure of Lowry *et al.* (1951) using as standard bovine serum albumin.

*Electrophoresis.* Gel electrophoresis of histones was performed by the method of Johns (1967) as described by Toczko *et al.* (1972).

*Reagents.* DNA was prepared from calf thymus according to Stern (1968). Sepharose 4B was from Pharmacia (Uppsala, Sweden); acrylamide, *NN*-bisacrylamide and TEMED were from Serva (Heidelberg, G. F.R.). Bovine serum albumin was from Edward Gurr Ltd (London, England). All other chemicals were from Polskie Odczynniki Chemiczne (Gliwice, Poland).

## RESULTS AND DISCUSSION

Chromatin depleted of particular histones was obtained by treatment with NaCl solution and with the sodium phosphate-urea system recommended by Bartley & Chalkley (1972), and isolated by column chromatography on Sepharose 4B. The elution profiles of chromatin preparations in 0.6 M-NaCl and in 0.8 M-sodium phosphate - 2 M-urea, pH 5.6, are presented in Fig. 1A and B, respectively. The electrophoresis of histones of the separated chromatin showed that only histone *f1* dissociated in 0.6 M-NaCl (Fig. 2B); all the lysine-rich histones dissociated in 0.8 M-sodium phosphate - 2 M-urea (Fig. 2C), the arginine-rich histones (*f3*, *f2a1*) remaining bound to DNA. The effect of urea concentration on dissociation of histones in 0.8 M-sodium phosphate buffer, pH 5.6, is presented in Fig. 3.

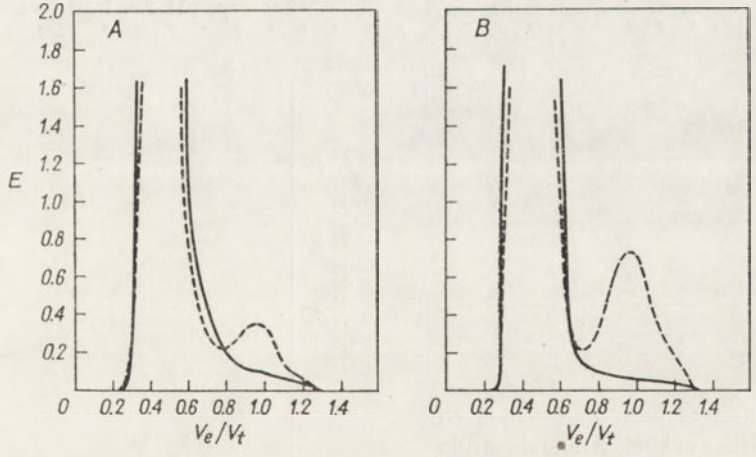


Fig. 1. Elution profiles from Sepharose 4B column of chromatin treated and eluted with: *A*, 0.6 M-NaCl; *B*, 0.8 M-sodium phosphate - 2 M-urea, pH 5.6.—, Extinction at 260 nm; — — —, Extinction at 230 nm. For details see Materials and Methods.

Derivatives of melting profiles of chromatin depleted of histone *f1*, and chromatin depleted of histone *f1*, *f2a2* and *f2b* showed for either chromatin preparation two melting bands in 0.25 mM-EDTA (Fig. 4A) and three melting bands in the presence

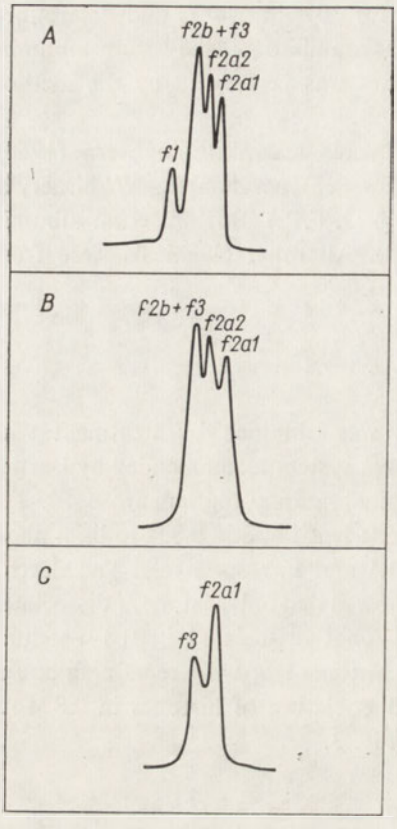


Fig. 2. Densitometer scans of electrophoretic patterns of acid-extracted histones from: *A*, whole chromatin; *B*, chromatin preparation after 0.6 M-NaCl treatment; *C*, chromatin preparation after 0.8 M-sodium phosphate - - 2 M-urea, pH 5.6, treatment.

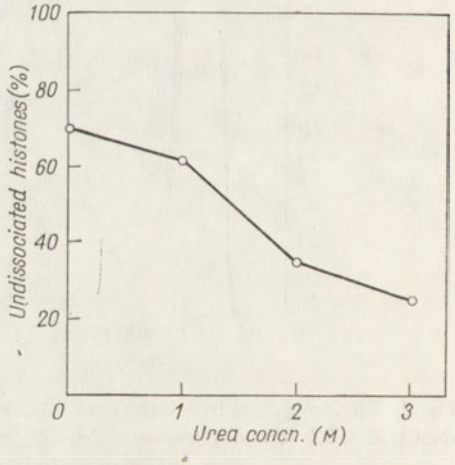


Fig. 3. Effect of urea concentration on the dissociation of histones from chromatin in 0.8 M-sodium phosphate, pH 5.6, for 12 h at 18 - 20°C.

of 3 M- and 5 M-urea (Fig. 4B, and 4C, respectively). The appearance of the third band may be interpreted as the result of splitting of a single thermal transition into two transitions. Denaturation properties of chromatin are dependent on the existence of several possible interactions between histones and DNA such as ionic

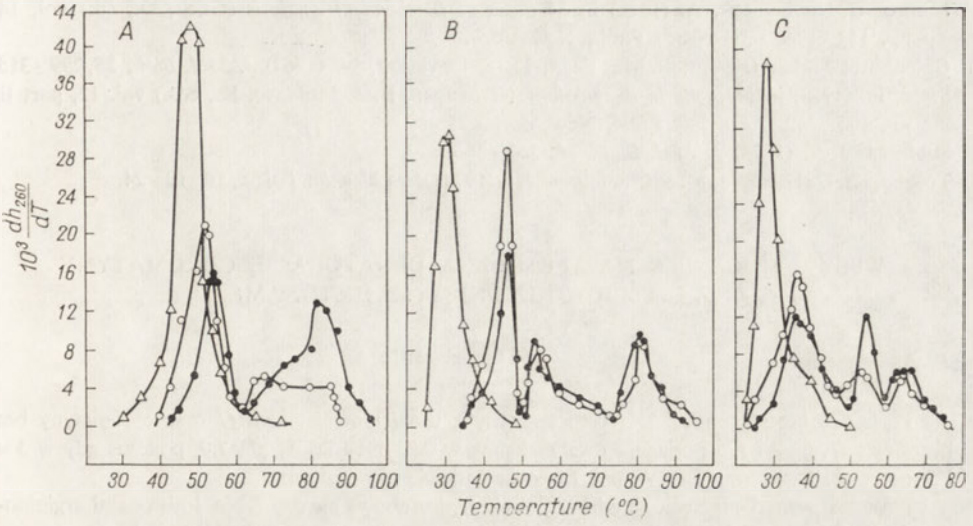


Fig. 4. Derivatives of melting profiles of: △, DNA; ●, chromatin depleted of histone *f1*; and ○, chromatin depleted of all lysine-rich histones. The melting was performed at pH 7.0: *A*, in 0.25 mM-EDTA; *B*, in 0.25 mM-EDTA - 3 M-urea and *C*, in 0.25 mM-EDTA - 5 M-urea. For measurements, Zeiss VSU 2-P (Jena, G.D.R.) spectrophotometer was used.

bonding, hydrogen bonding and hydrophobic forces. The nature of forces corresponding to particular melting bands is not clear. It seems, however, that urea-sensitive thermal transitions may be due mainly to hydrophobic interactions. Since there are no significant differences in the shape of derivatives of melting profiles in the presence of urea for chromatin preparations depleted of only histone *f1* and of all lysine-rich histones, it may be supposed that hydrophobic interactions between DNA and arginine-rich histones predominate over those with the lysine-rich histones. This supposition is consistent with the data reported by Bartley & Chalkley (1972) on the effect of urea on dissociation of histones from chromatin.

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## WPŁYW MOCZNIKA NA TERMICZNĄ DENATURACJĘ CHROMATYNY Z CZĘŚCIOWO USUNIĘTYMI HISTONAMI

Streszczenie

Pochodne hiperchromazji DNA chromatyny z usuniętym histonem *f1* oraz chromatyny bez histonów: *f1*, *f2a2* i *f2b* posiadają dwa maksima w 0.25 mM-EDTA, pH 7.0, podczas gdy w 3 M i 5 M-moczniku - 0.25 mM-EDTA, pH 7.0, obserwuje się trzy maksima.

Powyższe wyniki sugerują, że oddziaływania hydrofobowe między DNA i histonami argininowymi (*f3*, *f2a1*) są silniejsze niż między DNA i histonami lizynowymi (*f1*, *f2a2*, *f2b*).

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# PHOTOCHEMICAL TRANSFORMATION OF 5-HEXYLURIDINE, AND CYCLOBUTANE PHOTOADDUCTS OF 1-HEXENE WITH URIDINE AND 1-METHYLURACIL*

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1. Irradiation of 5-hexyluridine in neutral aqueous medium at 254 nm, or at wavelengths to the red of 265 nm, leads to dealkylation with formation of uridine which, in turn, undergoes photohydration at the 5,6 bond. Pyrimidine photodimerization does not occur under these conditions, and only to a minor extent, if at all, in an ice matrix. 2. The dealkylation reaction proceeds *via* an intramolecular photocyclization of the hexene radical to the pyrimidine 5,6 bond to form a 5,6-dihydro-5,6--cyclobutanyl substituted uridine. This intermediate then undergoes photodissociation to uridine and 1-hexene in high quantum yield, about 0.20. 3. An additional photoproduct in lower yield, with an alkaline u.v. absorption maximum at 348 nm, was also detected but its extreme lability rendered its identification difficult. 4. The cyclobutane photointermediate was obtained on a preparative scale by irradiation of uridine in the presence of 1-hexene. The photoadduct of 1-methyluracil with hexene was obtained in the same way. Both of these cyclobutane photoadducts were mixtures of diastereoisomers; these were separated analytically by gas chromatography, which revealed two diastereoisomers in the case of the 1-methyluracil-hexene photoadduct, and at least two in the case of the uridine-hexene photoadduct. 5. Mass spectroscopy and NMR spectroscopy of the 1-hexene adduct of 1-methyluracil confirmed the presence of two isomers, identified provisionally as *syn* and *anti*. 6. Additional physico-chemical properties of the 1-hexene photoadducts of uridine and 1-methyluracil are described and compared with the previously reported corresponding ethylene and propylene photoadducts. The biological significance of the results is discussed.

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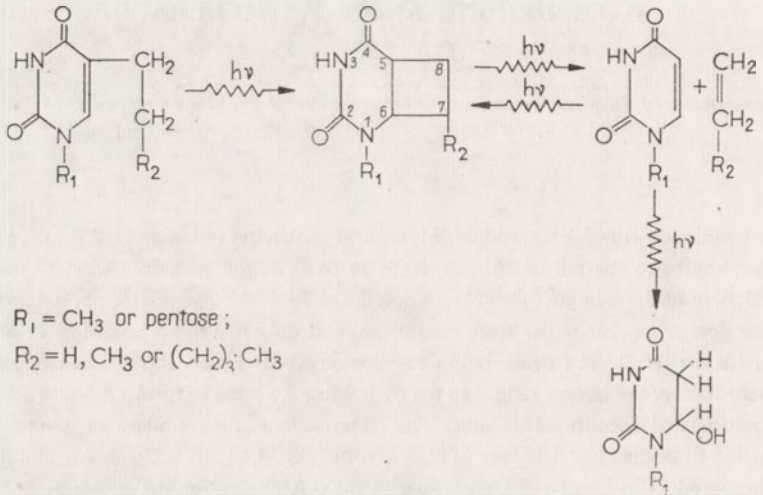
In previous investigations on the photochemistry of 5-EtUra[1] (Pietrzykowska & Shugar, 1968, 1970), a non-mutagenic analogue of thymine (Pietrzykowska & Shugar, 1967; Świerkowski & Shugar, 1969a) which can substitute for thymine in bacterial (Piechowska & Shugar, 1965) and phage (Pietrzykowska & Shugar, 1966, 1967) DNA, it was shown that both 5-EtUra and its nucleosides undergo predominantly photodimerization at wavelengths to the red of 265 nm; whereas at 254 nm the principal reaction is photodealkylation with resultant formation of uracil or uracil nucleosides and their photohydrates (Pietrzykowska & Shugar, 1968, 1970). Similar photodealkylation reactions are exhibited by two higher alkyl analogues, 5-PrUrd and 5-iPrUrd. The mechanism of this reaction involves intramolecular photocyclization of the 5-alkyl chain with the pyrimidine 5,6 bond to form a cyclobutane ring (Scheme 1), followed by photodissociation




Scheme 1

of the cyclobutane photointermediate with concomitant release of the pyrimidine ring and the hydrocarbon chain (Krajewska & Shugar, 1971, 1972). It was also found that the cyclobutane intermediates could be formed by the photochemical addition of an unsaturated hydrocarbon to the 5,6 bond of an excited pyrimidine (Krajewska & Shugar, 1971, 1972). It should be noted that the cyclobutane intermediates (Scheme 1) are formally analogous to pyrimidine photodimers and, as such, should be of value in studies on the properties of the biologically important photodimers.

[1] Most of the symbols and abbreviations used in this text are those recommended by the Commission on Biochemical Nomenclature of IUPAC-IUB (see *Eur. J. Biochem.* **15**, 203, 1970). The following additional non-usual abbreviations are employed: 5-HeUrd, 5-hexyluridine; 1-MeUra, 1-methyluracil; 5-EtUrd, 5-ethyluridine; 5-PrUrd, 5-propyluridine; 5-iPrUrd, 5-isopropyluridine; Urd·$H_2O$, photohydrate of uridine; 1-MeUra:1-hexene and Urd:1-hexene, 1:1 adducts of 1-hexene with 1-methyluracil and uridine. CDMS, carboxydimethanolsuccinate.

The present communication describes the photochemical transformation of a higher alkyl analogue, 5-hexyluridine, and the properties of its cyclobutane photointermediate, as well as that of the photoadduct of 1-MeUra with 1-hexene. Apart from the intrinsic interest of these reactions in relation to the photochemistry of 5-alkyluracils and their nucleosides, they take on added significance in the light of the recent demonstration of the presence in some phage DNA's of uracil residues with longer 5-alkyl chains (Brandon *et al.*, 1972; Marmur *et al.*, 1972).

## MATERIALS AND METHODS

Uridine was a product of Sigma Chemical Co. (St. Louis, Mo., USA). The sample of 5-hexyluridine, kindly supplied by Dr. M. Świerkowski, was prepared by a modification (Świerkowski & Shugar, 1969b) of the mercury procedure of Fox *et al.* (1956). We are indebted to J. Giziewicz for the preparation of 1-methyluracil (Shugar & Fox, 1952).

Three radiation sources were employed, as follows:

(A) A Phillips (Eindhoven, Holland) 6-watt germicidal lamp, with most of its emission at 254 nm. The lamp was surrounded by a 5-mm layer of 33% acetic acid to eliminate radiation below 230 nm. The intensity of this source 1.4 cm from the filter surface was about $3 \times 10^{16}$ quanta/cm$^2$/min, and was measured actinometrically with the use of an aqueous solution of uridine for which the quantum yield for photohydration is 0.021 (Shugar, 1960).

(B) A Zeiss (Jena, D.D.R.) medium-pressure therapeutic 700-watt mercury lamp with a Zeiss WG 7 filter (filter *a*) to cut off all radiation below 265 nm. For preparative purposes the same lamp formed part of a photochemical reactor surrounded by a 2-mm layer of a formamide solution of adenine (filter *b*) to eliminate radiation to the violet of 265 nm. The reactor was water-jacketed to prevent any excessive rise in temperature due to the heat from the lamp.

(C) A Phillips 40-watt germicidal lamp (254 nm), without filter, which was used for photodissociation of photoadducts on chromatograms, and for irradiation of frozen solutions.

For following the course of a photochemical reaction, irradiations were performed on $10^{-4}$ M solutions in 10-mm spectrophotometer cuvettes. When the irradiated solutions were to be chromatographed, $10^{-3}$ M solutions were irradiated in 1-mm spectro cuvettes.

Irradiations under anoxic conditions were carried out in a 10-mm cuvette fitted with a ground glass joint containing inlet and outlet tubes through which was passed a stream of nitrogen from which traces of oxygen were removed by first passing it through alkaline pyrogallol. Irradiations in the presence of pure oxygen were carried out in the same manner, the oxygen being first washed with water. For irradiation of frozen solutions, a 1-mm layer of $10^{-2}$ M solution was deposited in a Petri dish and irradiated with source C; the course of the reaction was followed by thawing the irradiated solution and diluting an aliquot to $10^{-4}$ M for spectrophotometry.

*U.V. absorption spectra* were obtained with the aid of a Zeiss (Jena, D.D.R.) VSU-2 instrument and a Unicam SP-8000 recording spectrophotometer, using 1-, 2- and 10-mm path length cuvettes, as well as semi-micro cuvettes with 10-mm path length.

*Melting temperatures* (uncorrected) were measured on a Boetius microscope hot stage.

*Proton magnetic resonance spectra* were obtained on a Bruker 90 MHz instrument at the University of Konstanz (G.F.R.), through the kindness of Professor W. Pfleiderer. Samples were dissolved in $CDCl_3$ with tetramethylsilane as internal standard.

*Mass spectra* were run on an LKB-9000 spectrometer, for which we are indebted to H. Gałązka.

*Gas chromatography* made use of a PYE-Unicam type 104 instrument with a flame ionization detector.

*Paper and thin-layer chromatography*: ascending, made use of Whatman paper no. 3*MM*, Merck (Darmstadt, G.F.R.) silica gels $HF_{254}$ and HR, as well as commercially available Merck plates Cellulose F 5718/0025 and Kieselgel F 254 5737/0025. The following solvent systems (all proportions v/v) were employed: (*A*) chloroform - methanol, 7: 3; (*B*) *n*-propanol - water, 7:1.5; (*C*) water-saturated *sec.* butanol; (*D*) water-saturated *n*-butanol; (*E*) *n*-butanol - glacial acetic acid - water, 80:12:30; (*F*) formic acid - *tert.* butanol - methyl ethyl ketone - water, 15:40:30: :15; (*G*) isopropanol - 1 M-ammonium acetate - sat. ammonium sulphate, 1:9:20; (*H*) upper phase of ethyl acetate - water - formic acid, 60:35:5; (*I*) *n*-butanol-formic acid - water, 8:1:2.

## RESULTS AND DISCUSSION

Irradiation of a $10^{-4}$ M solution of 5-HeUrd in neutral unbuffered medium with source B and filter *b* ($\lambda > 265$ nm) led to disappearance of the absorption maximum at 269 nm at a 7.5-fold lower rate than for uridine under identical conditions. When irradiation was at 254 nm (source A), the initial quantum yield for disappearance of 5-HeUrd was $7.0 \times 10^{-3}$, as compared to $21 \times 10^{-3}$ for uridine. The corresponding quantum yields for 5-EtUrd and 5-PrUrd are $4.3 \times 10^{-3}$, and $9.0 \times 10^{-3}$, respectively (Pietrzykowska & Shugar, 1970; Krajewska & Shugar, 1972). The rate of disappearance of 5-HeUrd was essentially unaffected when the concentration was increased to $10^{-3}$ M.

*Chromatographic analysis of 5-HeUrd photoproducts.* A $10^{-3}$ M solution of 5-HeUrd in a 1-mm cuvette was irradiated with source B, filter *b* until the optical density of $\lambda_{max}$ had decreased by 85%, and was then subjected to t.l.c. (Table 1). Amongst the photoproducts was Urd, identified chromatographically and by spectral analysis of the eluate, and showing that photochemical dehexylation had occurred. An additional product, not visible under a dark u.v. lamp, was converted by heating to Urd (see Pietrzykowska & Shugar, 1970) and was identified as Urd·$H_2O$. A third product, faintly visible under a dark u.v. lamp, was clearly revealed on irradiation of the chromatogram with source A. This product exhibited only end absorption

Table 1

*Thin-layer chromatography of 5-hexyluridine and its photoproducts*

T.l.c. plates and solvents as indicated.

| Carrier and solvents | $R_F$ values | | | | |
|---|---|---|---|---|---|
| | 5-HeUrd | Cyclobutane product | Urd | Urd·$H_2O$ | PP-348 |
| T.l.c.-cellulose F plates | | | | | |
| *A* | 0.91 | 0.84 | 0.09 | 0.03 | 0.91 |
| *B* | 0.82 | 0.76 | 0.30 | 0.22 | 0.82 |
| *C* | 0.86 | 0.80 | 0.38 | 0.28 | 0.86 |
| *D* | 0.89 | 0.71 | 0.21 | 0.14 | 0.89 |
| T.l.c.-silica gel $F_{254}$ plates | | | | | |
| *A* | 0.78 | 0.73 | 0.46 | 0.21 | 0.67 |
| *B* | 0.75 | 0.68 | 0.61 | 0.51 | 0.75 |
| *C* | 0.75 | 0.68 | 0.54 | 0.32 | 0.75 |
| *D* | 0.73 | 0.68 | 0.44 | 0.28 | 0.73 |

in the u.v. at neutral pH but at pH 12 it possessed a maximum at 240 nm (see, e.g. Fig. 4, below). Irradiation of this alkaline solution with source A led to disappearance of the 240 nm band and the appearance of a new band with a maximum at 262 nm and a minimum at 242 nm, corresponding to Urd, and confirmed by chromatography (Table 1); in addition, the irradiated solution was found by gas chromatography to contain substantial amounts of 1-hexene. All of the foregoing facts are consistent with the same scheme for photochemical transformation of 5-HeUrd as previously reported for 5-EtUrd, 5-PrUrd and 5-iPrUrd (Pietrzykowska & Shugar, 1970; Krajewska & Shugar, 1971, 1972) and illustrated in Scheme 1. We present further evidence, below, in support of this pathway.

When a $10^{-3}$ M solution of 5-HeUrd was initially irradiated at 254 nm (source A), a further photoproduct was revealed chromatographically. This product, hitherto unidentified, will be referred to as PP-348 from the fact that it exhibits an absorption maximum at 348 nm in alkaline medium.

*Spectral analysis of 5-HeUrd photoproducts.* A $10^{-4}$ M neutral solution of 5-HeUrd in a 10-mm cuvette was irradiated with source B, filter *a* ($\lambda > 265$ nm) until the absorbance of the main absorption maximum had decreased about 45% (Fig. 1). The solution was then brought to pH 12 (0.01 N-NaOH), leading to an increase in absorbance at about 265 nm which terminated after about 60 min; this increase was a measure of the amount of Urd·$H_2O$, which underwent alkaline dehydration. The absorption at 348 nm, taken as a measure of the amount of PP-348, attained its maximum value after 1 hour, and then decreased for 2 hours to zero. At this time the solution was irradiated with source A, leading to an increase in $A_{262}$ which attained its maximum value after about 35 min irradiation; this was regarded as indicating the amount of cyclobutane photoproduct.

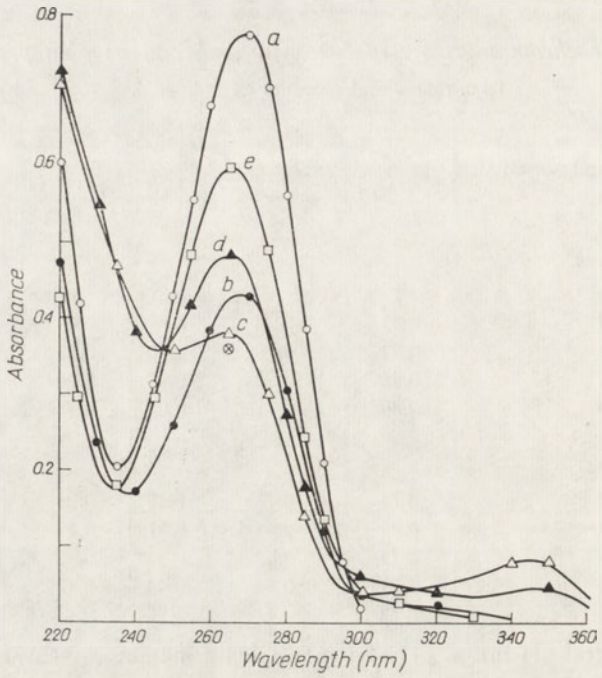



Fig. 1. Spectral examination of photolysis products of 5-hexyluridine: (*a*) absorption spectrum of $10^{-4}$ M-5-HeUrd in neutral aqueous medium; (*b*) spectrum following irradiation for 130 min with source B and filter *a* ($\lambda > 265$ nm); on alkalization of this irradiated solution to pH 12, the resulting modified spectrum exhibited an absorption maximum at the point denoted by ⊗, obtained by extrapolation to zero time, and finally stabilized to give absorption spectrum (*c*) after 1 hour; (*d*) spectrum following irradiation of irradiated alkalized solution for 29 min with source A (254 nm); (*e*) spectrum following neutralization of foregoing solution.

The foregoing analysis was then applied to an evaluation of the extent of formation of each of the photoproducts with increasing extent of photolysis of 5-HeUrd, both when irradiated at wavelengths to the red of 265 nm, and at 254 nm. The results are shown in Figs. 2A and 2B.

*Course of photolysis of 5-HeUrd at $\lambda > 265$ nm.* A comparison of Figs. 2A and 2B shows that more cyclobutane photoproduct is formed by irradiation at the longer wavelengths. This result might have been anticipated since the absorption of the cyclobutane intermediate is lower at the longer wavelengths, so that it is less susceptible to photodissociation (see Scheme 1). Of particular interest is the fact that formation of the cyclobutane photoproduct was not inhibited in the presence of oxygen.

The initial principal photoproduct on irradiation at $\lambda > 265$ nm (Fig. 2A) is the cyclobutane intermediate (in addition to PP-348), the quantity of which attains a plateau when about 40% of the 5-HeUrd has been photolysed. Shortly prior to this Urd·$H_2O$ begins to appear, in increasing quantity. This is undoubtedly due to attainment of some equilibrium at which further cyclobutane photoproduct is dissociated to Urd, and this in turn to Urd·$H_2O$. The course of photolysis is therefore analogous

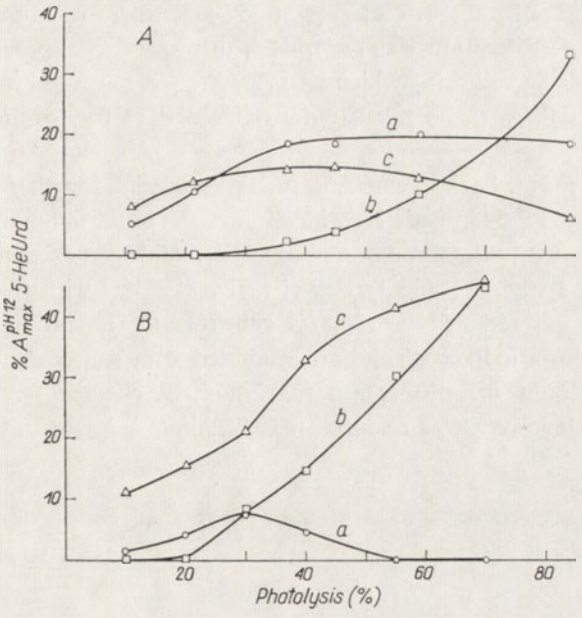
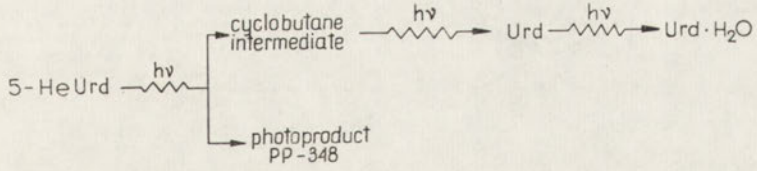



Fig. 2. Course of formation of various photoproducts, on irradiation of $10^{-4}$ M-5-HeUrd in neutral aqueous medium, as a function of % photolysis (*A*) with source B and filter *a* ($\lambda > 265$ nm), (*B*) with source A (254 nm): (*a*) cyclobutane photoproduct, (*b*) Urd·$H_2O$, (*c*) PP-348. The ordinate scale represents the absorbance of the $\lambda_{max}$ of the individual photoproducts at pH 12, expressed in % of the absorbance of $\lambda_{max}$ of the initial 5-HeUrd at pH 12.

to that for 5-EtUrd and 5-iPrUrd (Scheme 2). In agreement with this, the amount of Urd obtained by post-irradiation treatment (see above) increased with the radiation dose.

The photoproduct PP-348, detectable in the initial stages of photolysis (Fig. 2A), increased with irradiation time to attain a maximum at about 40% photolysis of 5-HeUrd, and its formation probably represents an alternative route for photochemical transformation of 5-HeUrd (Scheme 2).




Scheme 2

*Course of photolysis of 5-HeUrd at 254 nm.* Irradiation at the shorter wavelength led to the appearance of considerably less cyclobutane photoproduct, but markedly more Urd·$H_2O$ and PP-348. Again the initial photoproduct (in addition to PP-348) was the cyclobutane intermediate, which attained a low maximum at about 30% photolysis and then rapidly decreased (Fig. 2B). This is obviously

due to the rapid rate of photodissociation at 254 nm, where its absorption is relatively high. Urd·$H_2O$ formation was retarded with respect to appearance of the cyclobutane intermediate, following which its concentration increased rapidly (Fig. 2B). Formation of PP-348 was also much more marked than for irradiation at longer wavelengths.

*Photoproduct PP-348.* Lack of sufficient starting material, together with instability of the photoproduct PP-348, made identification of the latter impossible. It appears, nonetheless, of interest to list some of its properties. With one exception its mobility in various solvent systems (Table 1) was virtually identical with that of 5-HeUrd, suggesting that it has retained the 5-hexyl substituent. The product was isolated on a small scale, from a $10^{-3}$ M solution irradiated with source A to almost complete disappearance of the absorption maximum, by chromatography on Merck cellulose plates with solvent *C*. At neutral pH it exhibits a $\lambda_{max}$ with low absorbance

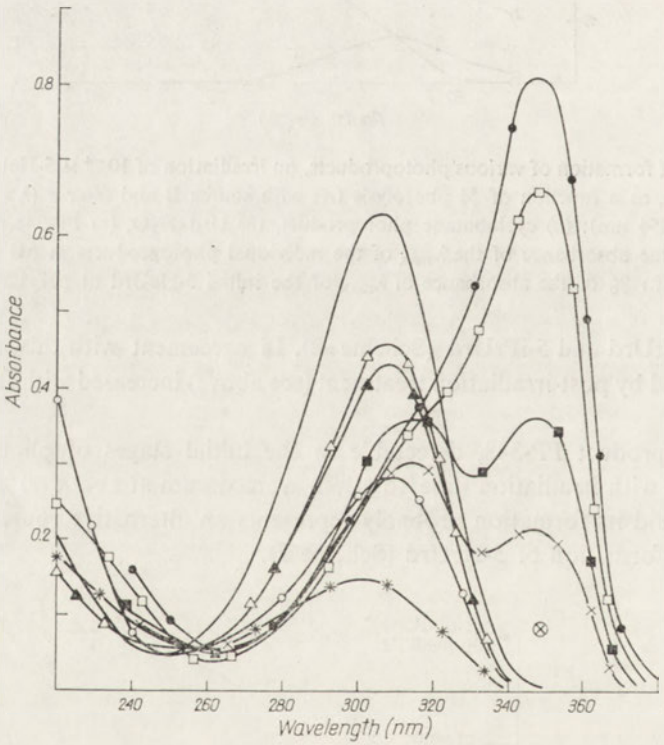



Fig. 3. Spectral properties, and lability at pH 6 and 12, of the unidentified photoproduct with alkaline absorption maximum at 348 nm (PP-348) formed from irradiated 5-hexyluridine: (○), absorption maximum at neutral pH; (⊗), location of absorption maximum immediately after bringing solution to pH 12, determined by extrapolation to zero time; (●), spectrum after 1 hour at pH 12; (——), spectrum following neutralization; (□), spectrum following alkalization once more to pH 12 and leaving at room temperature for 15 min; (■), following exposure to pH 12 for an additional 65 min; (△), spectrum immediately following neutralization; (▲), following 2 hours at neutral pH; (×), following alkalization to pH 12 and exposure to this pH for 40 min; (✳),following neutralization to pH 7.

at 307 nm; on alkalization to pH 12, a new $\lambda_{max}$ with high absorbance appeared at 348 nm. However, the spectral changes at both neutral and alkaline pH, exhibited in detail in Fig. 3, testify to the extreme lability of the photoproduct. It is worth noting that a similar photoproduct was observed in irradiated 5-EtUrd (Pietrzykowska & Shugar, 1970) although in appreciably lower yield.

*Photolysis of 5-HeUrd in frozen solution.* Irradiation of a frozen aqueous $10^{-2}$ M solution of 5-HeUrd with source C led to a relatively rapid decrease in absorbance of the absorption maximum, about 35% in one hour. Following thawing of the solution, dilution to $10^{-4}$ M and alkalization to pH 12, irradiation with source B demonstrated only feeble regeneration of the maximum varying from 5 to 10% of the initial substance, following degrees of photolysis of 15 to 70%. In view of the shortage of starting material, it was not feasible to attempt identification of the photoproducts, but it is clear from the foregoing that these were largely products other than photodimers or the cyclobutane intermediate.

*Preparation of cyclobutane photointermediate of 5-HeUrd (hexene photoadduct of Urd).* From Scheme 1 it is clear that formation of the cyclobutane photointermediate of 5-HeUrd should be possible *via* photochemical addition of 1-hexene to the 5,6 bond of Urd, as for the reported additions of ethylene and propylene (Krajewska & Shugar, 1971, 1972). Such a procedure would make available larger quantities of the intermediate for a comparison of its properties with those of the intermediate obtained from irradiated 5-HeUrd.

Because of the low solubility of 1-hexene in water, initial trials were carried out in ethanolic medium. Two identical $10^{-3}$ M solutions of Urd in anhydrous ethanol containing 0.5 M-1-hexene were irradiated, one at 254 nm (source A), the other at $\lambda > 265$ nm (source B, filter *b*). In both instances the rate of disappearance of Urd was twofold greater than in the absence of hexene. Trials conducted according to the scheme illustrated in Fig. 1 showed that there was relatively little addition of ethanol to the 5,6 bond of Urd (Moore & Thomson, 1956). Following about 80% photolysis of Urd, 25% of the product was the cyclobutane adduct for irradiation at 254 nm, and 31% at the longer wavelengths. The photoproduct, isolated on silica gel $HF_{254}$ with solvent *D*, was identical chromatographically and spectrally with the product obtained by irradiation of 5-HeUrd, and also underwent photodissociation to Urd and 1-hexene.

The foregoing led to the following scheme for isolation of the intermediate on a preparative scale: 300 mg Urd was dissolved at a concentration of $5 \times 10^{-3}$ M in anhydrous ethanol containing 0.5 M-1-hexene and irradiated in the reactor (source B, filter *b*). The irradiated solution was stirred from time to time and the course of the reaction followed by removal of an aliquot which was diluted for spectrophotometry. The absorbance of the $\lambda_{max}$ decreased almost linearly with time of irradiation for about 6 hours to 5% of the initial value. The irradiated solution was concentrated under reduced pressure and subjected to chromatography on $HF_{254}$ silica gel with solvent *D* ($R_F$ values of Urd and cyclobutane adduct 0.44 and 0.68, see Table 1), showing a 20% yield of the adduct. The entire solution was chromatographed in this way, the adduct eluted with anhydrous ethanol, and further chromatographed on

Whatman 3*MM* (which had been previously washed successively with 0.1 N-NaOH, then with water to disappearance of the alkaline reaction, again with 0.1 N-HCl, and finally with water until the reaction was neutral) with solvent *D*. The hexene adduct was eluted with anhydrous ethanol and concentrated under reduced pressure to yield a pale yellow oil. Numerous attempts to induce crystallization were unsuccessful. The product was readily soluble in ethanol and water and insoluble in ether and chloroform.

*Isolation of hexene photoadduct of 1-MeUra.* In view of the inability to crystallize the hexene photoadduct of Urd, attempts were undertaken to prepare the corresponding photoadduct of 1-MeUra. Initial trials were similar to those described in the previous section for Urd. Irradiation with source B, filter *b* of a $10^{-3}$ M solution of 1-MeUra in anhydrous ethanol containing 0.5 M-1-hexene led to a rate of decrease of the $\lambda_{max}$ fivefold greater than that in the absence of hexene. In addition, following 45 - 85% photolysis, the irradiated solution demonstrated the presence of 16 - 19% product which photodissociated to 1-MeUra and 1-hexene. The following preparative procedure was therefore adopted: 200 mg 1-MeUra in 400 ml anhydrous ethanol containing 0.5 M-1-hexene was irradiated in the reactor (source B, filter *b*) for about 3.5 hours, at which point the absorbance of the $\lambda_{max}$ had decreased about 50%. The irradiated solution was concentrated to small volume under reduced pressure and subjected to preparative t.l.c. on $HF_{254}$ silica gel with ethyl acetate as solvent ($R_F$ of 1-MeUra 0.08, and of hexene photoadduct 0.37). The photoadduct was eluted with 300 ml of methanol-chloroform (1:1, v/v), overall yield about 35%. The product was further purified by chromatography on HR silica gel, following which it readily crystallized from hot aqueous ethanol. In view of the possible presence of stereoisomers, several crops of crystals were collected.

*Properties of hexene photoadducts.* Under the microscope the 1-MeUra:1-hexene adduct was seen to melt in two stages, 88 - 91°C (majority of crystals) and 99 - 102°C. The product sublimed at 170 - 190°C. It was very soluble in chloroform, acetone and methanol, soluble in ethanol and only sparingly so in water.

Elementary analysis gave C, 63.11%, H, 8.76%, N, 13.06%. Calculation for a 1:1 adduct, $C_{11}H_{18}N_2O_2$ gave C, 62.83%, H, 8.63%, N, 13.32%.

From Figs. 4A and 4B it can be seen that, like the ethylene (Pietrzykowska & Shugar, 1970) and propylene (Krajewska & Shugar, 1972) photoadducts, the hexene photoadducts exhibit only end absorption at neutral pH. At alkaline pH the Urd adduct shows a maximum at 240 nm and the 1-MeUra adduct at 245 nm. The spectra in alkaline medium therefore correspond to those for the monoanions of 2,4-diketopyrimidines and their glycosides in which the 5,6 bond is saturated (Janion & Shugar, 1960). Because of the marked instability of the uridine adduct in 1 N-NaOH (see below), only the height of the absorption maximum is indicated in Fig. 4A. Spectral titration showed that the p*K* values for dissociation of the $N_3$ hydrogens of the adducts were 11.6 for the 1-MeUra adduct, values similar to those reported for propylene adducts (Krajewska & Shugar, 1972) and for the corresponding 2,4-diketo-5,6-dihydropyrimidine derivatives (Janion & Shugar, 1960).

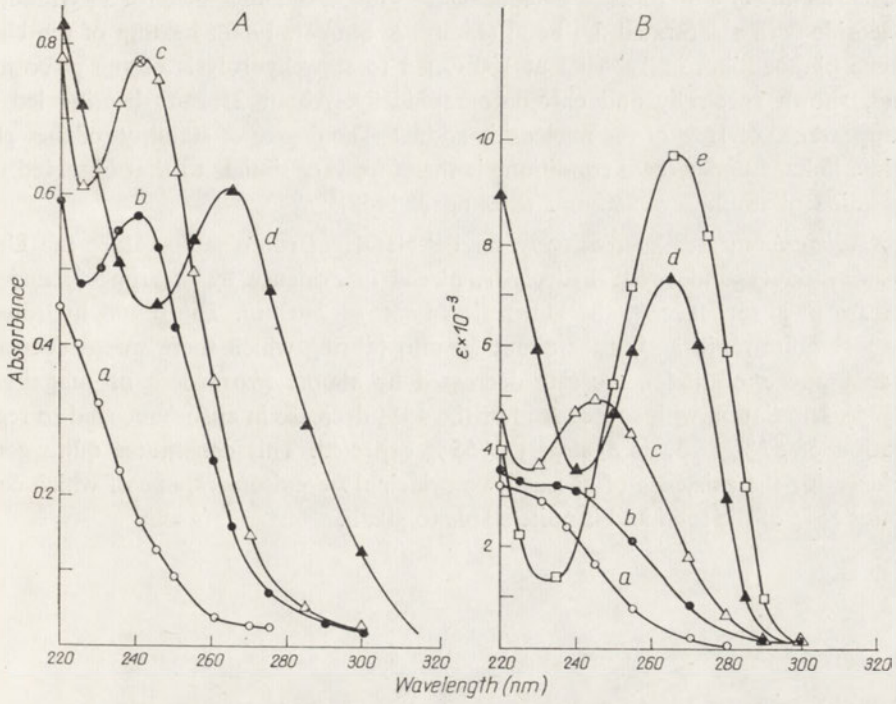



Fig. 4. Absorption spectra and photodissociation of 1:1 photoadducts of hexene with (*A*) uridine and (*B*) 1-methyluracil: (*A*): (*a*), absorption spectrum in neutral medium of Urd photoadduct; (*b*), at pH 12; (*c*), at pH 13; (⊗) absorbance of maximum at pH 14, obtained by extrapolation to zero time; (*d*), absorption spectrum following addition of 0.1 volume of conc. $NH_4OH$ to neutral solution and then irradiating with source A for 60 min. Curve *d* is identical with that for authentic Urd.

(B): (*a*) absorption spectrum in neutral medium of 1-MeUra photoadduct; (*b*) at pH 12; (*c*) at pH 13 - 14; (*d*) spectrum following irradiation of pH 12 solution with source A for 60 min; (*e*) following neutralization of irradiated solution. Note that curves *d* and *e* are identical with the alkaline and neutral absorption spectra of 1-MeUra.

*Photoadduct photodissociation.* As for the corresponding ethylene and propylene photoadducts (Pietrzykowska & Shugar, 1968, 1970; Krajewska & Shugar, 1971, 1972), the hexene adducts of Urd and 1-MeUra dissociate on irradiation at 254 nm, with the release of 1-hexene (shown by gas chromatography) and regeneration of uridine and 1-MeUra (Figs. 4A and 4B). For the 1-MeUra photoadduct, photodissociation led to quantitative regeneration of 1-MeUra, as previously observed for the propylene adduct of 1-MeUra (Krajewska & Shugar, 1972). This photodissociation reaction is, of course, completely analogous to the photodissociation of pyrimidine cyclobutane photodimers. The quantum yields for photodissociation in ammoniacal medium, pH > 11, were 0.19 for the uridine photoadduct and 0.29 for the 1-MeUra adduct. These values are comparable to those reported (Pietrzykowska & Shugar, 1970; Krajewska & Shugar, 1972) for Urd adducts of ethylene (0.30 at pH 12) and propylene (0.20 at pH 7 and 12).

*Acid lability of Urd:1-hexene photoadduct.* As might be anticipated for a pyrimidine nucleoside with a saturated 5,6 bond (Janion & Shugar, 1960), heating of the Urd-hexene photoadduct in 1 N-HCl at 100°C led to slow hydrolysis of the glycosidic bond, shown spectrally and chromatographically. About 2 hours heating led to disappearance of 15% of the nucleoside adduct. The degree of stability of the glycosidic linkage under these conditions is therefore very similar to that observed for thymidine photodimers (Sztumpf & Shugar, 1962).

*Alkaline lability of photoadducts.* In 1 N-NaOH at room temperature the Urd--hexene adduct underwent hydrolysis, placed in evidence by the time-dependent decrease in absorbance of the absorption band at 240 nm. The initial hydrolysis rate was quite rapid and, after about 30 min (during which there was a decrease in absorbance of 45%), the rate decreased by almost two orders of magnitude (Fig. 5). Irradiation with source A after the 45% decrease in absorbance led to regeneration of 57% Urd, as against the 55% expected. This constitutes quite good evidence for the existence of at least two principal stereoisomers, one of which constitutes 55% of the total and is quite stable to alkali.

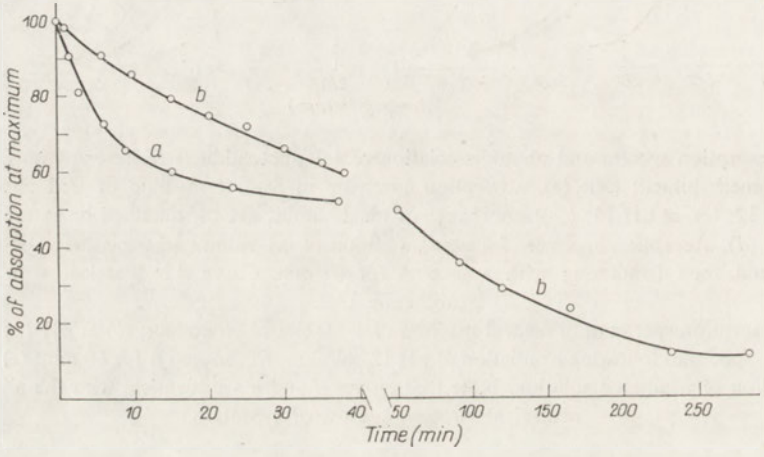



Fig. 5. Alkaline lability of hexene photoadducts of (*a*) Urd and (*b*) 1-MeUra, in 1 N-NaOH at 25°C, measured by the decrease with time of the absorbance of the absorption maxima for the two compounds.

Following partial alkaline hydrolysis of the uridine-hexene photoadduct, a small aliquot was chromatographed on a cellulose plate with solvent *B*, and the plate sprayed with *p*-dimethylaminobenzaldehyde (Fink *et al.*, 1956). A bright yellow spot appeared, testifying to ring-opening of the pyrimidine 3,4 bond, as in the case of the propylene adducts of Urd and 1-MeUra (Krajewska & Shugar, 1972), and as expected for a pyrimidine ring with a saturated 5,6 bond (Janion & Shugar, 1960).

The 1-MeUra:1-hexene photoadduct was more stable under the above conditions, the decomposition half-time being about 53 min (Fig. 5). Furthermore hydrolysis was in this case a single-stage process and, when terminated, no 1-MeUra was re-

generated on irradiation of the solution. The single-stage nature of the alkaline hydrolysis is of some significance in relation to the fact (see below) that 1-MeUra:1--hexene photoadduct consists of at least two stereoisomers.

*Chromatographic properties of photoadducts.* Notwithstanding the evidence cited above, viz. the two-stage process of alkaline hydrolysis of the Urd:1-hexene cyclobutane adduct, testifying to the existence of at least two stereoisomers, attempts to separate the isomers by t.l.c. with both polar and non-polar solvents were unsuccessful. The same applied to the 1-MeUra:1-hexene adduct.

However, the 1-MeUra:1-hexene photoadduct readily separated into two components when subjected to analytical gas-liquid chromatography on a strongly polar 1% CDMS column (2.1 m) with an argon flow rate of 40 ml/min at 179°C. The first crop of crystals obtained from the 1-MeUra:1-hexene preparation gave two well separated peaks with retention times of $R_{T_1}=48'$ and $R_{T_2}=53'42''$ in the proportion 54 to 46. The second crop of crystals from the mother liquors exhibited the same two peaks but in the proportion 25 to 75 (Fig. 6).

Gas-liquid chromatography was then applied to the Urd-hexene photoadduct, which was silylated with a 100-fold excess of *N*-trimethylsilylimidazole according

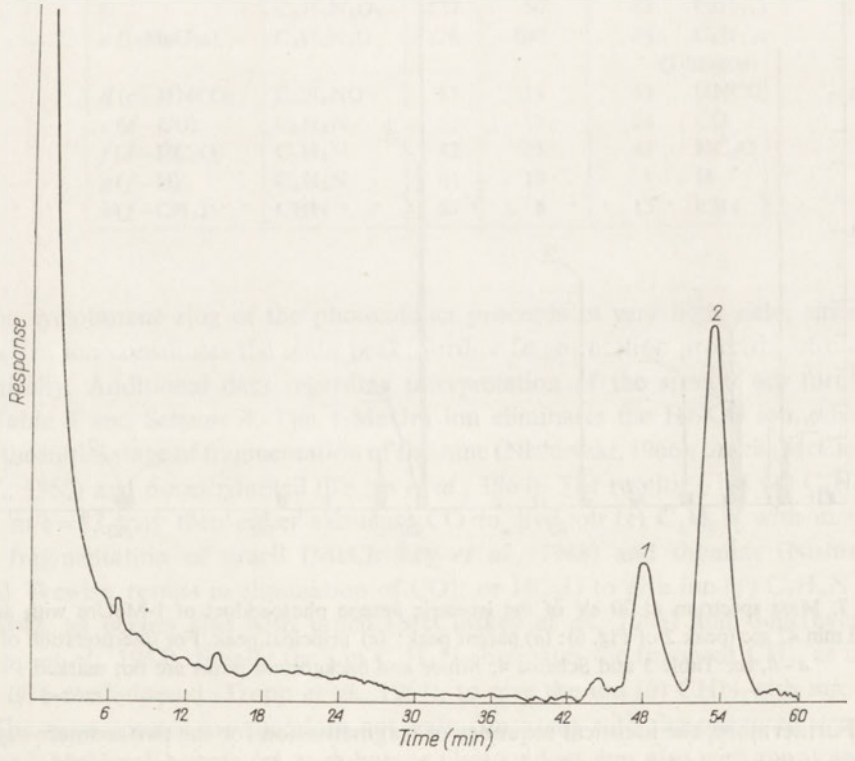



Fig. 6. Gas chromatogram of the 1 : 1 hexene photoadduct of 1-MeUra. Peaks *1* and *2* are presumed stereoisomers with retention times $R_{T_1}=48$ min and $R_{T_2}=53$ min 42 sec, respectively, and in the proportion 25% and 75%. This separation was achieved on a 9 ft column of 1% CDMS on Gaschrom Q 100/120 mesh, with a column temperature of 179°C and an argon flow of 40 ml/min.

to the procedure of Kulikowski & Shugar (1974), and then chromatographed on a strongly polar analytical column of OV-225 (198°C, argon 40 ml/min). The preparation gave 7 peaks with retention times considerably longer than that of the standard, phenanthrene (4.5 min), the principal peaks being:
1, $R_{T_1}=31$ min; 2, $R_{T_2}=57$ min; 5, $R_{T_5}=92$ min; 6, $R_{T_6}=98$ min; 7, $R_{T_7}=111$ min.

*Mass spectroscopy of 1-MeUra:1-hexene photoadduct.* The two well-separated peaks derived from the 1-MeUra:1-hexene photoadduct in the gas chromatograph were subjected directly to mass spectrometry. The mass spectra, identical for the two peaks, are shown in Fig. 7 and the corresponding data derived from these spectra are presented in Table 3. The identity of the mass spectra, pointing to the identity of the parent ions, of the two components proves that these are, in fact, stereoisomers.

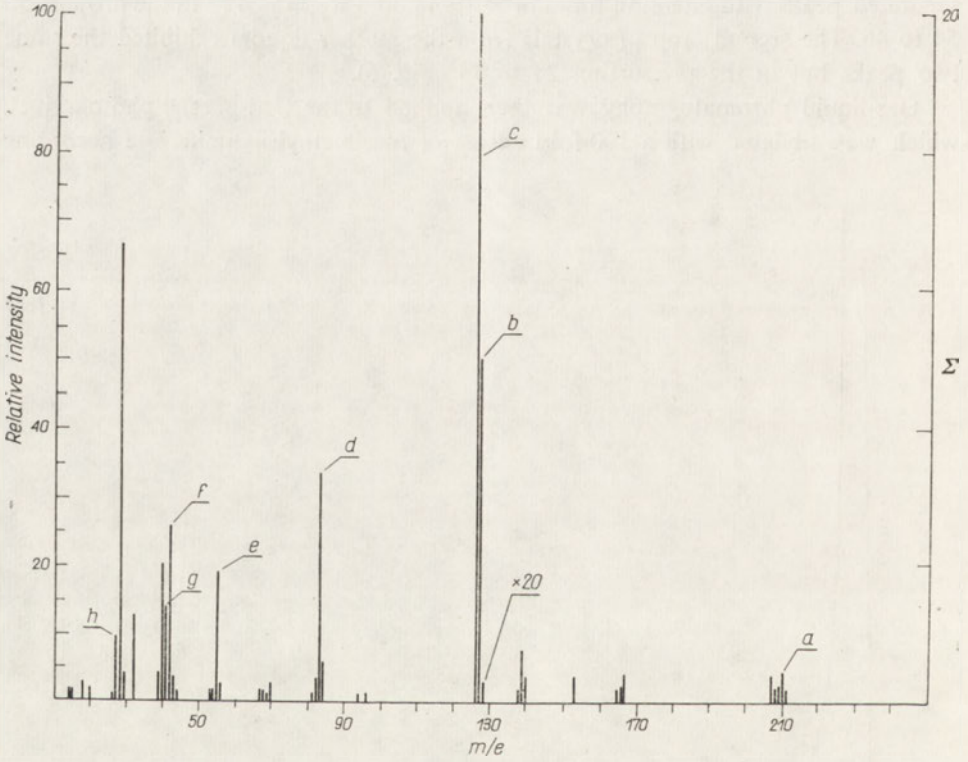



Fig. 7. Mass spectrum at 70 eV of the isomeric hexene photoadduct of 1-MeUra with an $R_{T_2}$ of 53 min 42 sec (peak *2* of Fig. 6): (*a*) parent peak; (*c*) principal peak. For interpretation of peaks *a* - *h*, see Table 3 and Scheme 4. Minor and background peaks are not marked.

Furthermore, the identical sequence of fragmentation for the two isomers suggests that positions 7 and 8 (Scheme 3 ) must be involved. For if isomerization were due to substituents on the pyrimidine ring, where the different positions are not equivalent, e.g. thymine (Nishiwaki, 1966) and 6-methyluracil (Tropp *et al.*, 1964), the fragmentation process would be different for the two isomers. Note that dissociation

Table 2

*Thin-layer chromatography of 1-methyluracil and its 1 : 1 photoadduct with 1-hexene*

T.l.c.-cellulose F plates were employed with solvents *E-I*.

| Compound | $R_F$ value with solvent | | | | |
|---|---|---|---|---|---|
| | *E* | *F* | *G* | *H* | *I* |
| 1-MeUra | 0.53 | 0.64 | 0.57 | 0.31 | 0.55 |
| 1-MeUra:1-hexene | 0.90 | 0.94 | 0.00 | 0.93 | 0.91 |

Table 3

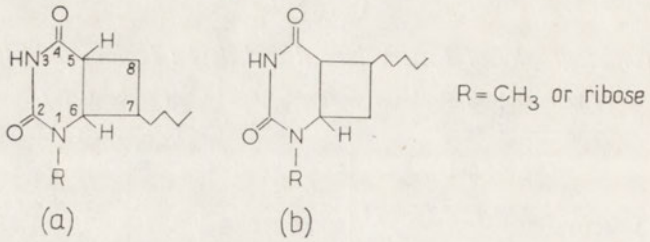
*Proposed identification of ions observed in the mass spectrum of the 1-hexene photoadduct of 1-MeUra*

| Ion | Composition | m/e | % of main peak | Fragment eliminated | |
|---|---|---|---|---|---|
| *a* ($M^+$) (parent ion) | $C_{11}H_{18}H_2O_2$ | 210 | 0.3 | $\bar{e}$ | |
| *b* | $C_5H_7N_2O_2$ | 127 | 50 | 83 | $C_6H_{11}$ |
| *c* (1-MeUra) | $C_5H_6N_2O_2$ | 126 | 100 | 84 | $C_6H_{12}$ (1-hexene) |
| *d* (*c*—HNCO) | $C_4H_5NO$ | 83 | 34 | 43 | HNCO |
| *e* (*d*—CO) | $C_3H_5N$ | 55 | 19 | 28 | CO |
| *f* (*d*—$HC_2O$) | $C_2H_4N$ | 42 | 25 | 41 | $HC_2O$ |
| *g* (*f*—H) | $C_2H_3N$ | 41 | 13 | 1 | H |
| *h* (*f*—$CH_3$) | CHN | 27 | 8 | 15 | $CH_3$ |

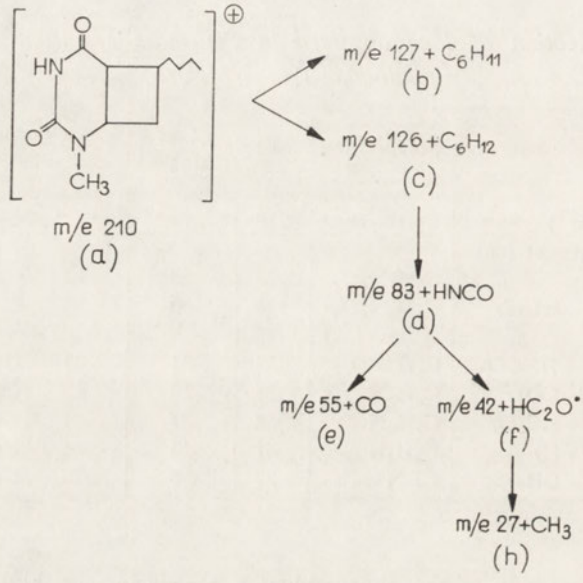
of the cyclobutane ring of the photoadduct proceeds in very high yield, since the 1-MeUra ion constitutes the main peak; further fragmentation proceeds, of course, identically. Additional data regarding interpretation of the spectra are furnished by Table 3 and Scheme 4. The 1-MeUra ion eliminates the HNCO ion, which is also the initial stage of fragmentation of thymine (Nishiwaki, 1966), uracil (McCloskey *et al.*, 1968) and 6-methyluracil (Tropp *et al.*, 1964). The resulting ion (*d*) $C_4H_5NO$ with m/e=83 may then either eliminate CO to give ion (*e*) $C_3H_5N$ with m/e=55 [the fragmentation of uracil (McCloskey *et al.*, 1968) and thymine (Nishiwaki, 1966) likewise results in elimination of CO]; or $HC_2O$ to give ion (*f*) $C_2H_4N$ with m/e=42, as occurs also with uracil (McCloskey *et al.*, 1968) and 6-methyluracil (Tropp *et al.*, 1964). Finally the ion $C_2H_4N$ eliminates the fragment $CH_3$, as in the case of 6-methyluracil (Tropp *et al.*, 1964), to give the ion (*h*) CHN with m/e=27.

The mass spectra are therefore not only consistent with the proposed structure of the 1-MeUra:1-hexene 1:1 cyclobutane photoadduct, but also with the stereoisomeric nature of the two components separated by g.l.c.

*Proton magnetic resonance spectroscopy*. The 90 MHz NMR spectrum of the 1-MeUra:1-hexene photoadduct in $D_2O$ proved to be rather complex because of




Scheme 3




Scheme 4

the presence of the mixture of diastereoisomers. However, the clear resolution of two methyl peaks at 2.95 and 3.02 ppm not only testified to the presence of two major diastereoisomers, but is also consistent with these isomers being *syn* and *anti*, such as (*a*) and (*b*) in Scheme 3.

## CONCLUDING REMARKS

The overall results of this study demonstrate that the photochemical transformation of 5-HeUrd proceeds principally *via* an intermediate involving the intramolecular cyclization of the 1,2 bond of the hexene substituent with the 5,6 bond of the pyrimidine ring to give a 5,6-dihydro-5,6-cyclobutanyluracil nucleoside as illustrated in Scheme 1. This intermediate is fully analogous to those formed by 5-alkyluracil nucleosides with shorter alkyl chains such as ethyl (Pietrzykowska & Shugar, 1968, 1970), propyl and isopropyl (Krajewska & Shugar, 1971, 1972). The absence of any

concentration or oxygen effects further indicate that photointermediate formation proceeds *via* an excited singlet state, as might be anticipated for an intramolecular photocyclization reaction.

The quantum yield for photodissociation of the intermediate, about 0.20, is somewhat lower than that expected for "half" of a pyrimidine photodimer, but is similar to the values reported for such photointermediates from 5-EtUrd, 5-PrUrd and 5-iPrUrd.

The absence of photodimer formation from 5-HeUrd is most likely due to steric effects involving the 5-hexyl substituent. In the case of 5-EtUrd, irradiation in aqueous medium at wavelengths to the red of 265 nm, or in frozen aqueous medium at 254 nm, leads predominantly to photodimerization. But dealkylation is the major reaction on irradiation in fluid medium at 254 nm, photodimerization accounting for only 10 - 15% of the photoproducts (Pietrzykowska & Shugar, 1968, 1970), the obvious conclusion being that intramolecular photocyclization *via* the excited singlet is so rapid as to effectively compete with intersystem crossing to the triplet state necessary for photodimerization. The complete absence of photodimerization for derivatives with higher 5-alkyl substituents, i.e. 5-PrUrd and 5-iPrUrd (Krajewska & Shugar, 1971, 1972) and 5-HeUrd suggest that this is due largely to the steric hindrance of the large alkyl chains.

The foregoing considerations are of biological significance in relation to the photochemistry of bacteriophages. Several examples have been reported recently of the isolation of phages, the DNA of which contain pyrimidines with fairly long 5-alkyl chains, e.g. the DNA of *Bacillus subtilis* phage SP-15 contains, in place of thymine, 12 mole % of 5-(4′,5′-dihydroxypentyl)uracil (Marmur *et al.*, 1972; Brandon *et al.*, 1972).

Disregarding the problem of isomerization, the cyclobutane photointermediate of 5-HeUrd is clearly identical with the 1:1 photoadduct of Urd and 1-hexene. Such 1:1 photoadducts of unsaturated hydrocarbons with the 5,6 bonds of pyrimidine rings appear to be quite general and include photoadducts of ethylene and propylene with thymidine (Z. Zarębska, in preparation) and ethylene with 1,3-dimethylthymine (Maleski & Morrison, 1972). More significant from a biological standpoint is the demonstration that ethylene photoadducts with thymine residues in poly(dAT) may be formed (Z. Zarębska, in preparation), pointing to the possibility of such photoadduct formation by irradiation of DNA or RNA in solution in the presence of ethylene. Photoadduct formation in nucleic acids may also be feasible by means of sensitization, as has been demonstrated in the case of free pyrimidines (Hyatt & Swenson, 1972).

The problem of distinguishing between stereoisomeric photoadducts poses a somewhat more difficult problem. It has already been pointed out that, with hydrocarbon chains longer than ethyl, the 1:1 adducts with uracil nucleosides may include at least 8 diastereoisomers (Krajewska & Shugar, 1972), i.e. twice as many as could arise from intramolecular photocyclization of the corresponding 5-alkyl nucleoside, where *syn-anti* isomerization is excluded. In the case of the 5-HeUrd:1-hexene photoadduct herein reported, the alkaline decomposition curve (Fig. 5) points to the pre-

sence of at least two isomers. For the 1-MeUra:1-hexene adduct, where the alkaline decomposition curve is one-step in nature, the presence of at least two isomers is testified to by the two-step melting of the crystalline product, as well as by the NMR spectral and gas chromatography data, the NMR data pointing to these isomers as being *syn* and *anti*. Unfortunately attempts to separate the 1-MeUra:1-hexene photoadduct isomers by preparative gas chromatography were unsuccessful, because of technical difficulties with the equipment.

With the 5-HeUrd:1-hexene photoadduct, where the presence of 7 peaks was revealed by gas chromatography of the silylated adducts, it is clear that identification of these would be possible only by isolation of the individual isomers by preparative gas chromatography.

Finally, it should be emphasized that the cyclobutane photointermediates and the 1:1 photoadducts herein described are simultaneously analogues of 5,6-dihydropyrimidines and their nucleosides, as well as of photodimers of pyrimidines or pyrimidine nucleosides. They should consequently be of some interest in clarification of the properties of the latter, the more so in that, as mentioned above, the photoadducts may be formed with thymine residues in nucleic acids.

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## FOTOCHEMICZNE PRZEKSZTAŁCENIE 5-HEKSYLOURYDYNY ORAZ CYKLOBUTANOWYCH FOTOADDUKTÓW 1-HEKSENU Z URYDYNĄ I 1-METYLOURACYLEM

### Streszczenie

1. Naświetlanie 1-heksylourydyny w obojętnych roztworach wodnych zarówno promieniowaniem UV powyżej 265 nm, jak i 254 nm prowadzi do dealkilacji z powstawaniem urydyny, która z kolei ulega fotouwodnieniu wiązania 5,6. Fotodimeryzacja pirymidyn w tych warunkach nie zachodzi; występuje ona prawdopodobnie w niewielkim stopniu w matrycy lodowej.

2. Reakcja dealkilacji przebiega poprzez wewnątrzcząsteczkową fotocyklizację rodnika heksenowego z wiązaniem 5,6-pirymidyny, prowadząc do powstania 5,6-dwuhydro-5,6-cyklobutanylopodstawionej urydyny. Związek pośredni ulega fotodysocjacji do urydyny oraz 1-heksenu z wysoką wydajnością kwantową (ok. 0,20).

3. Zaobserwowano powstawanie z niższą wydajnością dodatkowego fotoproduktu o maksimum absorpcji UV 348 nm w pH alkalicznym. Wyjątkowo duża labilność tego fotoproduktu utrudniła jego identyfikację.

4. Otrzymano w skali preparatywnej związek pośredni o strukturze cyklobutanowej drogą naświetlania urydyny w obecności 1-heksenu. W ten sposób otrzymano fotoaddukt 1-metylouracylu i heksenu. Obydwa fotoaddukty cyklobutanowe były mieszaninami diastereoizomerów, które rozdzielano przy pomocy analitycznej chromatografii gazowej. W przypadku fotoadduktu 1-metylouracylu i heksenu występowały dwa stereoizomery, a w przypadku urydyny i heksenu — co najmniej dwa.

5. Widma masowe i MRJ adduktów 1-heksenu i 1-metylouracylu potwierdziły obecność izomerów, przy czym oznaczono je prowizorycznie jako *syn* i *anti*.

6. Opisano inne własności fizykochemiczne fotoadduktów 1-heksenu i urydyny oraz 1-metylouracylu i porównano je z poprzednio opisanymi fotoadduktami etylenowymi i propylenowymi. Przedyskutowano aspekty biologiczne opisanych badań.

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# ROLE OF THE SOLVENT MEDIUM IN THE STABILIZATION OF HELICAL POLY(rU) BY POLYAMINES*

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The ordered structure formed by poly(rU) in the presence of a polyamine (spermidine) is destabilized on addition of various buffer ions, or of monovalent cations such as $Na^+$. This destabilizing effect is due in part to competition with the polyamine protonated amino groups for the polynucleotide phosphate sites. The results, together with other data culled from the literature, indicate that this phenomenon is quite general and encountered with natural nucleic acids, association of ribosomal subunits, etc. It must be taken into consideration in any studies on interaction of nucleic acids with polyamines.

The finding that polyamines are associated with DNA in the T-even bacteriophages stimulated numerous investigations on the stability of nucleic acids and synthetic polynucleotides by these biologically important and widespread compounds (for review see Cohen, 1971). In general polyamines provoke an increase in stabilization, usually expressed in terms of an increase in the $T_m$, of both natural nucleic acids (Tabor, 1961, 1962; Mandel, 1962; Goldstein, 1966) and synthetic polynucleotides (Szer, 1966a, b; Matsuo & Tsuboi, 1966; Higuchi & Tsuboi, 1966; Gabbay, 1966; Glaser & Gabbay, 1968). The interaction of spermidine and spermine with synthetic polyribonucleotides is more pronounced than that of diamines. The latter, in turn, more effectively interact than divalent cations (Szer, 1966b). Several proposals have been advanced as to the nature of the interactions between polyamines and nucleic acids, all of them based on the formation of specific complexes between the protonated amino groups of the polyamine and the negatively charged phosphates of the polynucleotide helix in such a manner that the polyamine is located in the narrow groove of the helix (Felsenfeld & Huang, 1961; Liquori *et al.*, 1967).

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Observations on the interaction of polyamines with poly(rU) analogues such as poly 5-ethyluridylic acid, poly 2′-*O*-methyluridylic acid and poly 2′-*O*-ethyluridylic acid (Świerkowski & Shugar, 1970; Żmudzka & Shugar, 1971; Kuśmierek *et al.*, 1973), raised some questions regarding the behaviour reported earlier for poly(rU) in the presence of polyamines (Szer, 1966b), and suggested that the nature of the solvent medium is of considerable significance with respect to these interactions. The present study reports on the influence of various ions on the interaction of poly(rU) with spermidine and relates these findings to others previously reported on the interaction of polyamines with both natural and synthetic polynucleotides.

## MATERIALS AND METHODS

The sodium salt of UDP, and spermidine·3HCl, were obtained from Calbiochem (Lausanne, Switzerland). Pancreatic ribonuclease, activity 1900 units/mg, was a product of Reanal (Budapest, Hungary). We are indebted to Dr. H. Matthaei for a sample of *M. luteus* polynucleotide phosphorylase, fraction III, prepared according to Matthaei *et al.* (1967).

Temperature profiles were run on a Zeiss (Jena, G.D.R.) VSU-2 spectrophotometer fitted with a thermostated cuvette carriage through which circulated a water--glycol mixture with the aid of a Hoeppler ultrathermostat. A thermistor in a dummy cuvette in the heating block was used to control temperatures to an accuracy of 0.1°C.

Analytical ultracentrifugations were carried out on a MOM Model G-120 ultracentrifuge (MOM, Budapest, Hungary) fitted with u.v. optics and with rotor temperature control to 0.1°C.

*Preparation of poly(rU)*. The polymer was prepared from UDP as described by Matthaei *et al.* (1967), with some modifications in the purification stage. The final polymer preparation was successively dialysed against 0.01 M-NaCl - 0.001 M-EDTA - 0.01 M-phosphate buffer, pH 7; 0.001 M-NaCl - 0.001 M-EDTA - 0.001 M-phosphate buffer, pH 7; and 0.001 M-phosphate buffer, pH 7. Residual low molecular weight fragments not removed by dialysis were eliminated by precipitation of the polymer as described by Rottman & Cerutti (1966). The poly(rU) preparation was washed with 85% aqueous ethanol and subjected once more to dialysis against decreasing concentrations of NaCl and pH 7 phosphate buffer, the final dialysis being against quartz redistilled water. The poly(rU) thus obtained exhibited no change in sedimentation pattern for a period up to 2 months, the longest storage period employed.

Several samples of poly(rU) prepared in the foregoing manner exhibited $s_{20}$ values varying from 5 to 6, all preparations showing a sharp boundary in the centrifuge. Hydrolysis of the polymer with ribonuclease (10 μg enzyme added to 1 ml of $\sim 10^{-4}$ M-polymer solution) gave an extinction coefficient for the polymer of $9.1 - 9.2 \times 10^3$ at 260 nm, assuming an extinction coefficient for 2′(3′)-UMP of $10^4$. Polymer concentrations were subsequently determined spectrophotometrically.

## RESULTS

In all that follows, the concentration of spermidine is expressed in terms of the ratio of the number of amino groups of the polyamine to the number of phosphate groups in the poly(rU) preparation. All polymer solutions, including those which were unbuffered, were at a pH not exceeding 7.5. Furthermore, all measurements

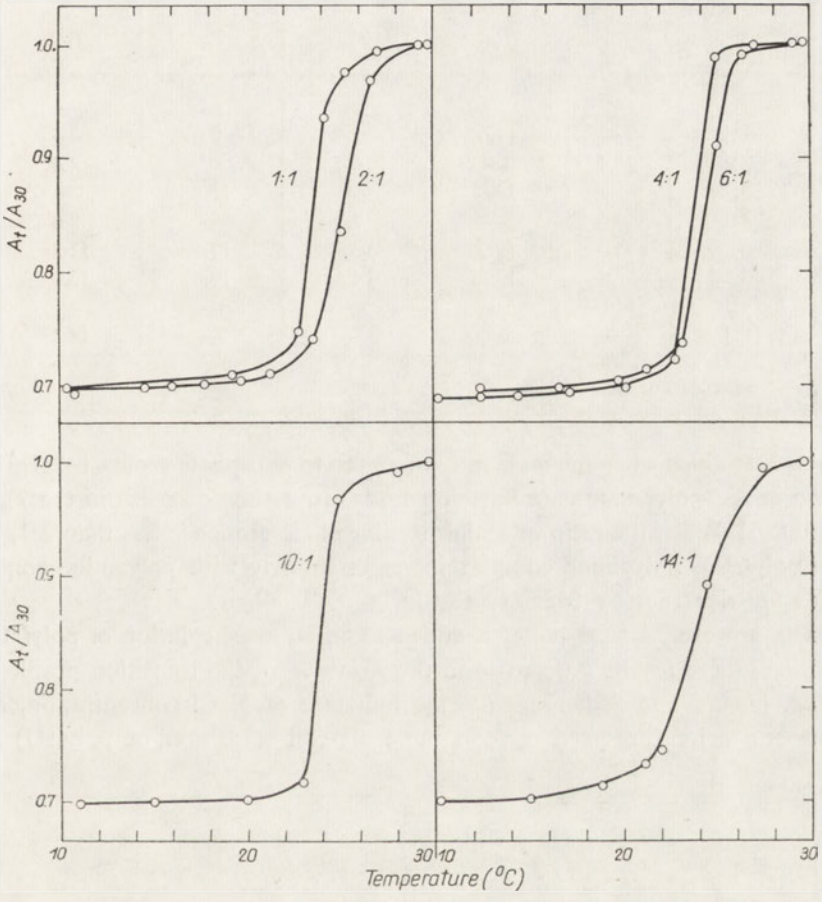


Fig. 1. Melting profiles of poly(rU) in aqueous unbuffered medium, pH 6 - 7, in the presence of different proportions of spermidine. The figures beside each profile indicate the molar ratio of amino to phosphate groups.

were conducted at temperatures which did not exceed 25°C. This ensured, bearing in mind the reported $pK$ values, as well as their temperature coefficients for spermidine (Hirschman *et al.*, 1967), that the latter was fully protonated under all experimental conditions herein reported.

The helix-coil transition, or temperature profile, measured by the change in absorbance at $\lambda_{max}$ (260 nm) of poly(rU) in the presence of various concentrations of spermidine, is shown in Fig. 1. It will be noted that the highest $T_m$ exhibited by

Table 1

*Influence of the solvent medium on the* $T_m$ *of poly(rU) - spermidine complexes*

| Molar ratio of spermidine amino groups to polynucleotide phosphate groups | $H_2O$ (pH 6.0) | 0.02 M-Tris-HCl, pH 7.0 | 0.05 M-Tris-HCl, pH 7.0 | 0.02 M-cacodylate, pH 7.0 | 0.02 M-phosphate, pH 7.0 |
|---|---|---|---|---|---|
| | $T_m$ | $T_m$ | $T_m$ | $T_m$ | $T_m$ |
| 0.7:1 | 14* | — | — | — | — |
| 1:1 | 23.2 | 11.6 | 1.8 | 3.6 | — |
| 2:1 | 25.4 | — | 6.4 | — | 4.4 |
| 3:1 | — | 14.8 | — | 12.4 | — |
| 4:1 | 24.6 | — | 7.8 | — | 8.6 |
| 6:1 | 24.0 | 19.0 | 10.8 | 17.6 | 11.2 |
| 10:1 | 23.9 | 19.8 | 14.6 | 19.6 | 13.0 |
| 14:1 | 23.8 | — | — | — | — |
| 15:1 | — | — | — | — | 14.2 |

* Taken from Szer (1966a).

poly(rU) is attained when the mole ratio of amino to phosphate groups is 2:1. Further increase in the polyamine concentration leads to a slow decrease in the $T_m$ value (see Table 1). When the ratio of amino to phosphate groups is less than 2:1, the $T_m$ of the poly(rU) - polyamine complex increases linearly with polyamine concentration, as noted earlier by Szer (1966a,b).

When, however, salt or buffer is added to an aqueous solution of poly(rU) and spermidine, there results a decrease in the $T_m$ value of the transition profile of the complex. Figs. 2 and 3 demonstrate the influence of NaCl concentration, and of various buffer ions, respectively, on the helix-coil transition of poly(rU) in the

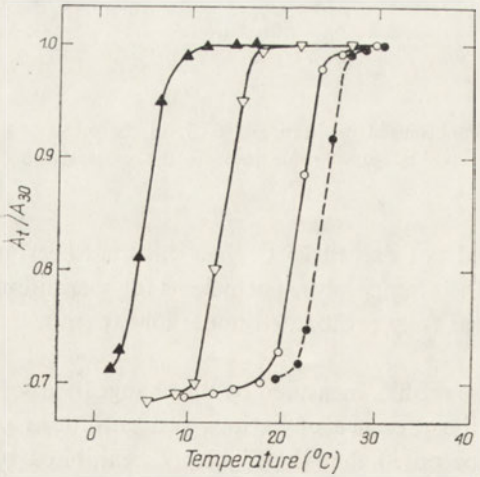


Fig. 2. Temperature profiles in neutral aqueous unbuffered medium of poly(rU) in the presence of 1 molar equivalent of spermidine, at different NaCl concentrations: (●), no salt; (○), 0.001 M-NaCl; (▽), 0.01 M-NaCl; (▲), 0.1 M-NaCl.

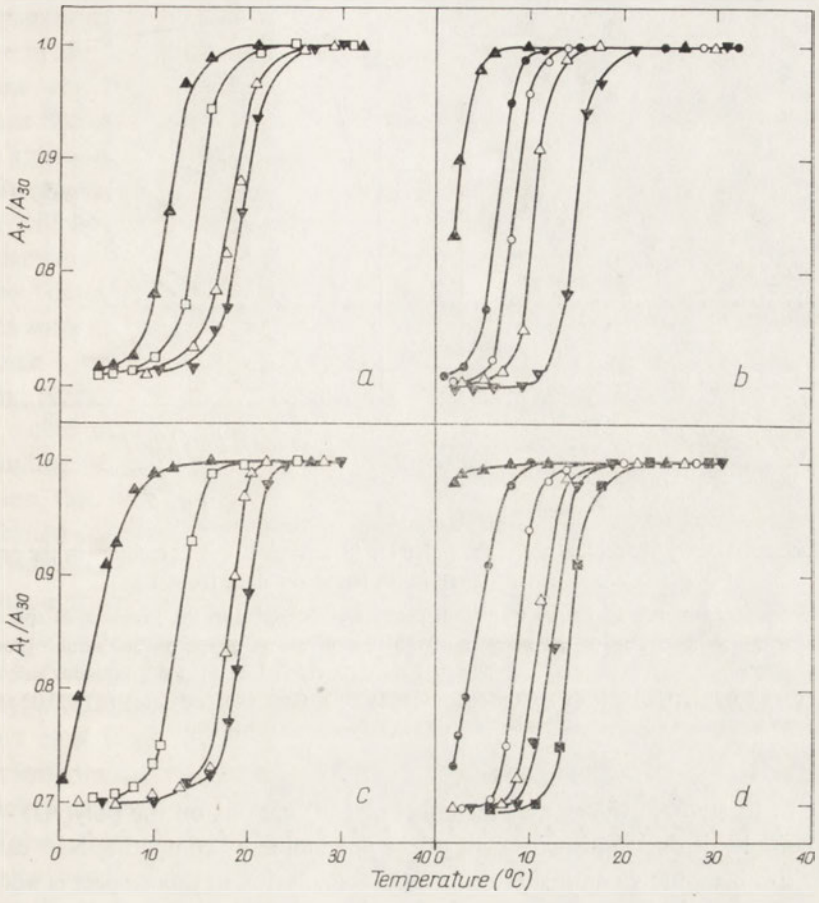


Fig. 3. Temperature profiles at pH 7.0 in the presence of various buffers of poly(rU) - spermidine complexes in the following molar proportions of amino to phosphate groups: (▲), 1:1; (●), 2:1; (□), 3:1; (○), 4:1; (△), 6:1; (▼), 10:1; (■), 15:1. (*a*), 0.02 M-Tris-HCl; (*b*), 0.05 M-Tris-HCl; (*c*), 0.02 M-cacodylate; (*d*), 0.02 M-phosphate.

presence of spermidine. From Fig. 4 it will be seen that the $T_m$ of the poly(rU)--spermidine complexes is linearly dependent on the logarithm of the NaCl concentration.

The influence of various buffer ions on the $T_m$ of the complexes, in the absence of added salt, is illustrated in Fig. 5 and Table 1.

## DISCUSSION

It is clear, from the foregoing results, that the extent of stabilization of polynucleotides by polyamines is dependent on both the ionic strength, and the nature of the ions, in the medium. It is, indeed, most surprising that this effect is so frequently overlooked, with resultant erroneous interpretations, particularly when extrapolations are done to biological systems.

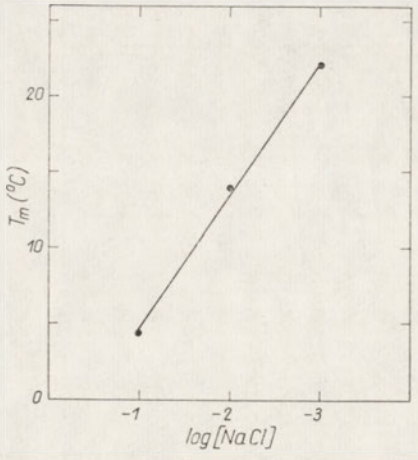


Fig. 4

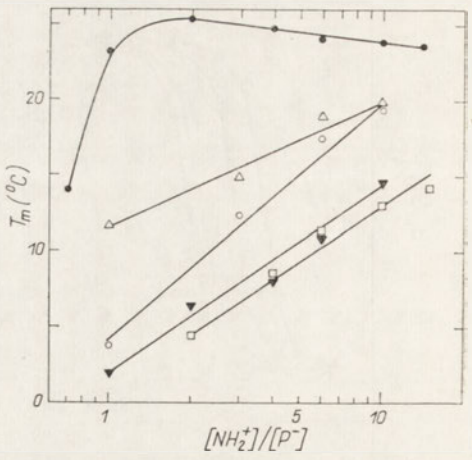


Fig. 5

Fig. 4. Dependence of $T_m$ on log[NaCl] for poly(rU) in aqueous neutral medium in the presence of 1 molar equivalent of spermidine, based on data from Fig. 2.

Fig. 5. Dependence of the $T_m$ of the transition profiles of poly(rU) in the presence of spermidine on the logarithm of the ratio of the number of molar equivalents of spermidine amino groups to polynucleotide phosphate groups at pH 7 (based on data from Fig. 3): (●), aqueous unbuffered medium; (△), 0.02 M-Tris-HCl; (▼), 0.05 M-Tris-HCl; (○), 0.02 M-cacodylate; (□), 0.02 M-phosphate.

It is, furthermore, evident that the effect of $Na^+$ cations on the poly(rU) - spermidine complex is due to displacement of the polyamine by competing $Na^+$ cations, and that this is a purely competitive effect. Our conclusion in this respect is not new. Apparently attention was first drawn to the competitive effect between mono- and divalent cations with polyamines by Eisinger *et al.* (1965) in a study on several polynucleotide systems, and was examined in some detail by Horacek & Cernohorsky (1968) for calf thymus DNA (see also Cohen, 1971). In the case of poly 2′-*O*-ethyluridylic acid which, in the presence of only 1 molar equivalent of spermidine, does not melt out even at 100°C, the addition of 0.01 M-$Na^+$ brings the $T_m$ down to 46°C (Kuśmierek *et al.*, 1973). Even with such a simple system as poly(rU), overlooking of this fact renders interpretation of results somewhat complex, e.g. Fig. 1 in a paper by Thrierr *et al.* (1972).

The "destabilizing" effect of salts on polynucleotide - polyamine complexes must be due to the greater affinity of these salts for the polynucleotide phosphate groups, notwithstanding that this leads to a decrease in helix stability. The dependence of this competitive effect on the physical dimensions of the competing cations was pointed out by Eisinger *et al.* (1965). An additional effect which must be taken into consideration is the nature of the buffer ions in the medium. In a study of the influence of spermine on the stability of DNA in the presence of cacodylate buffer and 0.01 M-NaCl, Hirschman *et al.* (1967) noted that, under their experimental conditions ($10^{-4}$

M-polyamine and $5 \times 10^{-3}$ M-buffer) about 12% of the polyamine behaved as though it were in the form of a stable complex with the buffer anions. This undoubtedly explains why the stabilizing effect of spermidine on poly(rU) in the presence of different buffers (Fig. 5) is least pronounced with phosphate buffer. It is probably to be anticipated that the interactions of basic polypeptides with polynucleotides, e.g. the reported stabilization of the helical form of poly(rU) by polylysine (Carroll, 1972), will be dependent on the same factors, and would have to be taken into consideration.

The "destabilization" of the poly(rU) - spermidine complex by monovalent cations such as $Na^+$ is also of interest in relation to the observed destabilization of ribosome couples by a variety of such ions, of which $Na^+$ is the most effective (Spirin, 1972).

It would obviously be desirable to measure directly the equilibrium constants for binding of polyamines to polynucleotides. The only reported attempt in this direction, that of Thrierr *et al.* (1972), who used dialysis equilibrium to measure the equilibrium constant between spermine and poly(rU), was too inaccurate to permit of the calculation of the constants.

Although not the objective of this study, it is of interest to consider the nature of the complex formed between poly(rU) and spermidine. Assuming the "hairpin" model for the helical form of poly(rU), so that the two strands are antiparallel, the only type of hydrogen bonding possible, with all the residues in the form *anti* as appears most likely from circular dichroism measurements (Rabczenko & Shugar, 1971), involves the $N_3$ and $C_4$, and the corresponding $C_2$ and $N_3$ of the complementary uracil residues (Fuller & Hodgson, 1967; Rabczenko & Shugar, 1971). This is different from that found in crystals of 1-methyluracil, where complementary hydrogen bonding is of the Watson-Crick type, involving the $N_3$ and $C_4$ of each residue (Voet & Rich, 1970). However, the non-Watson-Crick type of hydrogen bonding has recently been confirmed in the case of poly 2-thiouridylic acid fibres by X-ray diffraction measurements (W. Saenger, personal communication). Furthermore, both circular dichroism data, and temperature-dependent viscosity data in the neighbourhood of the $T_m$ value (Thrierr *et al.*, 1971, 1972), indicate that the helical structure of poly(rU), whether stabilized by polyamines or mono- or divalent cations, is the same.

In view of the foregoing, the molecular model of the poly(rU) - spermidine complex may be accepted as resembling that proposed earlier for the interaction of DNA with polyamines (Liquori *et al.*, 1967), as well as one of the models for the interaction of polyamines with polyribonucleotides proposed by Glaser & Gabbay (1968), and referred to as the interstrand model. According to these, the protonated spermidine amino groups separated by three carbon atoms form salt-like bonds with two consecutive phosphates in one strand, while the third protonated amino group (separated by four carbon atoms from the neighbouring amino group) would combine with a phosphate in the complementary strand. In the case of spermine the fourth amino group would form an ionic bond with the phosphate adjacent to that combining with the third amino group in spermidine.

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## WPŁYW SKŁADU ROZPUSZCZALNIKA NA STABILNOŚĆ STRUKTURY POLI(rU) POWSTAJĄCEJ W OBECNOŚCI POLIAMIN

### Streszczenie

Uporządkowana struktura poli(rU) powstająca w obecności poliaminy (spermidyny) jest destabilizowana przez jony buforowe oraz przez jednowartościowe kationy takie jak $Na^+$. Ten destabilizacyjny wpływ jest częściowo wynikiem zróżnicowanej kompetycji jonów soli i protonowanych grup aminowych w stosunku do grup fosforanowych polinukleotydu. Nasze wyniki wraz z danymi istniejącymi w literaturze świadczą o tym, że zjawisko to ma charakter bardziej ogólny i jest obserwowane w przypadku naturalnych kwasów nukleinowych, podczas asocjacji podjednostek rybosomalnych, itp. Wyniki te mogą przyczynić się do lepszego zrozumienia oddziaływań kwasów nukleinowych z poliaminami.

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# THE RELATIONSHIP BETWEEN DNA SYNTHESIS AND INCORPORATION OF [$^{14}$C]LYSINE INTO DIFFERENT HISTONE FRACTIONS IN EHRLICH ASCITES TUMOUR CELLS

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The effect of inhibition of DNA synthesis by hydroxyurea on [$^{14}$C]lysine incorporation into the main four histone fractions in Ehrlich ascites tumor cells, was examined *in vitro*. The radioactivity of lysine-rich histones, especially of histone *f1*, was preferentially decreased. The smallest decrease was observed for histone *f3*. The incorporation into other cellular proteins was but slightly affected.

The metabolic relation between the synthesis of particular histone fractions and synthesis of DNA found by some authors (Chalkley & Maurer, 1965; Ohly *et al.*, 1967; Sadgopal & Bonner, 1969) as well as the selectivity of their association with DNA (Clark & Felsenfeld, 1972; Šponar & Šormová, 1972) seem to indicate an essentially different role of the particular histone fractions in the DNA-histone complex, especially as regards the lysine- and arginine-rich fractions.

In the present work an attempt was made to determine the dependence on DNA synthesis of [$^{14}$C]lysine incorporation *in vitro* into four main histone fractions isolated from Ehrlich ascites tumour cells.

## MATERIALS AND METHODS

The mice bearing ascites tumour were kindly supplied by the Institute of Oncology in Warsaw. The tumour cells were further propagated in mice of BALB/c strain by intraperitoneal passages on every seventh day.

For experiments, ascitic fluid was withdrawn after 7 days of growth and centrifuged. The cells were washed in Hanks-Simms solution (Cameron, 1950) and immediately suspended in the same solution containing 25% of human serum, to the

density of 2000 cells/μl. The cells were preincubated with the indicated amounts of hydroxyurea, and then 0.05 μCi of [$^{14}$C]lysine/ml or 0.5 μCi of [$^{32}$P]orthophosphate/ml was added. The samples were incubated in duplicate at 37°C; the controls were preincubated without the inhibitor.

Cell nuclei were isolated according to Fischer & Harris (1962) using 0.1% Tween; on phase contrast microscopy they exhibited a high degree of purity. From the nuclei, four main histone fractions (*f1*, *f2a*, *f2b* and *f3*) were isolated by the method 1 of Johns (1964). Preliminary experiments showed that with the nuclei of ascites cells this method yielded preparations of fraction *f3* that gave amino acid analyses suggesting contamination by other proteins. Therefore the modification of MacGillivray (1968) applied for separation of fractions *f2a* and *f3* from solid human tissues, was used. The obtained fractions were subjected to further purification: fractions *f1* and *f3* according to Dick & Johns (1969); *f2a* by repeated solubilization in acidic ethanol (80% ethanol containing 0.25 N-HCl) and dialysis against ethanol; and *f3* by reprecipitation from aqueous solution.

The residue of nuclear proteins after isolation of histones was extracted twice with 5% trichloroacetic acid at 90°C for 10 min to remove nucleic acids, and delipidated.

The supernatant collected after separation of nuclei from the tumour cell homogenate, was clarified by centrifugation at 2000 *g* for 10 min and designated the cytoplasmic fraction.

DNA was separated from the isolated nuclei by the procedure of Schmidt-Thannhauser in the modification of Schneider (1945).

Starch-gel electrophoresis of histones was performed according to Johns *et al.* (1961).

Determinations of lysine, arginine, aspartic and glutamic acids were carried out as described by Fischer & Dörfel (1953). Protein was assayed by the microbiuret method of Siltanen & Kekki (1960) and DNA by the method of Burton (1956).

For radioactivity determinations, the protein and DNA solutions were dried on the planchettes and radioactivity was measured in window-less gas-flow counter (Tracer-Lab., Amsterdam, Holland). Sufficient counts were recorded to reduce the error to 2% or less.

Sodium [$^{32}$P]orthophosphate (carrier-free) in aqueous solution was obtained from the Institute of Nuclear Research (Świerk, Poland). [$^{14}$C]Lysine (spec. activity 85 mCi/mmol) was obtained from UVVVR (Prague, Czechoslovakia). Hydroxyurea was a gift from Zakłady Farmaceutyczne "Polfa" (Kraków, Poland). Standard preparations of calf thymus histone fractions were kindly supplied by Dr. E.W. Johns of the Chester Beatty Research Institute (London, England).

## RESULTS AND DISCUSSION

The four main histone fractions (*f1*, *f2a*, *f2b* and *f3*) obtained from Ehrlich ascites tumour cells, showed electrophoretic patterns closely similar to those of standard histone preparations from calf thymus. The analysis of amino acid com-

position of the histone fractions isolated in six series of experiments showed that the obtained preparations were fully comparable (Table 1), and their content of basic and acidic amino acids resembled closely that of standard histones, estimated in parallel determinations.

Table 1

*Amino acid content of histone fractions isolated from Ehrlich ascites tumour cells*

The results, expressed as percentage in the respective fraction, are mean values from 6 experiments ±S.D.

| Fraction | Lysine (%) | Arginine (%) | Aspartic acid (%) | Glutamic acid (%) | Lysine to arginine ratio | Basic to acidic amino acids ratio |
|---|---|---|---|---|---|---|
| *f1* | 28.26±1.22 | 2.71±0.32 | 3.21±0.45 | 4.91±0.39 | 10.56±1.28 | 3.83±0.12 |
| *f2a* | 10.27±0.65 | 10.93±0.48 | 4.52±0.56 | 6.59±0.72 | 0.94±0.03 | 1.91±0.22 |
| *f2b* | 13.04±0.72 | 6.04±0.43 | 4.53±0.50 | 7.63±0.61 | 2.17±0.11 | 1.57±0.11 |
| *f3* | 9.13±0.52 | 12.49±0.97 | 4.02±0.47 | 8.12±0.65 | 0.73±0.03 | 1.79±0.19 |

The inhibition of DNA synthesis by hydroxyurea lowered significantly the incorporation of [$^{14}$C]lysine into histones, and had no or a much smaller effect on its incorporation into other cellular proteins (Table 2). Such results could be expected since several authors found that inhibition of DNA synthesis by hydroxyurea (Zampetti-Bosseler *et al.*, 1969), cytosine arabinoside (Borun *et al.*, 1967) or 5-fluorodeoxyuridine (Sadgopal & Bonner, 1969) resulted in selective inhibition of histone synthesis, occurring probably at the level of messenger transcription as suggested by the observations of Borun *et al.* (1967) who found a temporal correlation of DNA replication, 7 - 9S RNA and histone synthesis in S-phase HeLa cells treated with cytosine arabinoside.

Although we were unable to find such experimental conditions under which only one histone fraction would be selectively affected, the presented data show that the incorporation of [$^{14}$C]lysine into the very lysine-rich fraction *f1* was inhibited to the greatest extent. The moderately lysine-rich fractions *f2a* and *f2b* were less affected. The smallest inhibition was observed with the arginine-rich fraction *f3* which at lower inhibitor concentration or shorter time of treatment was not affected et all. The effect of hydroxyurea on the incorporation into the non-histone nuclear protein and cytoplasmic protein was small and resembled that observed with histone *f3*. The effect of the time of treatment with 76 μg of the inhibitor/ml, is illustrated in Fig. 1.

The very similar extent of the inhibition in the nuclear acid-insoluble and cytoplasmic proteins might suggest a non-specific mechanism of this event, e.g. a general malfunction of cellular macromolecule synthesis under conditions of greater impair-

Table 2

*Incorporation of* [$^{32}$P]*orthophosphate into DNA, and of* [$^{14}$C]*lysine into proteins of Ehrlich ascites tumour cells treated and non-treated with hydroxyurea*

The cells were labelled by incubation *in vitro* for 60 min at 37°C with radioactive compounds, and DNA and proteins were isolated as described in Materials and Methods. The specific activity values of histone fractions were normalized for the lysine content.

| Expt. no. | Concn. of inhibitor (μg/ml) | Preincubation with inhibitor (min) | Control cells, specific activity (counts/min/mg protein) | | | | | Treated cells, inhibition of incorporation (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | DNA | *f1* | *f2a* | *f2b* | *f3* | DNA | *f1* | *f2a* | *f2b* | *f3* | nuclear acid-insoluble protein | cytoplasmic protein |
| 1 | 7.6 | 30 | 18 324 | 7 405 | 5 245 | 7 096 | 6 171 | 10.2 | 18.2 | 10.4 | 7.5 | 0 | 0 | — |
| 2 | 38 | 30 | 21 418 | 7 665 | 4 753 | 6 476 | 5 942 | 18.5 | 25.7 | 14.6 | 7.9 | 2.3 | 1.5 | 0 |
| 3 | 38 | 60 | 23 478 | 9 404 | 6 809 | 9 098 | 7 646 | 28.1 | 42.3 | 31.1 | 32.8 | 14.7 | 8.4 | 6.6 |
| 4 | 38 | 30 | 20 115 | 7 558 | 6 112 | 7 033 | 6 573 | 25.8 | 44.3 | 28.4 | 23.8 | 18.1 | 5.3 | 6.5 |
| | 38 | 120 | 22 192 | 9 400 | 6 793 | 7 754 | 6 862 | 38.7 | 55.4 | 36.2 | 33.3 | 20.1 | 9.8 | 7.8 |
| 5* | 76 | 30 | 8 916 | 2 938 | 1 883 | 2 963 | 2 511 | 17.2 | 31.3 | 16.5 | 6.4 | 1.6 | 0 | 3.6 |
| 6* | 76 | 30 | 10 235 | 4 985 | 3 862 | 4 213 | 3 511 | 15.9 | 37.2 | 25.7 | 19.5 | 6.3 | 3.8 | 0 |
| 7 | 76 | 30 | 23 282 | 9 960 | 6 403 | 7 509 | 7 905 | 22.8 | 52.6 | 35.2 | 41.1 | 12.7 | 13.4 | 15.9 |
| 8 | 76 | 30 | — | 8 234 | 5 641 | 7 392 | 6 484 | — | 37.8 | 24.3 | 20.6 | 11.8 | 0 | 3.4 |
| 9 | 76 | 30 | 19 642 | 8 369 | 4 978 | 8 514 | 7 215 | 21.4 | 35.9 | 21.3 | 20.6 | 15.2 | 8.4 | — |
| | 76 | 90 | 21 814 | 9 832 | 5 742 | 9 101 | 8 126 | 32.8 | 50.6 | 28.6 | 33.3 | 20.1 | 13.4 | 11.7 |
| 10 | 76 | 60 | 16 971 | 7 061 | 5 627 | 5 903 | 5 517 | 26.9 | 49.3 | 37.1 | 37.6 | 24.3 | 11.5 | 12.8 |
| 11 | 76 | 60 | 21 376 | 5 527 | 4 145 | 5 280 | 4 935 | 35.7 | 51.3 | 37.1 | 32.8 | 23.4 | 8.5 | 10.6 |
| | 76 | 180 | 22 743 | 7 285 | 4 405 | 5 592 | 6 302 | 48.7 | 58.3 | 41.4 | 38.3 | 35.2 | 17.6 | 15.4 |
| 12 | 76 | 180 | 17 491 | 8 881 | 7 196 | 7 520 | 6 015 | 52.3 | 61.7 | 40.8 | 42.1 | 32.3 | 23.6 | 17.8 |

* Incubation with label for 30 min instead of 60 min.

ment of DNA synthesis. If so, such a "malfunctional", non-specific inhibition could overlap the specific inhibition of the DNA-dependent histone synthesis. When the "non-specific" inhibition of [$^{14}$C]lysine incorporation into all the examined proteins is taken into account, the differences in the "specific" dependence of the synthesis of various histones on DNA synthesis become more apparent (Fig. 1).

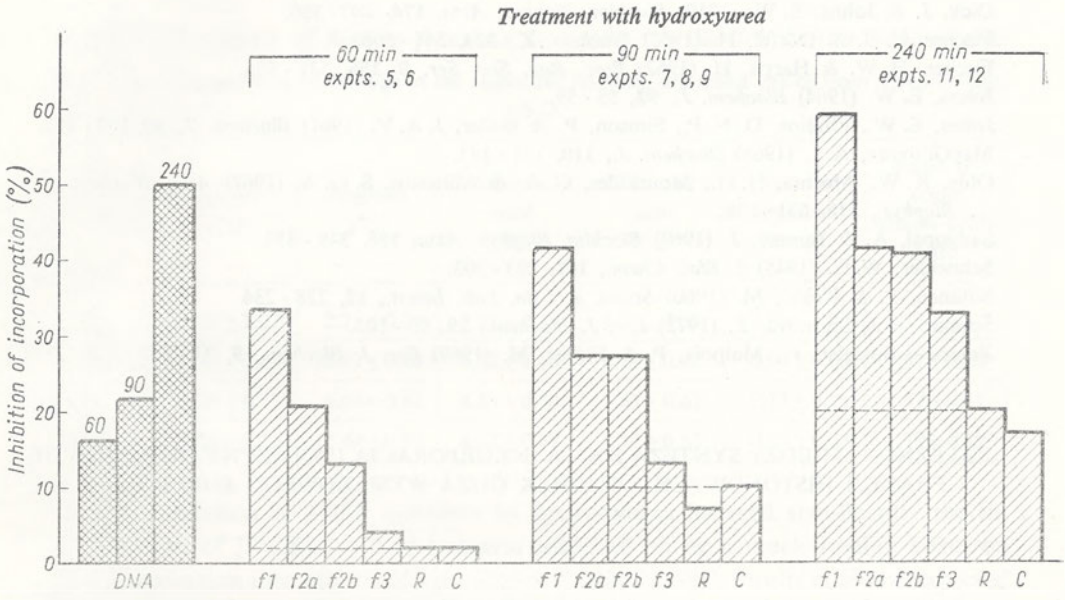


Fig. 1. Effect of hydroxyurea (76 μg/ml) on incorporation of $^{32}P_i$ into DNA and [$^{14}$C]lysine into the indicated histone fraction, the nuclear, non-histone protein (*R*) and cytoplasmic protein (*C*) of Ehrlich ascites tumour cells. The time of treatment with hydroxyurea is the sum of the time of preincubation and incubation. Average values of the indicated experiments (cf. Table 2) are given.

The presented results are in general agreement with the observations by Chalkley & Maurer (1965) on tobacco pith-cells and Sadgopal & Bonner (1969) on HeLa cells that the synthesis of lysine-rich histones is more dependent on DNA synthesis as compared with arginine-rich histones. However, in HeLa cells treated with 5-fluorodeoxyuridine or thymidine Sadgopal & Bonner found the most pronounced inhibition in moderately lysine-rich histones, whereas in our experiments with Ehrlich ascites tumour cells the most pronounced inhibition concerned the histone very rich in lysine. This observation may indicate that among the lysine-rich histones dependent on DNA synthesis, very lysine-rich histones of Ehrlich ascites tumour cells could be particularly strictly coupled with DNA synthesis.

The authors are grateful to Dr. E. W. Johns of the Chester Beatty Research Institute in London for standard calf thymus histone preparations, and to Prof. Dr. K. Dux and Dr. U. Bregula of the Institute of Oncology in Warsaw for the Ehrlich ascites-tumour bearing mice.

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## ZALEŻNOŚĆ MIĘDZY SYNTEZĄ DNA A INKORPORACJĄ [$^{14}$C]LIZYNY DO RÓŻNYCH FRAKCJI HISTONOWYCH KOMÓREK GUZA WYSIĘKOWEGO EHRLICHA

### Streszczenie

Badano *in vitro* wpływ hamowania syntezy DNA hydroksymocznikiem na inkorporację [$^{14}$C]lizyny do czterech głównych frakcji histonowych komórek guza wysiękowego Ehrlicha. Najbardziej zmniejszona jest radioaktywność histonów bogatych w lizynę, a zwłaszcza histonu *f1*. Aktywność właściwa histonu *f3* ulega obniżeniu w najmniejszym stopniu. Inkorporacja do innych białek komórkowych ulega tylko nieznacznym zmianom.

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B. SIELIWANOWICZ, EWA KOLANOWSKA and IRENA CHMIELEWSKA

# STUDIES ON THE INITIATION OF PROTEIN SYNTHESIS IN THE COURSE OF GERMINATION OF PEA SEEDS

## QUANTITATIVE CHANGES OF RNA FRACTIONS FROM EMBRYO AXES DURING INITIATION OF GERMINATION*

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RNA isolated from embryo axes of dry pea seeds and seeds germinated for up to 72 h, gave on polyacrylamide-gel electrophoresis the same bands, showing only small quantitative differences. The heterogeneous band of molecular weight $4 - 5.2 \times 10^5$, present mainly in the microsomal fraction, decreased during the first 24 h of germination, i.e. at the time of initiation of protein synthesis.

The results of our previous work (Sieliwanowicz & Chmielewska, 1973) indicated that initiation of RNA synthesis in embryo axis begins about 24 h after the initiation of protein biosynthesis. In the present work the RNA fractions of pea seeds were examined by polyacrylamide-gel electrophoresis. This technique has been successfully applied by Peacock & Dingman (1967, 1968a,b) for fractionation of RNA from various organisms, and by Loening (1967) for RNA of pea seedling root tips. These authors have demonstrated that, in addition to the RNA fractions of 28S, 18S and 4S, distinct bands of intermediate molecular weight could be obtained. Similar results were reported by Bofosoneanu & Vasu (1969) who separated by electrophoresis the nucleic acids from cotyledons and embryo axes of pea seeds.

## MATERIALS AND METHODS

For the experiments, pea (*Pisum sativum*) variety "Wczesna Kujawska" was used. Germination, labelling of RNA with [$^3$H]uridine and preparation of subcellular fractions were carried out as in the previous work (Sieliwanowicz & Chmielewska, 1973).

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

*Preparation of RNA.* RNA preparations were obtained from homogenates of embryo axes of pea seeds after different periods of germination, and from subcellular fractions of embryo axes of dry pea seeds. The extraction of RNA was performed at 0-5°C. To the material, 3 vol. of phenol-cresol mixture (10:1, v/v) saturated with water and cooled to 5°C was added and shaken vigorously to obtain a homogeneous emulsion. The phases were separated at 20 000 r.p.m. (5 min) and the water phase was treated again with phenol - cresol. The nucleic acids were precipitated with 2.5 vol. of anhydrous ethanol cooled to -20°C, collected by centrifugation at 3000 r.p.m. for 10 min and dissolved in 1 - 2 ml of 0.01 M-Tris-HCl buffer, pH 7.8, at 5°C. To this solution, deoxyribonuclease (100 μg/ml) was added and incubated for 1 h at 5°C. The reaction mixture was then deproteinized with phenol - cresol and from the supernatant RNA was precipitated with ethanol, washed twice with cold ethanol and dried over KOH under reduced pressure.

*Polyacrylamide-gel electrophoresis.* Polyacrylamide gel, 2.2%, containing 0.6% agarose was prepared according to Richards *et al.* (1965) using the buffer applied by Loening (1967) diluted in the ratio 1:25. To the gels a current of 1.4 mA/tube was applied for 1 h, then the RNA sample (80 μg) was layered over the gel. The electrophoresis was run at 4 - 6°C and terminated when the bromophenol blue marker was 1 - 2 mm from the end of the gel. The gels, after being fixed in 10% acetic acid, were stained for 16 - 20 h in 0.1% methylene blue in 0.4 M-acetate buffer, pH 4.7, and destained by repeated changes of the buffer. For scanning, Zeiss ERJ-65 densitometer (red filter) adapted for gels was used.

*Determination of radioactivity.* The radioactivity in polyacrylamide gels was determined according to Tishler & Epstein (1968) with dioxane scintillator containing in 1000 ml: 5 g PPO, 100 mg POPOP, 80 g naphtalene, 400 ml 1,4 dioxane, 400 ml xylene and 200 ml of anhydrous ethanol.

*Molecular weight of RNA fractions.* The approximate molecular weights of particular RNA fractions were calculated according to Peacock & Dingman (1968b).

*Reagents.* The reagents used were from the following sources: Bovine pancreas deoxyribonuclease (Schuchardt, Munchen, G.F.R.); reagents for polyacrylamide-gel electrophoresis (Serva, Heidelberg, G.F.R.); agarose, PPO and POPOP (Koch-Light Lab., Colnbrook, Bucks., England), bromophenol blue and methylene blue (B.D.H., Poole, Dorset, England); [$^3$H]uridine, spec. act. 4.6 Ci/nmol (The Radiochemical Centre, Amersham, England).

## RESULTS AND DISCUSSION

The electrophoretic patterns of all RNA preparations obtained from embryo axes of pea seeds (Fig. 1) showed the same RNA fractions irrespective of the period of germination; however, some quantitative differences could be observed. Approximate measurements of areas under the respective parts of densitometer tracings showed that during the first 48 h of germination the content of fraction *III* decreased from about 12% of the total amount of RNA in the gel, to 6% and after 72 h of

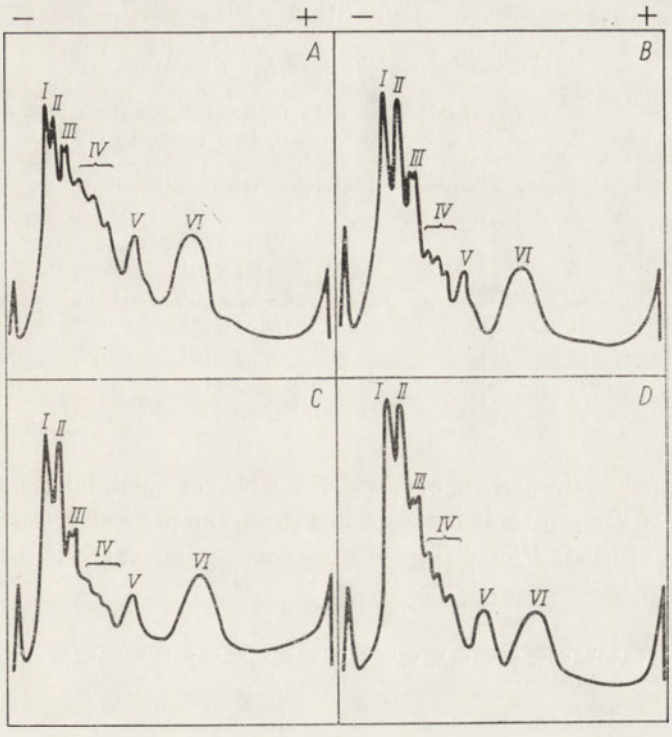


Fig. 1. Densitometric scan of electrophoretic patterns of RNA preparations from embryo axes of: *A*, dry pea seeds; *B*, seeds after 24 h of germination; *C*, seeds after 48 h of germination; *D*, seeds after 72 h of germination.

germination, somewhat increased. Fraction *IV* during the first 48 h decreased from 25% to 12%, and after 72 h, increased to 19%. The relative content of RNA in the remaining fractions was practically unchanged during the first 72 h of germination.

The average mobility of the individual bands did not change over the 72-h period of germination, therefore the molecular weight of the individual RNA fractions was calculated from their average electrophoretic mobilities (Table 1). For fractions *I* and *II* it corresponded to the value reported by Spirin & Gavrilova (1971) for rRNA subunits of higher plants' ribosomes, and for fraction *V*, to the low-molecular-weight RNA of larger ribosomal subunit. The molecular weight of fraction *VI* corresponded to that of tRNA (Wenkstern, 1970). It is more difficult to identify the ribonucleic acids of heterogeneous fractions *III* and *IV*. Loening & Ingle (1967) have demonstrated that RNA with the molecular weight of 400 000 daltons occurs in proplastids, and suggested that this fraction represents the proplastidic rRNA. On the other hand, Schultz *et al.* (1972) have observed on electrophoresis fractions of similar molecular weight isolated from ribosomes of dry wheat seed embryos. These authors have also shown that RNA with molecular weight of 150 000 daltons stimulated biosynthesis of protein in the *in vitro* system.

Table 1

*Molecular weight of RNA fractions*

The molecular weight of individual RNA fractions were calculated from their average electrophoretic mobilities according to Peacock & Dingman (1968b).

| Fraction no. | Molecular weight (daltons) |
|---|---|
| *I* | $1.16 \times 10^6$ |
| *II* | $8.1 \times 10^5$ |
| *III* | $4 - 5.2 \times 10^5$ |
| *IV* | $1.4 - 3 \times 10^5$ |
| *V* | $5.7 \times 10^4$ |
| *VI* | $2 \times 10^4$ |

The electrophoretic pattern obtained for RNA of subcellular fractions from embryo axes of dry pea seeds is shown in Fig. 2. The particular fractions differed in the content of bands *III* and *IV*, which were most pronounced in the microsomal and mitochondrial fractions.

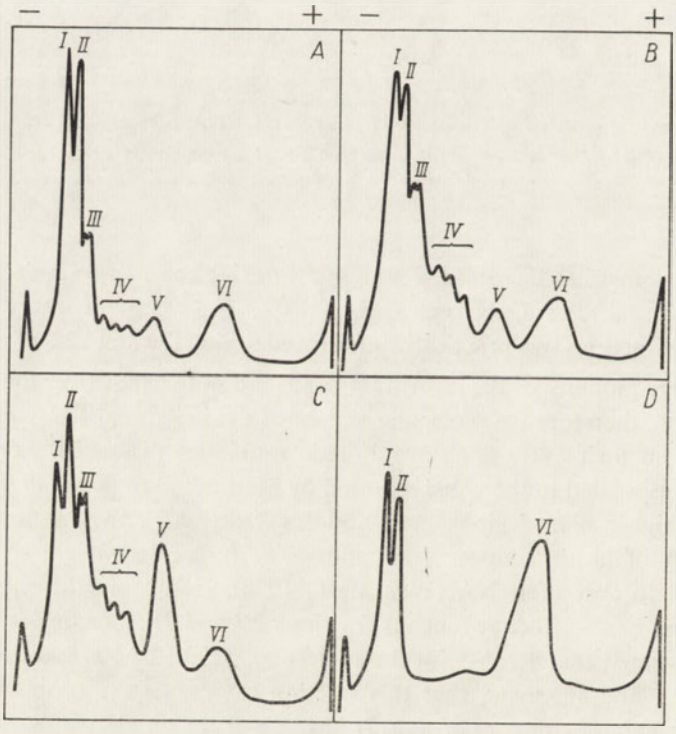


Fig. 2. Densitometric scan of electrophoretic patterns of RNA preparations from subcellular fractions of embryo axes of dry pea seeds. *A*, Crude nuclear fraction; *B*, mitochondrial fraction; *C*, microsomes; *D*, postmicrosomal supernatant.

The RNA synthesis in embryo axes of pea seeds was found to start only after 24 hours of germination, and the incorporation of [$^3$H]uridine increased in a continuous manner from the 24th to the 72nd hour of germination (Sieliwanowicz & Chmielewska, 1973).

The results of fractionation of the RNA preparations from embryo axes after 48 and 72 h of germination and 2 h of labelling with [$^3$H]uridine, are shown in Fig. 3. After 48 h of germination the radioactivity of RNA was found mainly in fractions *III* and *VI* (Fig. 3A). A comparison of the radioactivity incorporation and the amount of RNA indicated that fraction *III* was labelled more effectively. After 72 h of germination the labelling of fraction *III* was even more pronounced; moreover, labelling was observed also in fraction *V* (Fig. 3B). It should be noted that Weeks & Marcus (1971) isolated from dry wheat embryos a "messenger" fraction which stimulated amino acid incorporation *in vitro*, and the content of which decreased on initiation of protein synthesis.

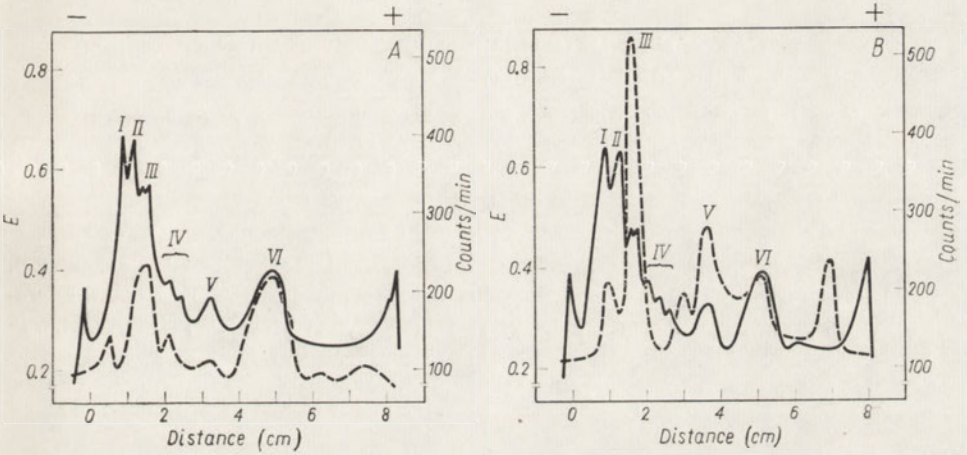


Fig. 3. Densitometric scan and radioactivity measurements of electrophoretic patterns of RNA preparations from pea seed embryo axes labelled for 2 h with [$^3$H]uridine. *A*, seeds after 48 h of germination; *B*, seeds after 72 h of germination. ——, E; - - -, radioactivity.

As it has been shown earlier (Sieliwanowicz & Chmielewska, 1973), the activity of protein synthesis in pea seed embryo axes increases gradually up to the 24th hour of germination, decreases between the 24th and 48th hour, then remains unchanged and, begining with the 66th hour, increases again. As the synthesis of RNA begins only after the 24th hour, the synthesis of protein, at least up to the 24th hour, is dependent on the preformed mRNA in resting seeds. The gradual decrease in the RNA content in fraction *III* during the first 48 hours of germination, and the intensive labelling of this fraction between the 48th and 72nd hour of germination may suggest that in embryo axes of both dry and germinating pea seeds, mRNA occurs in fraction *III*.

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## BADANIA NAD URUCHOMIENIEM SYNTEZY BIAŁKA PODCZAS KIEŁKOWANIA NASION GROCHU

### ZMIANY ILOŚCIOWE ZACHODZĄCE WE FRAKCJACH RNA Z OSI ZARODKOWYCH W POCZĄTKOWYCH FAZACH KIEŁKOWANIA

Streszczenie

Preparaty RNA izolowane z osi zarodkowych nasion spoczynkowych i nasion kiełkujących w czasie do 72 godzin, dzielą się elektroforetycznie na tę samą ilość pasm. Ilość RNA w heterogennym paśmie występującym głównie w mikrosomach o m.cz. 4 - $5.2 \times 10^5$ maleje w czasie pierwszych 24 godzin kiełkowania, to jest w czasie kiedy uruchamiana jest synteza białka.

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# RECENZJE KSIĄŻEK

B. T. Donovan, NEUROENDOKRINOLOGIE DER SÄUGETIERE. Eine Einführung von Bernhard Donovan. Übersetzt von G. E. K. Novotny. G. Thieme Verlag, Stuttgart; str. 255, cena 13.80 DM (Flexibles Taschenbuch).

Książka jest tłumaczeniem na język niemiecki angielskiego oryginału, który ukazał się w r. 1970. Tym niemniej książka nie straciła na aktualności i jest nadzwyczaj pożyteczna dla szerokiego kręgu badaczy zajmujących się neurobiologią w szerokim pojęciu tego słowa, tj. zarówno dla endokrynologów, neurofizjologów, neurochemików, jak i dla neuroanatomów oraz neuropatologów. Niektóre poruszane zagadnienia, tyczące się wpływu czynników zewnętrznych na zjawiska rozmnażania, mogą zainteresować socjologów ze względu na powiązania z regulacją urodzeń.

Monografia opracowana jest źródłowo na podstawie piśmiennictwa obejmującego około 400 pozycji, głównie z lat sześćdziesiątych. Zasadniczym celem, który sobie autor postawił, było przedstawienie wzajemnych zależności pomiędzy czynnościami układu nerwowego i układu hormonalnego u zwierząt ssących. Książka obejmuje więc zarówno zagadnienia tyczące się regulacji nerwowej układów hormonalnych, jak i badania nad wpływem hormonów na czynności nerwowe *in vivo* lub *in vitro*.

Już na samym początku książki autor podkreśla, że pomimo bardzo licznych danych wykazujących wpływ hormonów na czynności mózgu — mechanizm tego zjawiska pozostaje wciąż jeszcze niejasny. Myśl ta przewija się przez całą książkę. Jeden z pierwszych rozdziałów autor poświęca na omówienie morfologii mózgu szczura. Kilka schematów, w których przedstawiono główne drogi nerwowe pomiędzy podwzgórzem, strukturami układu limbicznego, jak i innymi częściami mózgu, ułatwiają czytelnikom mniej obeznanym z neuroanatomią zrozumienie dalszych rozdziałów.

Metodykę badań endokrynologicznych przedstawiono w sposób bardzo ciekawy i przydatny dla szerokiego kręgu eksperymentatorów. W dziewięciu rozdziałach autor omawia poszczególne układy hormonalne i ich współzależność z czynnością niektórych struktur mózgu. Hormony płciowe, które są przedmiotem własnych badań autora, zostały omówione bardziej szczegółowo od innych. Dla wskazania, jaki jest jeden z nowoczesnych sposobów podejścia do badań mechanizmów regulacji wydzielania hormonów, autor przytoczył model cybernetyczny wg Szwartz (1968, 1969). W modelu tym uwzględniono wzajemne zależności między działaniem poszczególnych hormonów żeńskich szczura a funkcją przysadki mózgowej. Model ten, uwidaczniający działanie mechanizmu sprzężenia zwrotnego, zmusza badacza do krytycznej analizy otrzymanych wyników i jasnego przedstawienia hipotez i poglądów, nie zaś jedynie do intuicyjnego ich przyjmowania. W książce poruszone jest także bardzo ważne zagadnienie rytmów biologicznych w odniesieniu do neuroendokrynologii, jak i problem nerwowo-humoralnej regulacji pobierania pokarmu i wody.

Książka zakończona jest omówieniem obecnych i przyszłościowych kierunków badawczych w endokrynologii. Jedynie multidyscyplinarne badania przy użyciu nowoczesnych metod oczyszczania i ilościowego oznaczania hormonów pozwolą w przyszłości wyjaśnić mechanizm działania szeregu hormonów. Zastosowanie hormonów znakowanych izotopami promieniotwórczymi w połączeniu z autoradiografią umożliwi lokalizację docelową działania hormonów.

Na podkreślenie zasługuje słuszna uwaga autora, że cała nasza wiedza neuroendokrynologiczna tycząca się ssaków opiera się na wynikach uzyskanych bądź na kilku gatunkach zwierząt laborato-

ryjnych, bądź też na danych klinicznych u człowieka. Już z obecnych osiągnięć wiadomo, że uogólnienie otrzymanych wyników jest niesłuszne. Nieodzowne jest więc poszerzenie badań nad innymi ssakami.

W przedmowie autor podkreślił, że książka jego obejmuje tylko ograniczony zakres tematyczny i że głównymi jej celami jest wypełnić lukę pomiędzy podręcznikami a czasopismami specjalistycznymi i zachęcić czytelnika do dalszych studiów. Wydaje się, że cel ten został osiągnięty.

*Stella Niemierko*

János Ladik, QUANTENBIOCHEMIE FÜR CHEMIKER UND BIOLOGEN. Akadémiai Kiadó, Budapest 1972; str. 252.

Omawiana książka stanowi jeszcze jeden przykład dążności współczesnych nauk biologicznych do opierania się na badawczych metodach rozwijanych przez współczesną fizykę. Z książki widać jednak jasno, że o ile metodami mechaniki kwantowej zdołano opisać nader ściśle atomy niektórych pierwiastków oraz wiele prostych związków chemicznych, to dla opisania tymi samymi metodami złożonych wielkich cząsteczek białek i kwasów nukleinowych aparat matematyczny, numeryczny i doświadczalny, jakim dysponuje współczesna nauka, jest jeszcze zbyt mało doskonały.

W pierwszym rozdziale autor przedstawia podstawy chemii kwantowej, wprowadzając czytelnika w terminologię i metody opisywania elektronowej budowy atomów przy pomocy mechaniki falowej. Dalsze rozdziały są poświęcone budowie elektronowej kwasów nukleinowych, białek, własnościom tych związków jako półprzewodników, oddziaływaniu DNA na promieniowanie oraz powstawaniu mutacji. Osobny rozdział poświęcono budowie elektronowej węglowodorów rakotwórczych i możliwościom ich oddziaływania ze składnikami komórki. W tym rozdziale przedstawiono teoretyczny schemat Pitota i Heidelbergera możliwości zaburzenia regulacji biosyntezy białka i DNA przy zadziałaniu czynników rakotwórczych. Ten podrozdział nazwano "naszkicowaniem jednolitej teorii powstawania nowotworów" i chociaż jest to dość daleko idąca symplifikacja nawet w stosunku do dziś już znanych faktów, to jednak jest ujęciem ciekawym i może stanowić stymulację do dalszych dociekań i uogólnień w tej dziedzinie. W przedostatnim rozdziale autor omawia elektronową strukturę porfiryn, ostatni zaś rozdział stanowi zbiór rozważań nad perspektywami penetracji metod mechaniki falowej do zagadnień biochemicznych i medycznych. Autor wprowadza szereg — ryzykownych zdaniem oceniającego — nowych terminów, np. "genetyka kwantowa", "gerontologia kwantowa", które w tej chwili nie mają sprecyzowanego znaczenia, a które w przyszłości mogą się okazać nie tylko zbędne, ale także i mylące. Bowiem operowanie elektronową strukturą związków wielkocząsteczkowych budujących żywą materię będzie zapewne w przyszłości niezbędne w każdej dziedzinie biologii i medycyny, podobnie jak obecnie operowanie pojęciem cząsteczki i chemicznej grupy funkcyjnej. Jest to jednak dość daleka przyszłość, gdyż — na co autor zwraca uwagę — poznanie elektronowej struktury rzeczywistych kwasów nukleinowych i białek wymaga znacznego udoskonalenia istniejących metod doświadczalnych, stosowanych obecnie metod matematycznych i metod numerycznych. Ciekawe są również — chociaż potraktowane bardzo ogólnikowo — uwagi ostatniego rozdziału dotyczące korelacji pomiędzy strukturą elektronową nowych syntetycznych leków a ich farmakologicznym działaniem. Chyba słuszny jest wniosek autora, że mechanika kwantowa pomoże w przyszłości w skutecznym poszukiwaniu nowych leków.

W sumie książka Jánosa Ladika jest ciekawa i stanowi pożyteczne i przystępne wprowadzenie do mechaniki kwantowej dla biochemików i biologów innych specjalności.

*Mariusz Żydowo*

M. Rocha e Silva, and J. Garcia Leme with the collaboration of Hanna A. Rothschild, CHEMICAL MEDIATORS OF THE ACUTE INFLAMMATORY REACTION. International Series of Monographs in Pure and Applied Biology, Modern Trends in Physiological Sciences (P. Alexander and Z. M. Bacq, eds.) Vol. 37. Pergamon Press, Oxford, New York, Toronto, Sydney, Braunschweig 1972; str. 265, cena Ł 6.00.

Profesor M. Rocha e Silva, kierownik Katedry Farmakologii Uniwersytetu w Sao Paulo, oddał wraz ze swoimi współpracownikami do rąk czytelnika cenną monografię o mediatorach ostrych reakcji zapalnych. Książka wyróżnia się oryginalnością ujęcia tematu i głębokim zaangażowaniem autorów w pracach badawczych tak złożonego zjawiska, jakim jest obrona organizmu przed agresorem, wyrażająca się u ssaków reakcją zapalną. Ostre reakcje zapalne stanowią przedmiot badań anatomów, biochemików, fizjologów, immunologów, patologów, farmakologów i klinicystów. Zdaniem autorów daje się odczuć pilna potrzeba integracji wyników badań prowadzonych przez różnych specjalistów, tak aby można było stworzyć hipotezę roboczą wyjaśniającą złożone mechanizmy reakcji zapalnych i pozwalającą na ukierunkowanie i zaprogramowanie dalszych badań tak *in vivo*, jak i *in vitro*. Już w pierwszych teoriach Virchowa i Cohnheima zapalenie traktowano jako reakcję stereotypową, która nie zależy od natury czynnika szkodliwego lecz od własności gatunkowych i indywidualnych osobnika. Narodziła się idea, że reakcja zapalna o charakterze stereotypowym zachodzi za pośrednictwem endogennych, aktywnie mobilizowanych czynników — którymi są chemiczne mediatory zapalenia. Dzisiaj hipotetyczny udział mediatorów w procesach zapalnych stał się wielostronnie i mocno ugruntowanym faktem. Autorzy przedstawiają szereg bezpośrednich dowodów na centralny udział w ostrej reakcji zapalnej układu kininotwórczego. Kininy z główną ich przedstawicielką — bradykininą odkrytą w 1949 r. przez autora książki prof. Rocha e Silva powstają, wg pierwotnej definicji, jako hormony tkankowe pod wpływem trypsyny i jadów węży z pseudoglobulinowej frakcji białek surowicy. Obecnie są już przedmiotem badań na poziomie molekularnym z użyciem czystych białek o naturze kininogenów, preenzymów i aktywnych enzymów oraz ich swoistych inhibitorów, stosowanych często jako leki przeciwzapalne. Na plan pierwszy w mechanizmie regulacji ostrych reakcji zapalnych wysuwa się czynnik Hageman (XII) i jego aktywacja, która zapoczątkowuje proces krzepnięcia krwi, fibrynolizy i generacji kinin. Na zasadzie sprzężenia zwrotnego produkty trawienia plazminą aktywnego czynnika XIIa skierowują łańcuch reakcji proteolitycznych w stronę układu kininotwórczego i zarazem wyłączają proces krzepnięcia. Szeroko w monografii dyskutowana jest również rola histaminy i innych drobnocząsteczkowych mediatorów w anafilaksji i ostrych zapaleniach alergicznych. Wnikliwie przedstawiono również problem skojarzonego działania mediatorów i licznych czynników modyfikujących ich działanie. W końcowym rozdziale książki znajdzie czytelnik wiele interesujących danych o lekach przeciwzapalnych, ich znaczeniu w badaniach podstawowych i w terapii. Autorzy omawiają wpływ tych związków na biogenezę i zwalnianie mediatorów, szczególnie histaminy, oraz biochemiczne aspekty ich działania przeciwzapalnego.

Bogatą treść książki autorzy zawarli w czterech rozdziałach: I. Naturalna historia rozwoju badań ostrych reakcji zapalnych. II. Metodyka pomiarów ostrych reakcji zapalnych. III. Mediatory reakcji zapalnych. IV. Przeciwzapalne działanie leków. Każdy rozdział kończy się starannie zebranym piśmiennictwem z podaniem tytułów cytowanych prac doświadczalnych, sympozjów, konferencji i prac przeglądowych. Cytowane są prace zarówno o znaczeniu historycznym, z drugiej połowy wieku XIX, jak i najnowsze, często kontrowersyjne, ale zawsze o poważnym znaczeniu naukowym. Pomimo bardzo starannej selekcji, bibliografia zajmuje około 50 stron.

Autorzy osiągnęli zamierzony cel, gdyż stworzyli dzieło, które zamyka dotychczasowy okres i otwiera nowe perspektywy badań mechanizmów ostrych reakcji zapalnych na różnych szczeblach rozwoju filogenetycznego.

*Wanda Mejbaum-Katzenellenbogen*

Cena zł 25–,

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