KOMITET BIOCHEMICZNY I BIOFIZYCZNY
POLSKIEJ AKADEMII NAUK

COMMITTEE OF BIOCHEMISTRY AND BIOPHYSICS
POLISH ACADEMY OF SCIENCES

ACTA BIOCHIMICA POLONICA

QUARTERLY

Vol. 19
No. 3

WARSZAWA 1972
PAŃSTWOWE WYDAWNICTWO NAUKOWE

ADDRESS

ul. Banacha 1, Warszawa 22
(Zakład Biochemii A.M.)
Poland

Państwowe Wydawnictwo Naukowe — Warszawa, Miodowa 10

Nakład 1759+131 egz. Ark. wyd. 10,0 ark. druk. 7,625+0,125 kredy
Papier druk. sat. kl. III, 80 g. 70×10
Oddano do składania 4.V.72 r. Podpisano do druku 30.IX.72 r.
Druk ukończono w październiku 1972
Zam. 422 A-103 Cena zł 25.—

ACTA BIOCHIMICA POLONICA
Vol. 19 1972 No. 3

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CHANGES IN NET HYDRATION OF DNA DURING THERMAL HELIX-COIL TRANSITION

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1. The non-rotational hydration of calf thymus DNA molecules was calculated from measurements of longitudinal T_1 and transversal T_2 magnetic relaxation times. The relaxation times were measured by the spin-echo technique at resonance frequency of 14 MHz. 2. At temperatures increasing in the range of 20 - 100°C, dehydration of native DNA molecules was demonstrated. At a temperature close to the T_m value, measured spectrophotometrically at 260 nm, the DNA molecule became practically completely dehydrated. The degree of thermal dehydration of DNA was a function of DNA concentration. The observed phenomenon gives some experimental support to the view that water dipoles bound to the DNA molecule participate in stabilization of the secondary structure of DNA.

A number of experimental data suggest that factors attacking the structure of molecular hydration lead to a weakening of the DNA stability. Destabilization of the DNA structure occurs in non-aqueous solution (Herskovits, Singer & Geiduschek, 1961; Herskovits, 1963), and in aqueous solutions in the presence of large amounts of electrolytes (Emanuel, 1960) or high concentration of bases, nucleosides or nucleotides (Ts'o, Helmkamp & Sander, 1962). All these and other compounds influence hydration of DNA molecules because of the arrangement of their own hydration layers.

It is well known that acid titration influences the thermal helix-coil transition of DNA (Cox & Peacocke, 1958; Peacocke & Preston, 1959). Lewin & Munroe (1965) observed a strong fall of the melting point, T_m, during alkali and acid denaturation of DNA without any change in the hyperchromicity. In some regions of pH, the extinction did not change, whereas the T_m value was very sensitive to changes of pH.

According to the hypothesis of Lewin (1967), all these phenomena can be explained by the assumption that forces stabilizing the secondary structure of DNA are influenced by water bridges in hydration shells. The water bridges may be disrupted just before or during DNA denaturation.

Direct experimental evidence for the influence of water dipoles on the structural stability of DNA molecules could be obtained by demonstration of the DNA dehydration just before or during helix-coil transition. Such evidence for the relationship between the degree of net hydration and stability of the helical state of DNA macromolecules has been presented by Lubas & Wilczok (1970). The hydration of DNA in solutions of various ionic strength was calculated from relaxation time values measured by the spin-echo technique.

In the present work, changes in the degree of hydration during thermal denaturation of DNA, measured by the spin-echo technique, were observed also for solutions of various DNA concentrations.

The method for determining the DNA hydration from measurements of relaxation times by means of the spin-echo technique has been described in detail in a previous paper (Lubas & Wilczok, 1966). The longitudinal and transversal magnetic relaxation times, T_1 and T_2, of an aqueous DNA solution were found to be lower than those for the solvent (Lubas & Wilczok, 1966). This decrease, which may be related to the amount of water bound to the DNA molecules and to the exchange of individual water dipoles between the free and bound water, made possible the determination of the hydration of DNA. The dependence of relaxation times T_1 and T_2 on temperature indicated that DNA underwent a phase transition similar to that observed spectrophotometrically at 260 nm (Lubas, Wilczok & Daszkiewicz, 1967; Lubas & Wilczok, 1970). Large differences were found to exist between T_1 and T_2 values for the native or denatured state of DNA. This phenomenon may be also directly related to changes in non-rotational hydration of DNA, thus making it possible to calculate the DNA hydration during the thermal helix-coil transition.

MATERIALS AND METHODS

DNA preparations. Calf thymus DNA isolated by the detergent method of Kay, Simmons & Dounce (1952) was dissolved in 0.15 M-NaCl. Three preparations of DNA were made, and designated as A, B and C. The buoyant density of DNA determined by ultracentrifugation in CsCl was 1.699 g/cm^3. This value corresponds to the guanine and cytosine content of 46 mol%. The molecular weight was calculated using the formula of Doty, McGill & Rice (1958). The intrinsic viscosity of the DNA solutions was determined using the viscometer described by Eigner (1960). The melting temperature, T_m, hyperchromicity, ΔT, and heterogeneity represented by the value of 2σ, were calculated from the extinction-temperature profiles determined at 260 nm. The characteristics of the DNA preparations used are given in Table 1. The absence of paramagnetic ions in the DNA solutions was checked spectrophotometrically.

Measurements of relaxation times. The longitudinal spin-lattice relaxation time, T_1, and transversal spin-spin relaxation time, T_2, were measured at four DNA concentrations in 0.15 M-NaCl (Table 1). The concentration was calculated from

the extinction at 260 nm, assuming that 18 extinction units correspond to 1 mg of DNA.

Table 1

Characteristic data of the DNA preparations

Preparation	Melting temperature T_m (°C)	Hyperchromicity (%)	Heterogeneity 2σ (°C)	Intrinsic viscosity (dl/g)	$10^{-6}\times$ ×mol. wt.	DNA concentration for T_1 and T_2 measurements (mg/cm³)
A	89.0	38	8,0	75.0	7.70	1.08 2.17
B	89.0	38	8.0	57.5	6.00	2.90
C	89.5	38	8.5	87.0	8.66	5.38

Measurements of T_1 and T_2 were carried out as described by Lubas & Wilczok (1966) and Lubas *et al.* (1967) with the spin-echo apparatus in a magnetic field of 3288.1 Oe, corresponding to a resonance frequency of 14.0 MHz.

Denaturation of DNA obtained by increasing the temperature in the head of the spin-echo apparatus was found to be much slower than the denaturation in a water bath. At every fixed temperature, both T_1 and T_2 were measured. The denaturation was completed within 6 - 9 hours, and cooling of the DNA samples lasted 2 - 4 hours, with interruptions for measurements of the relaxation times. The characteristic dependence of the extincion at 260 nm on temperature, was used as a criterion for completeness of DNA denaturation.

RESULTS AND DISCUSSION

No differences in denaturation patterns were found between completely denatured DNA and that denatured directly in the head of the spin-echo apparatus (Lubas, 1969). Therefore the measurements of T_1 and T_2 values when the sample is cooled down can be regarded as characteristic for a denatured DNA sample.

The results of measurements of T_1 and T_2 values for DNA concentration of 2.90 mg/cm³ have been published previously (Lubas *et al.*, 1967). The results for DNA concentrations of 1.08, 2.17 and 5.38 mg/cm³ in 0.15 M-NaCl, plotted against increasing and decreasing temperatures, exhibit a similar pattern. In typical curves for DNA concentration of 5.38 mg/cm³ (Fig. 1), three parts may be distinguished: the first one where the increase of T_1 and T_2 values with the temperature is slow, the steeper second part corresponding to a more rapid change of relaxation time, and the third part related to cooled down samples which exhibit at respective temperatures higher values of relaxation times than the heated up ones.

The relaxation times at 20°C for the 0.15 M-NaCl solvent are listed in Table 2. The values of both T_1 and T_2 of the solvent are practically equal to those for distilled water exposed to air (Simpson & Carr, 1958; Meiboom, 1961). The mean values at 20°C are $T_1 = T_2 = 2.7$ seconds.

Table 2

T_1 *and* T_2 *measurements at* 20°C *for* 0.15 M-NaCl *solutions used as solvent*

Solvent for DNA	Sample no.	T_1 (s)	T_2 (s)
A	1	2.73	2.69
	2	2.71	2.70
	3	2.708	2.69
B	1	2.71	2.70
	2	2.70	2.65
C	1	2.72	2.73
	2	2.70	2.67
	3	2.67	2.71

The dependence of T_1 and T_2 for 0.15 M-NaCl solution on temperature is shown in Fig. 2. The relationship between T_1 and T_2, and temperature has in this case an almost linear character, and no differences are observed between the values

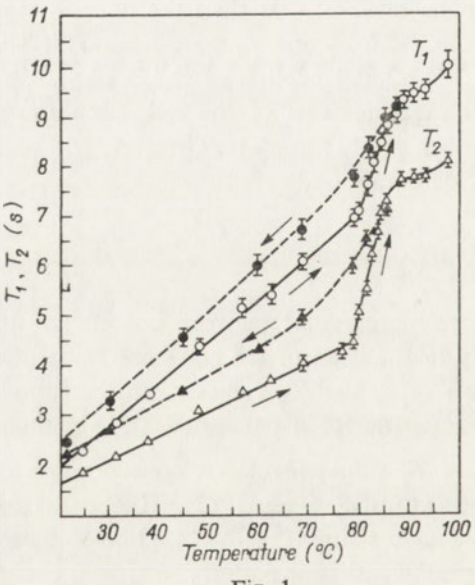

Fig. 1

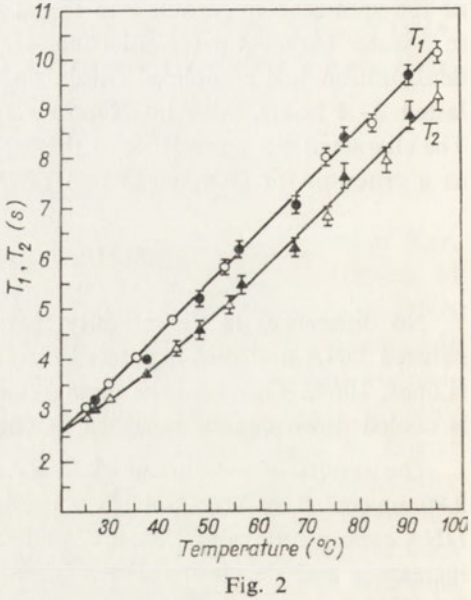

Fig. 2

Fig. 1. Dependence of relaxation times of DNA solution in 0.15 M-NaCl on temperature. DNA concentration 5.38 mg/cm³. Circles, T_1; triangles, T_2; ○, △, at increasing temperature; ●, ▲, at decreasing temperature. Vertical bars represent the S.E.M.

Fig. 2. Dependence of relaxation times of the 0.15 M-NaCl solution on temperature. Symbols as in Fig. 1. Vertical bars represent the S.E.M.

for the heated and cooled samples. The T_1 and T_2 values for the DNA solution (cf. Fig. 1) are smaller than the respective values for the solvent at all measured temperatures.

The dependence of relaxation times of the DNA solution upon the temperature may be interpreted in terms of changes in hydration of DNA macromolecules. This permits to determine the non-rotational hydration, i.e. this part of the total hydration shell within which the water dipoles have no freedom of movement in the high frequency field. The exchange of protons between the free and non-rotationally bound water molecules can influence the relaxation times in a way which may be described according to the theory of Zimmermann & Brittin (1957) by the following equations:

$$\frac{1}{T_1} = \frac{1}{T_{1w}} + \frac{cw}{T_{1n}}; \qquad \frac{1}{T_2} = \frac{1}{T_{2w}} + \frac{cw}{T_{2n}} \tag{1}$$

where T_1 and T_2 represent the measured longitudinal and transversal relaxation times of DNA solution; T_{1w} and T_{2w}, relaxation times of the solvent; T_{1n} and T_{2n}, relaxation times of protons of water molecules non-rotationally bound by DNA; c, DNA concentration; and w, non-rotational hydration of DNA expressed in weight percent.

The equations (1) contain three unknown variables, i.e. T_{1n}, T_{2n} and w. To overcome this difficulty, we can express T_{1n} and T_{2n} by equations used in the theory of Kubo & Tomita (1954) and Solomon (1955). They expressed T_{1n} and T_{2n} as functions of the mean correlation time τ for water molecules bound non-rotationally to DNA, and simultaneously equal to the correlation time of the DNA macromolecule itself.

It should be mentioned that the distribution function is introduced sometimes for a quantitative interpretation of the molecular motional relaxation (Resing, Thomson & Krebs, 1964; Clifford & Sheard, 1966). However, such an approach to the molecular motion is necessary only when all water molecules adsorbed on, and arranged around, the surface of a given solid are taken into consideration, and then the ordered water molecules do not form a typical hydration shell. Resing *et al.* (1964) and Clifford & Sheard (1966) found it necessary to introduce the distribution of correlation times when considering the results obtained for the adsorbed water molecules in terms of the theory of relaxation. They based their interpretation on the difference between dependence of T_1 and T_2 values of water protons on the temperature in the low temperature range and on differences between T_1 and T_2 at room temperature.

We have not found any anomalous effect in T_1 and T_2 dependences on the temperature. Both relaxation times for DNA solutions (Lubas *et al.*, 1967) have similar values at room temperature and their behaviour with the increase of temperature was almost identical. If we considered only the non-rotational part of water molecules characterized by relaxation times T_{1n} and T_{2n}, the molecular movement of this part of water could be described by only one, mean correlation time, τ, according to Solomon's (1955) equations.

On the other hand, our treatment does not exclude the possibility that all other water layers may be arranged around the DNA molecule in such a way that the continuous energy binding spectrum and a distribution of correlation times have to be used for the description of their movement. However, these water dipoles are bound to DNA only rotationally and are expected to exhibit free movement in a high frequency field, i.e. their correlation times resemble those of bulk, liquid water. One has to take into consideration that some distribution of correlation times of water dipoles bound non-rotationally to DNA molecules may also occur, but it would result mainly from heterogeneity of DNA macromolecules in a given DNA sample and not from the distribution of energy of water binding. Generally, the problem of this kind of distribution determined by heterogeneity of DNA cannot be solved at the present stage of DNA investigation. Evaluation of such distribution can be attempted by studying nuclear relaxation processes in a DNA solution containing very homogeneous fractions of DNA with the use of the nuclear magnetic resonance apparatus operated at various resonance frequencies. A similar problem concerning proteins has been discussed by Blicharska, Florkowski, Hennel, Held & Noack (1970). In our case the freedom of movement and mobility of DNA molecules can be only characterized by the mean correlation time τ of a statistical molecule (similarly as the mean molecular weight is used to characterize a given preparation).

This permits to apply Solomon's equations in their raw form for determination of T_{1n} and T_{2n}. The non-rotational hydration of DNA can thus be described as

$$w_1 = k_1 T_{1n}; \quad w_2 = k_2 T_{2n} \tag{2}$$

where the experimental constants k_1 and k_2 are expressed as

$$k_1 = \frac{1}{c}\left(\frac{1}{T_1} - \frac{1}{T_{1w}}\right); \qquad k_2 = \frac{1}{c}\left(\frac{1}{T_2} - \frac{1}{T_{2w}}\right) \tag{3}$$

respectively. The k_1 and k_2 values were calculated by substituting into equations (3) the appropriate experimental relaxation times of the DNA solution (T_1, T_2) and of 0.15 M-NaCl (T_{1w} and T_{2w}) at a given temperature. The equations (2) may be solved graphically and values of non-rotational hydration, w, and correlation time, τ, can be determined subsequently for each chosen temperature. In the temperature range below 80°C, the w values were determined at intervals of 5°C; at all higher temperatures, at intervals of 2°C.

Typical calculations of the non-rotational hydration of DNA are presented in Fig. 3 as a function of temperature. With the increase in temperature, a strong dehydration of DNA molecules occurred. This dehydration involves apparently two distinct phases: the first phase of slow dehydration within the range of temperature from 20 to 80°C, and the second phase of fast dehydration at temperatures higher than 80°C. The temperature at which the slow phase of dehydration is completed and at which the fast process of dehydration begins, seems to correspond to the temperature at which the beginning of DNA melting is observed by spectrophotometric measurements at 260 nm.

The fast process of dehydration is completed in the range of temperatures corresponding to that of the helix-coil transition. At a temperature equal to the T_m (measured spectrophotometrically), molecules of DNA seem to be completely dehydrated, as the non-rotational hydration becomes close to zero.

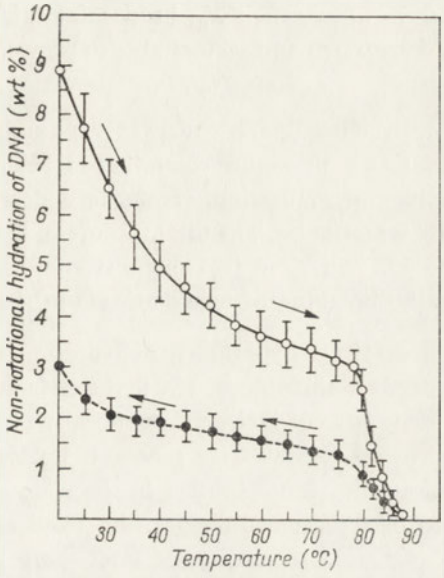

Fig. 3. Dependence of non-rotational hydration of DNA molecules on temperature. DNA concentration 5.38 mg/cm³. ○, Native DNA at increasing temperature; ●, denatured DNA at decreasing temperature. Vertical bars represent the S.D.

When the DNA sample is cooled, rehydration of DNA molecules can be observed (Fig. 3); however, values of non-rotational hydration of denatured DNA obtained in this way are always considerably lower than those determined for native DNA.

It is commonly known that an increase in temperature evokes changes in extinction of the DNA solution, which are usually measured at 260 nm. These changes are observed at temperatures ranging from 70 to 100°C, depending on the ionic strength of the solution and on the GC content of the DNA used. These changes can be attributed to the rupture of hydrogen bonds between bases of the complementary strands and to the transition of the DNA helix into disordered coil. The question how and for what purpose the thermal energy administered to the DNA solution at elevated temperature is utilized before the hydrogen bonds become disrupted, is of great interest. On the basis of our results is seems reasonable to conclude that this energy is used mainly for dehydration of DNA molecules. This supports the hypothesis that water dipoles bound to DNA molecules play an important role in the stabilization of the secondary and tertiary structure of DNA.

As it has been demonstrated by Rownd, Lanyi & Doty (1961), Wilczok & Mendecki (1966) and Wardas (1967), changes in the DNA structure brought about by heating of the DNA solution at temperatures below the helix-coil transition

are almost completely reversible. Also at temperatures close to T_m, changes in the DNA structure are to some extent reversible (Wilczok & Mendecki, 1966; Wardas, 1967). We suppose that when calf thymus DNA is heated from 20 to 80°C in 0.15 M-NaCl solution, the administered energy is mainly used for dehydration of DNA and for rupture of some of the hydrogen bonds. The rupture of all hydrogen bonds may occur at temperatures higher than T_m, because at this temperature the DNA molecule is completely dehydrated and factors able to prevent denaturation of DNA are not operative.

As it has been shown by Wilczok & Mendecki (1966) and Wardas (1967), heating of the DNA solution up to a temperature somewhat higher than T_m, but lower than that necessary for complete denaturation, results in a decrease of the amount of reversibly denatured DNA molecules. The total rupture of hydrogen bonds, resulting in separation of the double helix into two single strands, seems to be much more difficult to repair than the breaking of water bridges in the DNA hydration sites.

According to Lewin's (1967) hypothesis, dehydration of the DNA molecule may be visualized to proceed in three steps. The first step is the labilization of weaker water bridges in the wide groove of DNA to a point at which the value of the total sum of all stabilization forces for the wide groove is lower than zero. The second step involves further rupture of water bridges in the wide groove accompanied by a decrease in the value of repulsive forces arising from the enforced helical deconvolution and from increased separation of inter-chain phosphate groups. The last step is the rupture of the strongest water bridges in the wide groove and water bridges in the narrow groove, resulting in extensive deconvolution and labilization of the helix to a point at which the sum of the stabilization forces for the narrow groove will be lower than zero. Just at the end of this step, rupture of hydrogen bonds takes place, which leads to the helix-coil transition.

Taking into account these considerations and our results (see Fig. 1), it is reasonable to conclude that during the phase of slow dehydration, rupture of water bridges in the wide groove of DNA takes place, and in the phase of fast dehydration, rupture of water bridges in the narrow groove occurs.

Quite close but slightly different values of DNA hydration were observed at the starting temperature 20°C for various DNA concentrations. A valid estimation of the thermal dehydration of DNA measured at different concentrations can be carried out only when relative values of DNA dehydration are compared. These relative values are defined as ratios w_t/w_{20}, where w_t represents the hydration at a temperature t, and w_{20}, hydration at 20°C. The dependence of the relative decrease of hydration on temperature is presented in Fig. 4.

Although the values of non-rotational hydration at 20°C are similar for various DNA concentrations, the character of DNA dehydration at elevated temperatures depends markedly on DNA concentration. In the temperature range of 20 - 80°C an increase in DNA concentration leads to larger dehydration. At the lowest DNA concentration used, 1.08 mg/cm^3, the relative hydration decreases to 73% at 80°C,

whereas at the highest DNA concentration, 5.38 mg/cm³, the hydration decreases to 33% of the initial value.

To interpret this phenomenon, the following hypothesis is proposed. The value of the non-rotational hydration of DNA molecules at room temperature indicates full saturation of the primary sites of DNA hydration, introduced and discussed by Falk, Hartmann & Lord (1963). Therefore, the non-rotational hydration does not depend on the number of water molecules present in the solution and available to the statistical DNA macromolecule. At low DNA concentrations the excess of water molecules in solution can be bound to DNA molecules only rotationally, participating in an exchange of protons between the non-rotationally and rotationally bound water dipoles. An increase in temperature leads to liberation of non-rotationally bound water dipoles. The number of water molecules available to the statistical macromolecule is large when the DNA concentration is low. Therefore the probability of reconstruction of broken water bridges is enhanced relative to that for highly concentrated DNA solution, where the number of free water molecules is smaller. Thus it seems possible to assume that at a given temperature the degree of dehydration will be lower at a lower DNA concentration. At temperatures above 80°C, the hydration of DNA decreases more abruptly in the samples with low concentration, reaching at 89°C values close to zero.

The rehydration of denatured DNA with decreasing temperature is also dependent on DNA concentration. Figure 5 shows w_t/w_{20} ratios as a function of temperature.

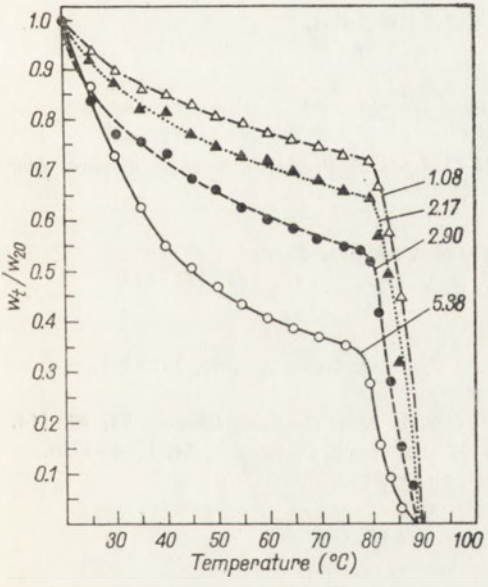

Fig. 4

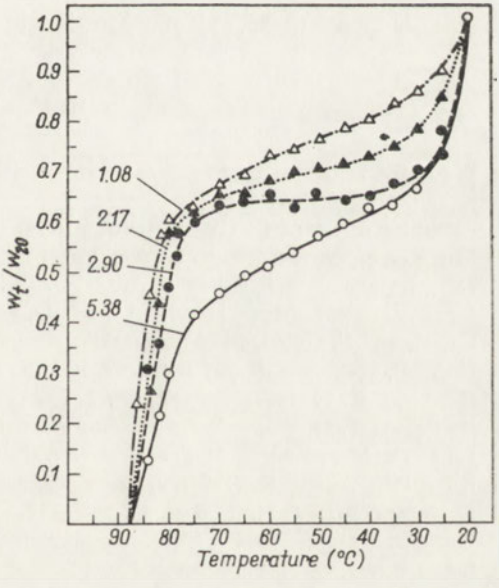

Fig. 5

Fig. 4. Dependence of relative DNA hydration, w_t/w_{20}, on increasing temperature for various DNA concentrations (mg/cm³) indicated in the Figure.

Fig. 5. Dependence of relative DNA hydration on decreasing temperature for various DNA concentrations (mg/cm³) indicated in the Figure.

As it may be seen, water bridges in hydration sites of single strands of denatured DNA are more readily restored at low DNA concentration.

The ratio of non-rotational hydration for denatured DNA, w_d, to the hydration for native DNA, w_n, as a function of temperature is shown in Fig. 6. It may be seen

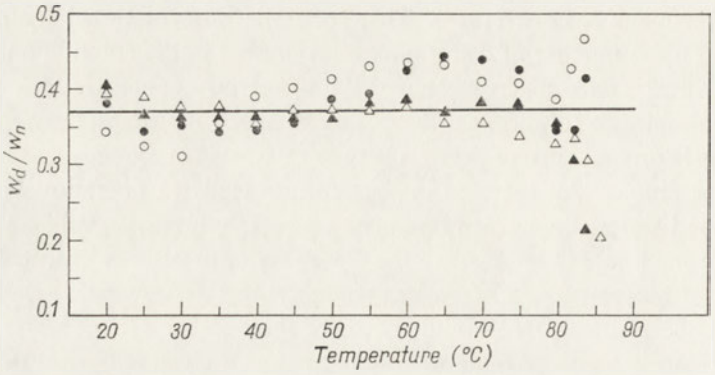

Fig. 6. Dependence of the ratio of hydration for denatured and native state of DNA, w_d/w_n, on temperature for the following DNA concentrations: △, 1.08 mg/cm³; ▲, 2.17 mg/cm³; ●, 2.90 mg/cm³; and ○, 5.38 mg/cm³.

that this ratio is practically independent of the temperature and DNA concentration, and ranges from 0.205 to 0.465. The mean value of DNA hydration in the denatured state is equal to 36.7% of hydration in the native state.

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ZMIANY UWODNIENIA SIECIOWEGO DNA W TRAKCIE TERMICZNEGO PRZEJŚCIA FAZOWEGO SPIRALA-KŁĘBEK

Streszczenie

1. Badano nierotacyjne uwodnienie cząsteczek DNA izolowanego z grasicy cielęcej, wyliczone na podstawie pomiarów magnetycznych czasów relaksacji protonów, podłużnego T_1 i poprzecznego T_2. Czasy relaksacji mierzono techniką echa spinowego przy częstości rezonansowej 14 MHz.

2. Przy wzroście temperatury od 20 do 100°C wykazano odwodnienie cząsteczek natywnego DNA. W temperaturze bliskiej wartości T_m, zmierzonej spektrofotometrycznie przy 260 nm, cząsteczka DNA jest praktycznie zupełnie odwodniona. Stopień termicznego odwodnienia DNA jest funkcją stężenia DNA. Zaobserwowane zjawisko daje pewne eksperymentalne potwierdzenie poglądu, że dipole wody związane z DNA biorą udział w stabilizacji jego drugorzędowej struktury.

Received 25 October, 1971.

ACTA BIOCHIMICA POLONICA
Vol. 19 1972 No. 3

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DETERMINATION OF CITRATE AND CoA BY ATP : CITRATE OXALOACETATE-LYASE

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1. A method for determination of citrate and CoA in mitochondrial preparations by ATP : citrate oxaloacetate-lyase (EC 4.1.3.8) is described. 2. The variation coefficients were 2.5 and 4.0% for citrate and CoA, respectively. The recovery of citrate added to mitochondria was 94 - 97%, and that of CoA 90 - 95%. 3. Intermediates of Krebs cycle, glutamate, aspartate and nucleotides were without effect on citrate determination. 4. In the presence of maleate the content of CoA and formation of citrate from 2-oxoglutarate in rat kidney cortex mitochondria was decreased. No such effect was observed in liver mitochondria.

Several methods have been described for determination of citrate in biological material. Chemical methods are not simple, sensitive and specific enough for the use in biochemical studies (Pucher, Sherman & Wickery, 1936; McArdle, 1955; Krans, 1965). The available specific enzymic assays of citrate (Dagley, 1965) are based on coupled reactions, either catalysed by bacterial citrate lyase (EC 4.1.3.6), malate dehydrogenase (EC 1.1.1.37) and lactate dehydrogenase (EC 1.1.1.27), or (Siebert, 1965) by aconitate hydratase (EC 4.2.1.3) and isocitrate dehydrogenase (EC 1.1.1.41). ATP citrate lyase (ATP : citrate oxaloacetate-lyase, EC 4.1.3.8) has not so far been used for determination of citrate in biological material although it is recommended by Srere (1961) and Michal & Bergmeyer (1965) for determination of CoA.

The present paper describes the method for determination of both citrate and CoA with this enzyme, as applied for measuring citrate and CoA contents in liver and kidney mitochondria and for measuring citrate formation from 2-oxoglutarate in the presence of maleate.

MATERIALS AND METHODS

Reagents. ATP disodium salt, adjusted before use to pH 7.2 with KOH; NADH and malate dehydrogenase were from Boehringer (Mannheim, West Germany),

CoA, I grade, from Sigma Chem. Co. (St. Louis, Mo., U.S.A.), 2-mercaptoethanol and tris from British Drug Houses (Poole, Dorset, England), DL-isocitrate and *cis*-aconitate from Fluka A. G. (Buchs, Switzerland); citric acid, potassium citrate and other reagents were products of P.P.H., Polskie Odczynniki Chemiczne (Gliwice, Poland).

Principle of the method. The oxaloacetate formed in the ATP citrate lyase reaction was measured by coupling with malate dehydrogenase reaction. In this reaction the quantity of citrate or CoA was related to the decrease in extinction at 340 nm as NADH was converted to NAD^+.

Preparation of ATP citrate lyase. The enzyme was obtained from liver of white Wistar rats. The animals before being killed were starved for 48 hours and then refed with white bread for 72 hours. The enzyme was purified according to Srere (1962) except that reextraction of zinc acetate precipitate and the last step with alumina C-γ gel were omitted. Specific activity of the freshly prepared enzyme was 3 to 4 µmol/min/mg of protein. The activity was assayed by the method of Srere (1959) as modified by Szutowicz & Angielski (1970). The preparation was free of NADH oxidase (EC 1.6.99.3), malate dehydrogenase (EC 1.1.1.37), lactate dehydrogenase (EC 1.1.1.27), ATPase (EC 3.6.1.3), L-aspartate - 2-oxoglutarate aminotransferase (EC 2.6.1.1) activity. The enzyme, when kept as a suspension in 0.25 saturated ammonium sulphate with 2 mM-dithiothreitol at 0 - 4°C (about 20 mg of protein/ml), lost about 20 - 25% of its activity per week, and was used within two weeks. Before use the enzyme preparation was diluted with 10 mM--2-mercaptoethanol to contain 0.3 - 0.4 unit per 0.01 ml.

Citrate determination. The assay mixture contained in the final volume of 1.6 ml: 62 mM-tris-HCl buffer, pH 7.6, 3 mM-ATP, 3 mM-$MgCl_2$, 9 mM-2-mercaptoethanol, 0.06 mM-CoA, 0.06 mM-NADH, 10 i.u. of malate dehydrogenase, and the sample tested. The reaction was started by the addition of 0.3 to 0.4 unit of ATP citrate lyase. The rate of NADH oxidation was followed for about 20 min. As a control, the rate of NADH disappearance in the absence of citrate was measured. The amount of NADH oxidized was calculated by extrapolation.

CoA determination. This was carried out by a modification of the method of Michal & Bergmeyer (1965). The assay mixture contained in the final volume of 1.6ml: 62 mM-tris-HCl buffer, pH 7.6, 3 mM-ATP, 19 mM-potassium citrate, 9 mM-$MgCl_2$, 9 mM-2-mercaptoethanol, 0.06 mM-NADH, 10 i.u. of malate dehydrogenase, and the sample tested. The reaction was started by the addition of 0.2 unit of ATP citrate lyase and the reaction was followed for at least 10 min. When mitochondrial CoA was determined, the volume of the mixture was reduced from 1.6 ml to 1.0 ml.

Mitochondrial experiments. Rat liver and kidney cortex mitochondria were prepared according to Hogeboom (1955). The animals before being killed were starved for 24 h, except in the experiment presented in Fig. 4.

The incubation medium in the experiments on the effect of maleate contained in a final volume of 2.0 ml: 125 mM-sucrose, 25 mM-tris-HCl buffer, pH 7.4, 10 mM--KCl, 5 mM-$MgCl_2$, 5 mM-2-oxoglutarate, 1 or 5 mM-maleate where indicated; and kidney cortex or liver mitochondrial protein corresponding to 15 - 20 mg and

30 - 35 mg, respectively. The reaction mixture was preincubated in 25 ml plastic centrifugal tube for 5 min at 23°C. The reaction was started by adding the mitochondrial suspension and the incubation was carried out at the same temperature with continuous agitation at 80 rev./min for 10 min. The reaction was stopped by adding 0.15 ml of ice-cold 60% perchloric acid. The sample was put to ice immediately, and centrifuged at 0 - 2°C. The supernatant was neutralized to pH 6.0 - 6.5 with 2 N-KOH containing 0.02 M-2-mercaptoethanol, centrifuged, and the supernatant taken for determinations of citrate and CoA.

To check the recovery of citrate in the incubation mixture containing 2-oxoglutarate, after incubation a known amount of citric acid was added to the mixture just prior to addition of perchloric acid.

To study the determination and recovery of CoA, freshly prepared liver mitochondria were used. The suspension containing about 30 mg mitochondrial protein/ml, was deproteinized with 60% perchloric acid (final concentration 4%) and neutralized as above.

Protein was determined by the biuret method (Gornall, Bardawill & David, 1949).

RESULTS

Determination of citrate. As shown in Fig. 1, the amount of NADH oxidized was equivalent to the amount of citric acid added up to 60 nmol per sample. The efficiency of citric acid recovery was calculated to be 97 - 102%. The day-to-day

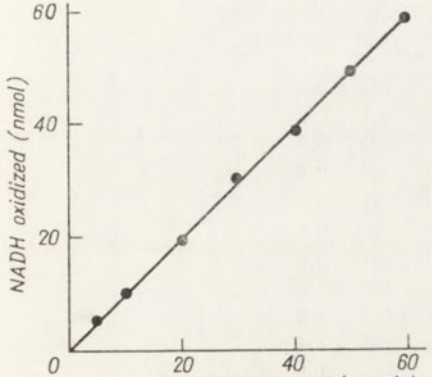

Fig. 1

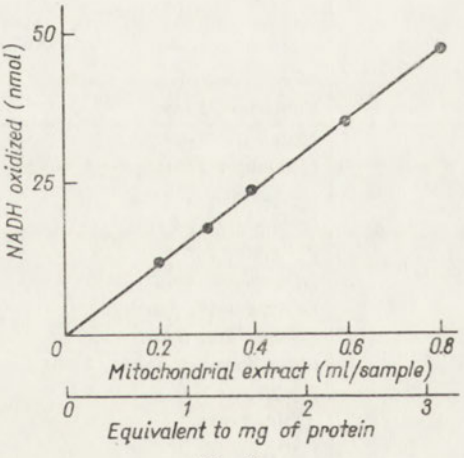

Fig. 2

Fig. 1. Determination of citric acid with ATP citrate lyase isolated from rat liver. The incubation mixture contained in 1.6 ml: lyase, 0.3-0.4 unit; malate dehydrogenase, 10 units; 3.0 mM-ATP; 0.06 mM-CoA; 62 mM-tris buffer, pH 7.6; 0.06 mM-NADH; 3.0 mM-$MgCl_2$; 9.0 mM-2-mercaptoethanol; and various amounts of citric acid.

Fig. 2. Citrate determination in rat liver mitochondria. Conditions of the assay as in Fig. 1. Perchloric acid extract of liver mitochondria corresponded to 0.75 - 3.0 mg of protein.

precision was calculated for series of 11 determinations. The variation coefficients were 4.6% for 5 nmol, 2.6% for 10 and 20 nmol and about 1.0% for higher amounts of citric acid per sample.

The amount of NADH oxidized was also proportional to the amount of liver mitochondrial perchloric-acid extract corresponding up to 3 mg of protein (Fig. 2). The variation coefficient for the extract corresponding to 1.5 mg of mitochondrial protein and containing 23 nmol of citrate was 2.7%. The recovery of citric acid, added in the amount of 5 or 10 nmol per 1 mg of mitochondrial protein, to the incubation mixture just before perchloric acid addition, was calculated to be 94 - 97%. The recovery was independent of the amount of extract used for determinations.

As it is shown in Table 1, many organic anions and nucleotides were without effect or exerted negligible interference with the determination of citrate. However, in the presence of 1 mM-$CaCl_2$ or 2 mM-ADP, which are known to be relatively strong inhibitors of ATP citrate lyase, it was necessary to prolong the time of reaction from 20 to 30 min. Table 2 shows that 1 mM-ADP and 1 mM-$CaCl_2$ inhibited the lyase, about 40 and 60%, respectively.

Determination of CoA. It is shown in Fig. 3 that the amount of NADH oxidized strictly corresponded to the amount of CoA added up to 64 nmol per sample. The

Table 1

The effect of different compounds on determination of citrate with ATP citrate lyase

The values represent the mean of 3 determinations ±S.E.M. Each sample contained 30 nmol of citric acid. The incubation time was 20 min.

Additions	Recovery (%)
None, control	100 ± 0.7
Fumarate, 5 mM	101 ± 0.5
Malate, 5 mM	99 ± 0.4
Malonate, 5 mM	98 ± 0.5
cis-Aconitate, 5 mM	99 ± 0.5
DL-Isocitrate, 5 mM	97 ± 0.4 *
Pyruvate, 5 mM	101 ± 0.4
2-Oxoglutarate, 5 mM	103 ± 1.0
L-Glutamate, 5 mM	103 ± 1.0
L-Aspartate, 5 mM	96 ± 1.0 *
Phosphoenolpyruvate, 5 mM	99 ± 1.1
P_i, 20 mM	102 ± 0.5
ADP, 2 mM	80 ± 0.9 (100 ± 1.4) **
IDP, 2 mM	95 ± 1.1 *
GDP, 2 mM	95 ± 1.1 *
AMP, 2 mM	97 ± 1.9
$CaCl_2$, 1 mM	83 ± 0.5 (98 ± 0.5) **

* Value significantly different from the control value at $P < 0.05$ (t test).

** In parentheses, the value after 30 min incubation.

Table 2

The effect of ADP and $CaCl_2$ on the activity of ATP citrate lyase

The incubation mixture contained: 50 mM-tris-HCl buffer, pH 7.4, 3 mM-ATP, 0.4 mM-CoA, 5 mM-$MgCl_2$, 10 mM-2-mercaptoethanol, the enzyme (0.01 mg of protein), citrate, ADP and $CaCl_2$ as indicated in the table, in the total volume of 0.25 ml. The reaction was started by the addition of CoA and carried out at 37°C for 10 min and was stopped by adding 1.15 ml of ice-cold 10 mM-phosphate buffer, pH 7.4, containing 0.12 mM-NADH and 2 i.u./ml of malate dehydrogenase. After 5 min incubation at 0°C, 0.2 ml of 0.5 M-NaOH was added and samples left in refrigerator for about 2 hours. The precipitate formed was removed by centrifugation and in the clear supernatant the extinction was read at 340 nm against water. To the control sample CoA was not added.

Details of the assay procedure were as described by Szutowicz & Angielski (1970).

Addition	Citrate (mM)	
	2.0	0.5
	Inhibition (%)	
None, control	0	0
ADP, 1 mM	36	43
$CaCl_2$, 1 mM	57	59

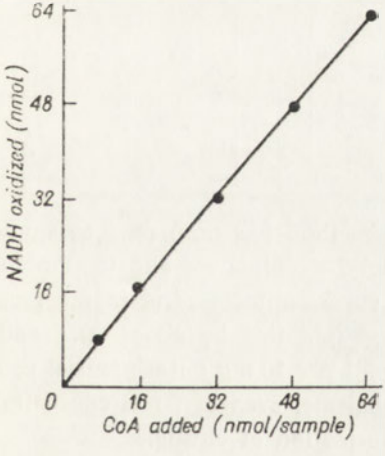

Fig. 3

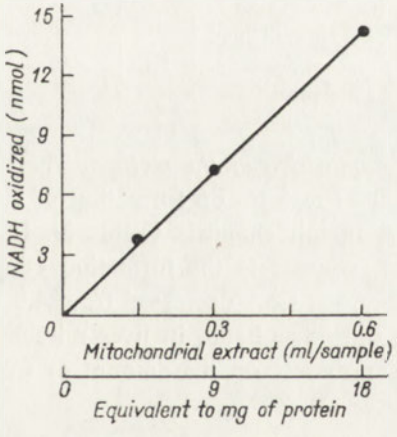

Fig. 4

Fig. 3. Determination of CoA with ATP citrate lyase. Conditions of the assay as in Fig. 1, except that $MgCl_2$ was 9 mM and 19 mM-citrate was added, and the amount of CoA varied.

Fig. 4. CoA determination in rat liver mitochondria. Conditions of the assay as in Fig. 3. In this experiment, mitochondria were prepared from livers of non-starved rats. Perchloric-acid extract of liver mitochondria corresponded to 4.5 - 18.0 mg of protein.

variation coefficient for 11 day-to-day paired determinations of about 20 nmol of CoA was 3.2%.

The amount of NADH oxidized was proportional with the amount of liver mitochondria perchloric-acid extract corresponding up to 18 mg of protein (Fig. 4). The variation coefficient for within-day precision test was calculated to be 4.0%. The amount of CoA in the mitochondria was 8.2 nmol/10 mg of protein. The recovery of exogenous CoA added to mitochondria was 90 - 95%.

Table 3

Determination of citrate and CoA by the enzymic assay and the effect of maleate on citrate formation from 2-oxoglutarate in rat kidney cortex and liver mitochondria

Conditions of experiments: the incubation mixture contained in the final volume of 2.0 ml: 125 mM-sucrose, 25 mM-tris-HCl buffer, pH 7.4, 10 mM-KCl, 5 mM-$MgCl_2$, 5 mM-2-oxoglutarate, maleate as indicated in the table, and 15 - 20 mg of the kidney cortex or 30 - 35 mg of the liver mitochondrial protein. After 10 min incubation at 23°C, the mixture was deproteinized and in the supernatant the content of citrate and CoA were determined and expressed in nmol/10 mg of mitochondrial protein. The results are mean values of three experiments, run in duplicates.

Treatment	Kidney cortex mitochondria			Liver mitochondria		
	CoA	Citrate	Net citrate formation	CoA	Citrate	Net citrate formation
Control (without incubation)	7.3	26	—	3.1	39	—
Incubation with 2-oxoglutarate	4.0	176	150	2.3	86	47
Incubation with 2-oxoglutarate and 1 mM-maleate	1.1	40	14			
5 mM-maleate				2.4	81	42

Application of the method. The described method has been used to study the effect of maleate on formation of citrate from 2-oxoglutarate and the amount of CoA in mitochondria (Table 3). In kidney cortex mitochondria in the presence of 1 mM-maleate the formation of citrate was inhibited by about 90% and the amount of CoA decreased from 4.0 to 1.1 nmol per 10 mg mitochondrial protein. On the other hand, in liver mitochondria, maleate, even at 5 mM concentration, had no effect on the amount of CoA and formation of citrate.

DISCUSSION

The presented results proved that determination of citrate and CoA with ATP : citrate oxaloacetate-lyase assures sufficient accuracy and reproducibility for the use of this method in biochemical investigations. The main advantage of the elaborated method is the possibility of determining both citrate and CoA in a single procedure using one enzyme.

It is known that ATP citrate lyase shows very high specificity to reduced CoA, whereas 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), phosphate acyltransferase (EC 2.3.1.8) or acyl-CoA synthetase (EC 6.2.1.2) used in other methods, are rather less specific (Michal & Bergmeyer, 1965). In the proposed procedure, mercaptoethanol is used to protect thiol groups of CoA.

Determination of citrate with bacterial citrate lyase (EC 4.1.3.6) is, may be, simpler than with ATP citrate lyase. However, in the former method 2-oxoglutarate and glutamate interfere with citrate determination (Dagley, 1965) whereas they are without effect if ATP citrate lyase is employed in the assay (see Table 1). The method based on isocitrate dehydrogenase gives the sum of citrate, *cis*-aconitate and D--isocitrate, and the assay must be supplemented with an additional determination of isocitrate and calculations. The choice of a suitable method depends therefore on the kind of experiments to be performed.

The experiments with maleate are presented to show suitability of the proposed method in enzymic studies. It has been previously reported (Angielski & Rogulski, 1964) that maleate in kidney cortex mitochondria interferes with succinyl-CoA metabolism. It has been suggested that the reaction catalysed by succinyl-CoA : 3--oxoacid CoA-transferase (EC 2.8.3.5) is a possible site of maleate action (Angielski, Rogulski, Szutowicz, Pacanis & Wójcikowski, 1968). The results of the present work support this suggestion. The decrease in CoA content may be explained by irreversible formation of maleyl-CoA which would decrease the efficiency of CoA-dependent reactions, such as that catalysed by 2-oxoglutarate dehydrogenase (EC 1.2.4.2). This in turn would result in inhibition of citrate formation from 2-oxoglutarate in the presence of maleate.

The lack of any effect of maleate in rat liver mitochondria may be connected with the absence in this organ of succinyl-CoA : 3-oxoacid CoA-transferase activity (Stern, Coon, del Campillo & Schneider, 1956). However, it should be pointed out that the presence of maley-CoA has not yet been demonstrated in kidney or kidney mitochondria treated with maleate.

This work was supported in part by the Polish Academy of Sciences, Department of Medical Sciences. The authors are indebted to Professor Dr. Irena Mochnacka for her helpful suggestions in the preparation of this manuscript.

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OZNACZANIE CYTRYNIANU I CoA ZA POMOCĄ LIAZY ATP CYTRYNIANOWEJ

Streszczenie

1. Opisano metodę oznaczania cytrynianu i CoA w preparatach mitochondrialnych przy użyciu liazy ATP cytrynianowej z wątroby szczura.

2. Współczynnik zmienności przy oznaczaniu cytrynianu wynosił 2.5%, a przy CoA 4.0%. Odzysk cytrynianu dodanego do mitochondrii był w granicach 94 - 97%, a CoA 90 - 95%.

3. Metabolity cyklu Krebsa, glutaminian, asparaginian i nukleotydy nie miały wpływu na oznaczanie cytrynianu.

4. Maleinian powoduje obniżenie stężenia CoA i zmniejszenie produkcji cytrynianu z 2-ketoglutaranu w mitochondriach kory nerek. Nie stwierdzono tego efektu w mitochondriach wątroby.

Received 16 December, 1971.

ACTA BIOCHIMICA POLONICA

Vol. 19 1972 No. 3

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HUMAN PLACENTA ARYLSULPHATASE B PURIFICATION AND SEPARATION INTO SUBFRACTIONS

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1. A method is presented for purification and separation into subfractions of human placenta arylsulphatase B. 2. Three different forms of the enzyme, α, β and γ, with molecular weight of, respectively, 71 500, 60 000 and 48 000 and pI values of 7.6, 7.9 and 8.1, have been obtained. 3. The pH optimum for the activity with 2-hydroxy--5-nitrophenyl sulphate as substrate was at pH 5.8 for all three subfractions. 4. The K_m values for subfractions α, β and γ were, respectively 1.04, 1.96 and 0.65 mM.

So far, multiple forms of arylsulphatase B (one of the arylsulphate sulphohydrolases, EC 3.1.6.1) have been found in ox tissues (Wortman, 1962; Błeszyński, 1967; Allen & Roy, 1968) and rabbit cornea (Wortman, 1962). Separation of the enzyme into subfractions has been achieved by DEAE-cellulose or CM-Sephadex chromatography. There is no evidence for the occurrence of arylsulphatase B subfractions in other species, e.g. in bream tissues (Działoszyński, Kuik & Leźnicki 1966), red kangaroo liver (Roy, 1971), rat liver (Leźnicki & Błeszyński, 1970) or human brain (Harinath & Robins, 1971). However, the B enzyme isolated from various species exhibited different behaviour on ion-exchange chromatography.

In the previous work concerning arylsulphatases of human placenta, we did not achieve the separation of sulphatase B into subfractions, although DEAE--cellulose was used as a convenient procedure for separation of sulphatase A from sulphatase B (Gniot-Szulżycka & Działoszyński, 1967).

In the present work, three subfractions of placental arylsulphatase B were obtained and some of their properties were determined.

MATERIAL AND METHODS

Purification procedure. The course of purification is outlined in Table 1. The first three steps closely resemble those previously described (Gniot-Szulżycka& Działoszyński, 1967). Their main purpose is the extraction and separation of soluble sulphatases A and B from human placenta tissue.

Stage 1. Fresh human placentae (5000 g) from healthy women were homogenized in an equal volume of 0.2 M-ammonium acetate, pH 7.0, containing heparin. Acetone, toluene and ethyl acetate were added to the tissue pulp to the final concentration of, respectively, 10%, 5% and 5%. The mixture was autolysed at 0 - 4°C for 72 h and then filtered. The clear filtrate was adjusted to pH 5.0 with acetic acid; then acetone was added at −5°C up to 60%. The precipitate containing sulphatase A and B was dissolved in water and dialysed against water. Some insoluble proteins were discarded.

Stage 2. The dialysed solution (2200 ml) was adjusted to pH 7.0 and solid ammonium sulphate was added up to 2.0 M concentration at −5° to −9°C. After 20 h the precipitate containing sulphatase A was collected and discarded. The concentration of ammonium sulphate in the supernatant was raised to 2.8 M. After 20 h the precipitate containing sulphatase B was collected, made into a slurry with water and dialysed until free from sulphate ions (final volume 680 ml). Some insoluble proteins were discarded and the clear supernatant dialysed against 0.5 M-tris-HCl buffer, pH 7.5.

Stage 3. To the obtained solution, wet DEAE-cellulose pulp (45 g dry wt.) equilibrated with 0.5 M-tris-HCl buffer, pH 7.5, was added. At this pH and ionic strength sulphatase B was not adsorbed on DEAE-cellulose, whereas most of accompanying albumin was strongly adsorbed. After 1 h the mixture was filtered

Table 1

Purification of arylsulphatase B from human placenta

Stage of the purification procedure	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg of protein)	Yield (%)	Purification
1. Acetone preparation	49 500	300 000	6	100	—
2. Ppt. at 2.0 - 2.8 M-ammonium sulphate	30 600	260 000	8.5	87	1.4
3. Filtrate after DEAE-cellulose treatment at pH 7.5	3 760	215 000	57	72	9.5
4. DEAE-cellulose chromatography, pH 7.5	1 460	139 000	95	46	16
5. Sephadex G-200 gel filtration, pH 7.5	237	103 000	436	34	72.5
6. Dialysis, pH 6.2	36	56 700	1575	19	262.5
7. CM-cellulose chromatography, pH 6.8	14.4	2 500	174	0.83	29
	10.7	3 420	320	1.14	53
	5.7	20 810	3660	6.95	610

through Schott G-2 funnel (Φ 13 cm) and then the DEAE-cellulose cake was washed with 0.05 M-tris-HCl buffer, pH 7.5.

Stage 4. The filtrate was concentrated to a volume of 80 ml, then dialysed against 0.05 M-tris-HCl buffer, pH 7.5, and chromatographed at 10°C on DEAE-cellulose column equilibrated with the same buffer. The enzyme B was eluted at V_0 of the column, whereas the bulk of accompanying proteins were eluted with a linear NaCl concentration gradient (Fig. 1).

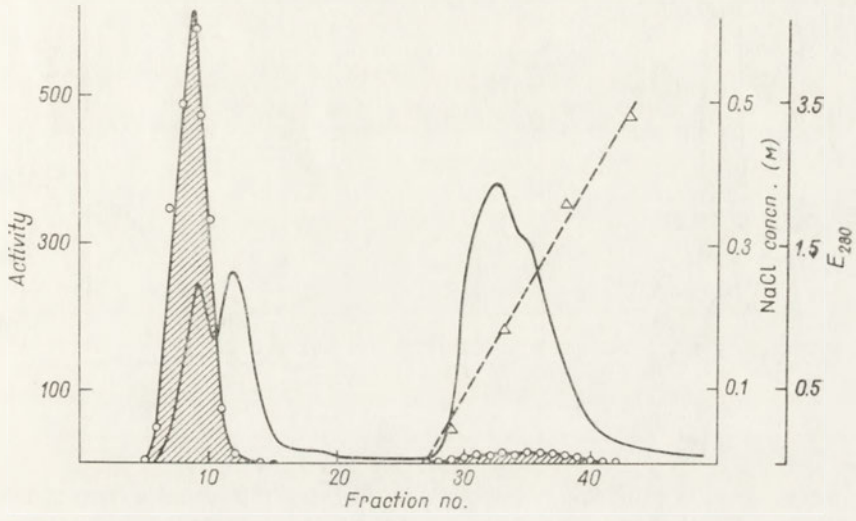

Fig. 1. DEAE-cellulose column chromatography of placenta arylsulphatase B preparation (step 4, Table 1). The chromatography was carried out at 10°C on a column (3.5×33 cm) equilibrated with 0.05 M-tris-HCl buffer, pH 7.5, and eluted with the same buffer. Fractions of 14 ml were collected. ○, Enzymic activity expressed in μg of 4-nitrocatechol liberated by 0.1-ml samples after 30 min incubation at 37°C; ——, E_{280}; △, NaCl gradient.

Stage 5. The enzymically active fractions were pooled, concentrated to 10 ml and dialysed against 0.1 M-tris-HCl buffer, pH 7.5.The dialysed solution was applied to a Sephadex G-200 column, which was equilibrated and eluted with 0.1 M-KCl - - 0.05 M-tris-HCl buffer, pH 7.5, at 10 - 14°C. Two active peaks were obtained (Fig. 2), the second being eluted together with the haemoprotein fraction showing high absorption at 405 nm. Attempts at obtaining better separation on Sephadex G-150 were unsuccessful.

Stage 6. The active fractions from Sephadex G-200 chromatography were pooled, concentrated and dialysed for 2 days against 0.05 M-tris-acetate buffer, pH 6.2, at 0 to 2°C. The copious red precipitate which appeared during the dialysis was centrifuged off.

Stage 7. The obtained clear supernatant was again dialysed against 0.05 M-tris--HCl buffer, pH 6.8, and applied to a CM-cellulose column equilibrated with the same buffer. Elution was made with a linear NaCl gradient made from 300 ml of

0.05 M-NaCl solution in 0.05 M-tris-HCl buffer, pH 6.8, and 300 ml of 0.05 M-tris-HCl buffer, pH 6.8, and fractions of 6 ml were collected. By this procedure, three subfractions of placental sulphatase B were obtained (Fig. 3); subfraction α was distinctly separated from the two others, called β and γ.

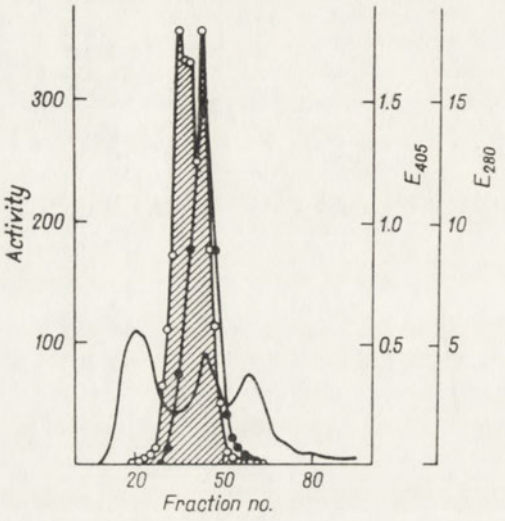

Fig. 2

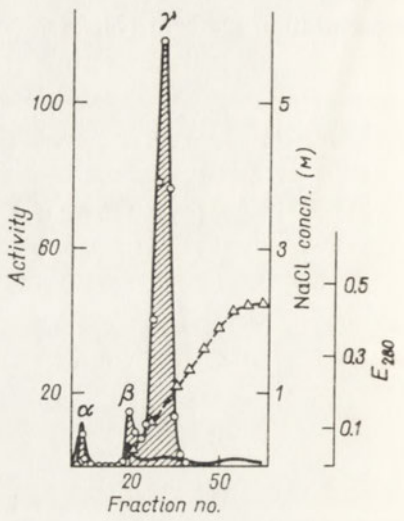

Fig. 3

Fig. 2. Sephadex G-200 gel filtration of placenta arylsulphatase B preparation (step 5, Table 1) The column (3×50 cm) was equilibrated and eluted at 10 - 14°C with 0.1 M-KCl - 0.05 M-tris-HCl buffer, pH 7.5. The flow rate was 0.7 ml/min; 3-ml fractions were collected. ○, Enzymic activity expressed in μg of 4-nitrocatechol liberated by 0.1-ml samples after 30 min incubation at 37°C; ——, E_{280}; ●, E_{405}.

Fig. 3. CM-cellulose chromatography of placenta arylsulphatase B preparation (step 7, Table 1). The column (2.2×20 cm) was equilibrated with 0.05 M-tris-HCl buffer, pH 6.8, and eluted at 10 - -14°C with a linear NaCl gradient. Fractions of 6 ml were collected. ○, Enzymic activity expressed in μg of 4-nitrocatechol liberated by 0.1-ml samples after 30 min incubation at 37°C; ——, E_{280}: △, NaCl gradient.

When the purification procedure was repeated starting with 8500 g of placental tissue, similar results have been obtained.

Concentration procedure. Protein solutions at different stages of the purification were concentrated in dialysing bags in a ventilated chamber cooled at the top.

Isoelectric point determination. This was done by the method of Lampson & Tytell (1965), which is based on stepwise pH gradient elution from CM-Sephadex G-50 column. However, since phosphate ions are known to have a strong inhibitory effect on sulphatase B, the phosphate buffer was replaced by 0.125 M-veronal-acetate buffer (Michaelis, 1931) and 0.1 M-glycine-NaOH buffer. The following proteins were used as standards: haemoglobin (Mann Research Lab., New York, N.Y., U.S.A.); ribonuclease ex bovine pancreas (Koch-Light Lab., Colnbrook, Bucks., England); cytochrome *c* and lysozyme (Reanal, Budapest, Hungary).

Samples of the proteins (1% solutions in 0.125 M-veronal-acetate buffer, pH 6.0) were additionally dialysed against the same buffer and applied to CM-Sephadex C-50 columns equilibrated with the above buffer. The columns were eluted with 5-ml portions of 0.125 M-veronal-acetate buffer or glycine-NaOH buffer, the pH being increased by 0.2 unit. In the collected 5-ml fractions, pH and protein were determined. In the case of sulphatase B, enzyme activity was determined in 0.2-ml portions of each fraction.

Immunoelectrophoresis. Protein fractions from the successive steps of the purification procedure were examined immunoelectrophoretically as previously described (Gniot-Szulżycka & Komoszyński, 1970). Polyvalent horse anti-human plasma serum and rabbit anti-human placenta proteins serum were used.

Thin-layer gel chromatography. Sephadex G-200 of superfine grade was suspended in 0.1 M-KCl - 0.05 M-tris-HCl buffer, pH 7.5, and kept for 3 h in boiling water bath. The swollen gel was spread on 20×40 cm glass plate and equilibrated for 12 h in t.l. gel chamber.

The following proteins were used as reference compounds: horse myoglobin (Koch-Light Lab., Colnbrook, Bucks., England); albumin from bovine serum (Sigma, St. Louis, Mo., U.S.A.), ovoalbumin (Serva, Heidelberg, West Germany); and γ-globulin (Mann Research Lab., New York, N.Y., U.S.A.).

Sulphatase B α, β and γ subfractions and 1% solutions of the reference proteins were applied with capillaries on the starting line. The plate was developed at an angle of 30° for 5 h.

To detect protein and the enzymic activity, the replica technique was employed. For the enzymic activity, Whatman 3 *MM* filter paper soaked with 30 mM-2-hydroxy--5-nitrophenyl sulphate in 1.5 M-acetate buffer, pH 5.8, was used. After incubation for 2 h at 37°C, the paper was alkalized. Red spots of liberated 4-nitrocatechol marked the position of enzyme activity. To detect protein, 1% alcoholic solution of bromophenol blue was used.

Determination of sulphatase B activity. The substrate, 2-hydroxy-5-nitrophenyl sulphate, dipotassium salt, was prepared according to Roy (1958) and crystallized several times from glass-distilled water. The activity of the enzyme was determined by the spectrophotometric method of Robinson, Smith & Williams (1951), at substrate concentration of 10 mM in 0.5 M-acetate buffer, pH 5.8. After 10 min incubation at 37°C, the reaction was stopped with 10% solution of NaOH, and the liberated 4-nitrocatechol measured at 510 nm.

Protein determination. This was carried out by the spectrophotometric method of Warburg & Christian (1941), protein content being calculated from the Kalckar equation: $1.45\,E_{280} - 0.74\,E_{260}$.

RESULTS

The first steps of purification of human placental arylsulphatase B resulted in removal of the bulk of inactive protein and in separation of sulphatase B from

sulphatase A (Table 1). Large losses in sulphatase B activity occurred beginning with step 4, and were concomitant with losses of haemoprotein present in the placental extract.

Separation by CM-cellulose chromatography gave three subfractions, α, β and γ, their specific activities being, respectively, 174, 320 and 3660 nmol of hydrolysed 2-hydroxy-5-nitrophenyl sulphate/min/mg protein.

The overall yield was about 9% of the initial activity; the most highly purified subfraction γ corresponded to 78% of the total recovered activity, subfraction β to 13% and α to 9%.

Immunoelectrophoretic patterns of the three subfractions with horse anti-human plasma proteins serum (Fig. 4) showed that neither of them was immunoelectro-

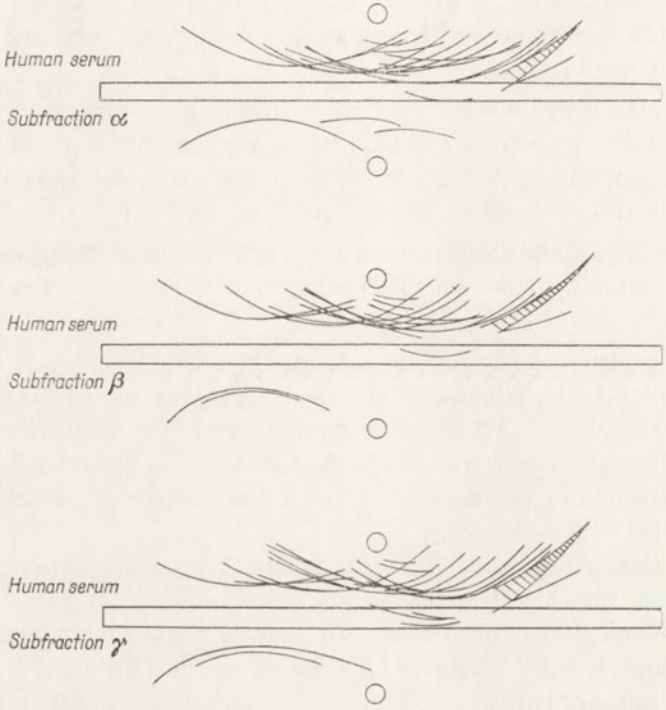

Fig. 4. Immunoelectrophoresis of the isolated human placenta arylsuphatase B subfractions α, β and γ with horse antiserum to human plasma proteins.

phoretically pure. Three different precipitation lines were present in subfraction α, and two with identical mobility in subfractions β and γ. Similar results were obtained with rabbit anti-human placenta proteins serum.

Further purification was not performed due to insufficient amount and pronounced instability of the enzyme.

The pI values estimated by the method of Lampson & Tytell (1965) were 7.9 and 8.1, respectively, for subfractions β and γ (Fig. 5) and 7.6 for subfraction α

(Fig. 6). When subfraction *a* was stored for 24 h dissolved in 0.125 M-veronal-acetate buffer, pH 6.0 (30 µg of protein/ml), its elution pattern became changed and three distinct peaks appeared with maxima at pH 6.8, 7.4 and 7.9 (Fig. 6). On the other hand, storage of subfractions *β* and *γ* under the same conditions had no effect on their elution pattern.

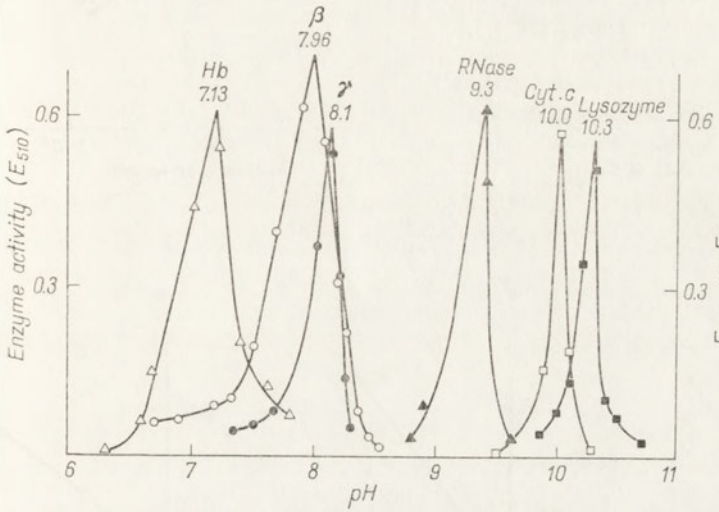

Fig. 5

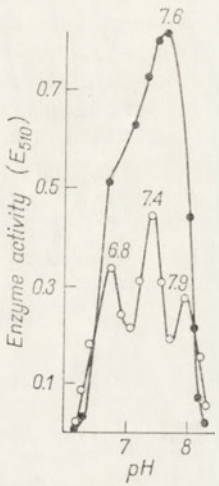

Fig. 6

Fig. 5. Isoelectric point determination of human placenta arylsulphatase B subfractions *β* and *γ*. Standard proteins (1% solutions in 0.125 M-veronal-acetate buffer, pH 6.0) and sulphatase B subfractions were applied to CM-Sephadex G-50 columns (1 × 10 cm) equilibrated with 0.125 M-veronal-acetate buffer, pH 6.0. The column was eluted with 5-ml portions of the buffer, the pH being raised by 0.2 unit; 0.125 M-veronal-acetate buffer was applied up to pH 9.6, and then 0.1 M-glycine-NaOH buffer. Activity of the enzyme subfractions was determined by measuring the amount of liberated 4-nitrocatechol at 510 nm (○, ●). Haemoglobin and cytochrome *c* were determined by the method of Lowry *et al.* (1951) by measuring the extinction at 600 nm (△, □), and lysozyme and RNase by measuring the extinction at 280 nm (▦, ▲).

Fig. 6. Isoelectric point determination of arylsulphatase B subfraction *a*. Conditions as described for Fig. 5, protein concentration 30 µg/ml. ●, Enzymic activity of freshly diluted subfraction *a*; ○, enzymic activity after 1-day storage at 4°C.

Worth noting is the fact that when acetate-veronal buffer was used instead of phosphate buffer (employed by Lampson & Tytell, 1965), the method seemed to give more accurate results.

The pI values of haemoglobin and ribonuclease were identical with those determined by isoelectric focusing (Ui, 1971) and for lysozyme and cytochrome *c* as determined by Alderton, Word & Fevold (1945) and Barlow & Margoliash (1966), whereas the pI values determined by Lampson & Tytell (1965) were consistently by 0.4 - 0.6 unit lower than those reported in the literature.

The molecular weight determined by thin-layer gel chromatography on Sephadex G-200 (Fig. 7 and Table 2) was 71 500 for subfraction *a*, 60 000 for subfraction *β* and 48 000 for subfraction *γ*. The molecular weight of the enzymic subfraction

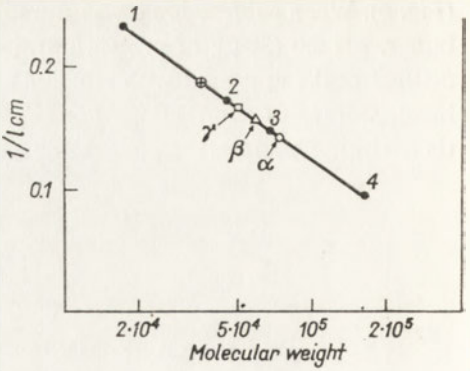

Fig. 7. Estimation of molecular weight of human placenta arylsulphatase B subfractions *α*, *β* and *γ* by thin-layer gel filtration on Sephadex G-200 superfine. Conditions: 4°C, 20×40 cm plate, 30° angle, 5 h 0.05 M-tris-HCl buffer, pH 7.5, containing 0.1 M-KCl, *1*, Myoglobin; *2*, ovoalbumin; *3*, serum albumin; *4*, *γ*-globulin; ⊗ the subfraction from Sephadex G-200 gel filtration (Fig. 2) containing the haemoprotein with maximum absorption at 405 nm.

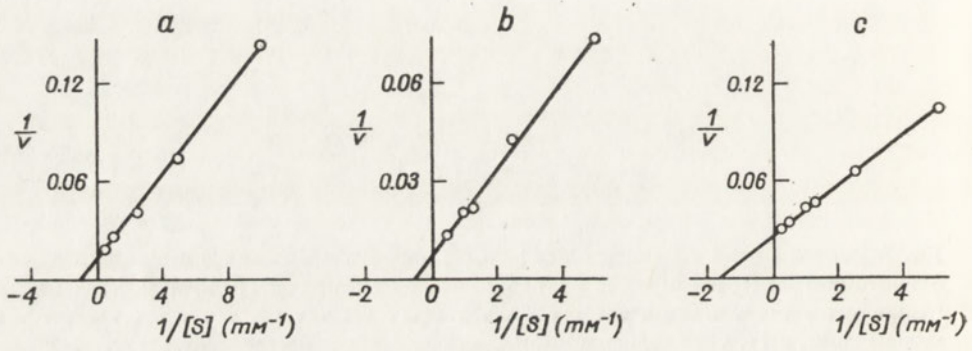

Fig. 8. Lineweaver-Burk plots for the decomposition of 2-hydroxy-5-nitrophenyl sulphate by arylsulphatase B: *a*, subfraction *α* (35.7 μg protein); *b*, subfraction *β* (21.5 μg protein); and *c*, subfraction *γ* (0.9 μg protein). Conditions and procedure as described in Methods section. Incubation 10 min. *v* is expressed as nmol of liberated 4-nitrocatechol/min/mg protein.

Table 2

Molecular weight and K_m *values of placental arylsulphatase B subfractions*

Mean results from 10 - 15 determinations ±S.D. are given. The differences in K_m values of subfractions *α* and *β*, *α* and *γ*, and *β* and *γ*, are significant with $P<0.01$, 0.01 and 0.05, respectively.

Subfraction	Molecular weight	K_m (mM)
α	71 000±2180	1.04±0.076
β	60 000± 987	1.96±0.34
γ	48 000±2590	0.65±0.09

from Sephadex G-200 chromatography containing the haemoprotein with maximum absorption at 405 nm (cf. Fig. 2), was about 36 000.

All sulphatase B subfractions after 10 min incubation at 37°C in the presence of 10 mM-substrate and 0.5-acetate buffer, showed optimum activity at pH 5.8.

The Michaelis constants calculated from Lineweaver-Burk plots (Fig. 8 and Table 2) for subfractions α, β and γ were found to be, respectively, 1.04, 1.96 and 0.65. The differences between the K_m values are statistically significant.

DISCUSSION

Up to now, the most purified and extensively studied arylsulphatase B fractions were those obtained from ox tissues. The two subfractions isolated from ox liver had the same K_m value of 1.84 and 1.94 mM (Allen & Roy, 1968) and a molecular weight of 45 000 (Roy & Jerfy, 1970). Similarly, the subfractions isolated from ox brain did not differ in the affinity for the substrate, the K_m being 1.0 and 1.2 mM (Błeszyński, Leźnicki & Lewosz, 1969) and the molecular weight about 48 000 (Lewosz, 1971). Human brain arylsulphatase B has an apparent molecular weight of 51 300 (Harinath & Robins, 1971).

Unlike the enzyme subfractions obtained from ox tissues, subfractions of arylsulphatase B from human placenta differed in the affinity for substrate and molecular weight. Although the differences in K_m values do not seem to be large, they are statistically significant (see Table 2).

The molecular weight of the isolated subfractions showed considerable differences; the subfraction associated with haemoprotein had a mol.wt. of 36 000, and the pure subfractions 48 000, 60 000 and 71 500. It seems probable that subfractions of arylsulphatase B are composed of one or two active subunits of 24 000 mol.wt., which can associate with each other and with component(s) of 12 000 mol.wt. The most active isolated subfraction γ would be composed of two 24 000 subunits, the less active subfraction β of two subunits and a 12 000 component, and the least active subfraction α of two subunits and two 12 000 components.

The interaction between the subunits and the component(s) could be favoured by the high isoelectric point and probably the great dipole moment of the enzyme.

However, it still remains an open question whether these subfractions occur in placenta *in vivo* and participate in regulation of enzyme activity, or whether they are artefacts arising during the purification procedure.

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OCZYSZCZANIE I ROZDZIAŁ NA PODFRAKCJE ARYLOSULFATAZY B ŁOŻYSKA LUDZKIEGO

Streszczenie

1. Opisano metodę oczyszczania i rozdziału na podfrakcje arylosulfatazy B łożyska ludzkiego.
2. Uzyskano trzy różne formy enzymu różniące się punktem izoelektrycznym, ciężarem cząsteczkowym i powinowactwem do siarczanu 2-hydroksy-5-nitrofenolu. Ciężary cząsteczkowe podfrakcji α, β i γ wynoszą odpowiednio 71 500, 60 000 i 48 000; punkty izoelektryczne 7.6, 7.9 i 8.1; K_m 1.04, 1.96 i 0.65.
3. Podfrakcja γ stanowi 78% ogólnej aktywności końcowej.

Received 12 January, 1972.

ACTA BIOCHIMICA POLONICA
Vol. 19 1972 No. 3

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MUTATIONS RENDERING *SALMONELLA TYPHIMURIUM* RESISTANT TO 3-AMINOTRIAZOLE IN THE PRESENCE OF HISTIDINE

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Mutants *amtA* and *amtB* of *S. typhimurium* resistant to aminotriazole were isolated. Locus *amtA* was mapped on the *Salmonella* chromosome between genes *cysB* and *pyrF*. The mutants did not differ substantially from wild-type strain in the ability to produce arylamine intermediates in purine biosynthesis, and in the activity of succinyl-AMP synthetase. No difference was found between the wild-type strain and the investigated mutants in the catalase activity and sensitivity of this enzyme to aminotriazole.

Aminotriazole has been reported to inhibit in *S. typhimurium* synthesis of histidine and purines (Hilton, Kearney & Ames, 1965; Hulanicka, Kłopotowski & Bagdasarian, 1969). It was therefore expected that in mutants resistant to aminotriazole in the presence of histidine, biosynthesis of purines should be insensitive to aminotriazole.

The results to be presented have shown that most of the strains resistant to 3-aminotriazole in the presence of histidine were mutated in one locus, designated *amtA*, situated between genes *cysB* and *pyrF*. The behaviour of these mutants ruled out permeability as a cause of the resistance. Neither have we found evidence that the resistance is due to a change in regulation of purine biosynthesis.

MATERIALS AND METHODS

Organisms. All bacterial strains used were *Salmonella typhimurium* LT-2 and its derivatives, obtained from other laboratories or isolated during this study (Table 1). Mutant L4 of phage P22 isolated by Smith & Levine (1967) was used for transduction.

Media. Medium C of Vogel & Bonner (1956) supplemented with 0.2% glucose was used as minimal medium. Concentration of added amino acids was 0.1 mM,

Table 1

Origin and genotype of the used strains of Salmonella typhimurium

Collection number	Genotype	Source
LT-2	wild-type	B. N. Ames
	cysB403	K. E. Sanderson
	pyrF146	K. E. Sanderson
	trpC109	P. E. Hartman
SA535	*serA13 HfrK5*	K. E. Sanderson
SL863	*purE801 trpA8 hisD27 hagG501 hagS521 mtl⁻ ofi⁻ colE130*	B. A. D. Stocker
TA827	*hisBHAFIE612*	B. N. Ames
TK801	*amtA1*	NG mutagenesis of LT-2
TK806	*amtA32 hisBHAFIE612*	NG mutagenesis of TA827
TK810	*amtA54 serA13 HfrK5*	NG mutagenesis of SA535
TK811	*amtB58 serA13 HfrK5*	NG mutagenesis of SA535
TK812	*amtA60 serA13 HfrK5*	NG mutagenesis of SA535
TK815	*amtA80 pyrF146*	NG mutagenesis of *pyrF146*
TK831	*amtA32 purE801 hisD27 hagG501 hagS521 mtl⁻ ofi⁻ colE130*	by transduction of SL863 with phage grown on TK806
TK833	*purE801 hisD27 hagG501 hagS521 mtl⁻ ofi⁻ colE130*	same as TK831

and that of serine 2 mM. Uracil and adenine (0.2 mM) were added when required. Minimal media supplemented with adenine contained 0.01 mM-thiamine. Biomed dry broth, 0.5% in 0.5% sodium chloride solution was used as rich medium. Solid media were prepared by adding agar (2%); those for testing aminotriazole-resistant mutants contained 40 mM-aminotriazole and 0.1 mM-L-histidine.

Cultures. Media were inoculated with washed bacteria to contain 10 μg of cells/ml. Bacteria were cultivated at 37°C in a reciprocating shaker and growth was followed turbidimetrically at 420 nm. Optical density readings were converted to μg of dry weight per ml using a calibration curve.

Transduction. Broth culture, 0.2 ml (about 4×10^8 cells) of recipient strains was mixed on agar plates with 0.1 ml of phage suspension (about 2×10^9 plaque-forming units). Phage was grown by inoculating actively growing bacterial culture at cell density of about 2×10^8 cells/ml with 2×10^7 plaque-forming units of phage.

Conjugation. Mating was performed on agar plates by mixing 0.1 ml aliquots of broth cultures of donor and recipient strains.

Isolation of mutants. Overnight broth culture, 0.1 ml (about 2×10^8 cells) was spread on agar medium containing 40 mM-aminotriazole, 0.1 mM-L-histidine and supplements required for growth. After three days of incubation at 37°C, resistant colonies appeared. To ensure independent origin of mutants, separate single aminotriazole-resistant colonies were picked up and restreaked twice on nutrient agar medium. Few crystals of nitrosoguanidine were put on inoculated plate and the

induced mutants were isolated from the dense ring of resistant colonies growing around inhibition zone produced by nitrosoguanidine.

Determination of arylamine intermediates in purine biosynthesis. Cells of the mutant *purE801* (strain SL863 or its derivatives) grown on minimal medium containing the required supplements were washed three times with isotonic sodium chloride by centrifugation. Washed cells were incubated for 2 h at 37°C at the density 0.5 mg dry weight of cells/ml in a medium containing 20 mM-potassium-phosphate buffer, pH 7.4, 5 mM-ammonium sulphate, 130 mM-sodium chloride and 0.4% glucose. After incubation, the mixtures were chilled and the cells were discarded by centrifugation. Arylamine compounds formed were then determined in supernatants by the modified method of Bratton-Marshall (Hulanicka *et al.*, 1969); the modification enables to determine the arylamine intermediates in the presence of aminotriazole.

Cell-free extracts. Logarithmically grown cells were harvested and washed three times with isotonic saline by centrifugation. Packed cells, 5 g wet weight, were suspended in 1 ml of 50 mM-potassium-phosphate buffer, pH 7.2. After 2 min sonication in the cold, cell debris was discarded by centrifugation. The supernatants were passed through Sephadex G-50 columns equilibrated with 1 mM-potassium--phosphate buffer, pH 7.2. Protein in the extracts was determined according to Lowry *et al.* (1951).

Enzyme assay. The activity of adenylosuccinate synthetase was determined after Lieberman (1963) by measuring $\Delta E_{280\ nm}$ of the incubation mixture containing in the final volume of 0.7 ml: glycine buffer, pH 7.9, 100 µmol; magnesium chloride, 4 µmol; ATP, 0.04 µmol; sodium phosphoenolpyruvate, 0.4 µmol; potassium aspartate, 0.5 µmol; GTP, 0.01 µmol; IMP, 0.4 µmol; phosphoenolpyruvate kinase 20 units, and enzymic protein, 0.4 - 0.6 mg. After 30 min incubation at 37°C the reaction was stopped by the addition of 0.5 ml of 7% perchloric acid. The insoluble material was removed by 30 min centrifugation at 10 000 rev./min. The control was treated in the same way except that the enzyme was added after the addition of perchloric acid.

The catalase activity was assayed in intact cells. Cell-free extracts were not used because they did not produce hydrogen peroxide, which is essential for aminotriazole effect on catalase (Margoliash, Novogrodsky & Schejter, 1960). The incubation mixture contained in 1.0 ml of Vogel-Bonner medium C: 0.5 mg of glucose, 0.10 - 0.12 mg of hydrogen peroxide and 50 µg dry weight of bacteria. The reaction was started by the addition of bacterial suspension; after incubation at 37°C, it was stopped with 2.0 ml of titanous sulphate reagent followed by colorimetric determination of hydrogen peroxide (Egerton, Everett, Minkoff, Rudrakanchana & Salooja, 1954). The enzyme activity was expressed as k, velocity constant of first--order reaction.

Chemicals. Sodium ATP, GTP, IMP, phosphoenolpyruvate and phosphoenolpyruvate kinase were products of Sigma Chemical Comp. (St. Louis, Mo., U.S.A.). Aminotriazole was purchased from Eastman-Kodak (Rochester, N.Y., U.S.A.). Other chemicals were commercial products of reagent grade.

RESULTS

Isolation and properties of amt *mutants. Salmonella typhimurium* mutants resistant to aminotriazole were isolated on agar plates containing 40 mM-aminotriazole and 0.1 mM-L-histidine, and inoculated with about 2×10^8 bacteria. After three days of incubation a few dozens of separate colonies appeared on a background of weak growth of sensitive cells. The number of resistant colonies could be increased by using mutagens.

We have designated the mutants resistant to aminotriazole, *amt*. On the basis of genetic classification, at least two groups of *amt* mutants, A and B, were isolated; over 90% of all these mutants were *amtA* and only three were *amtB*.

Growth of an *amtA* mutant and its parent strain in liquid cultures is shown in Fig. 1. The *amt*$^+$ parent strain grew very slowly in the presence of aminotriazole.

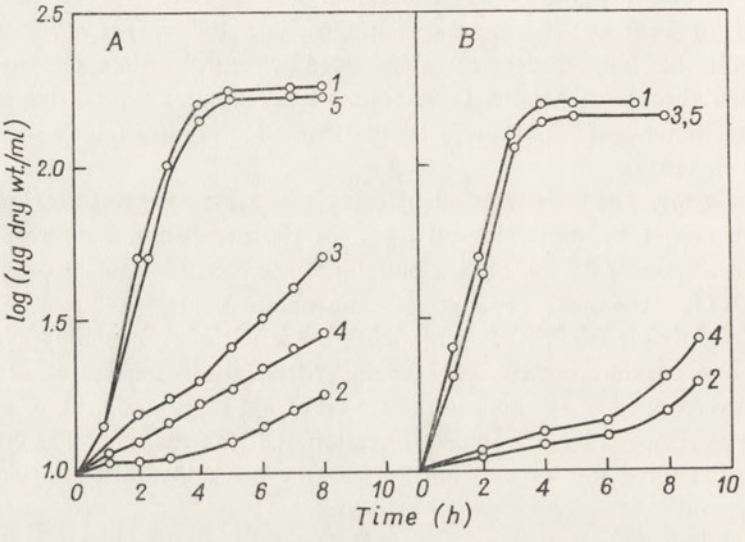

Fig. 1. Effect of histidine and adenine on growth of *S. typhimurium HfrK5 ser13 amt*$^+$ parent strain (*A*), and mutant *HfrK5 ser13 amtA54* (*B*). The control minimal medium contained 2 mM-serine (*1*). 10 mM-Aminotriazole was added alone (*2*), with 0.4 mM-histidine (*3*), with 0.4 mM-adenine (*4*), with histidine and adenine (*5*).

Histidine or adenine separately had little effect on the growth rate, but together they reversed completely inhibition by aminotriazole. Similarly, mutant *amtA* could not grow at a normal rate when aminotriazole or aminotriazole and adenine were present. However, unlike the parent strain, it grew well in the presence of aminotriazole and histidine in the culture medium.This histidine-dependent resistance of the mutant to aminotriazole rules out impermeability of *amt* cells to aminotriazole as a cause of the resistance, as in such a case bacteria would be resistant under any conditions.

It was postulated that *amt* mutations may render purine biosynthesis insensitive

to aminotriazole. To test this supposition, formation of arylamine precursors of purines was studied in the strains with genetic block in *purE* gene, carrying wild-type or mutant allele of gene *amtA*.

For this purpose, the strain carrying two mutations was constructed using phage grown on strain *amtA32* (TK806) to transduce the strain carrying mutations *trpA* and *purE* (SL863). *Trp*$^+$ transductants were then selected; *amtA* and *trp* were proved to be contransducible. As the presence of purine auxotrophy precludes direct examination for *amt*$^-$ character, these transductants were subsequently transduced to *pur*$^+$ with phage grown on wild-type strain LT-2. Recombinants *pur*$^+$ *trp*$^+$ were tested for *amt* character. Synthesis of purines was then studied with two strains carrying mutations *amtA32 purE801* and *amtA*$^+$ *purE801*. It has been shown that formation of arylamines in these mutants was inhibited by 94 and 84%, respectively, by adenine (Table 2). This demonstrates that almost all arylamines determined are precursors of purines. Since 10 mM-aminotriazole inhibited arylamine production by either strain more than 65%, it can be concluded that in this respect there was no physiologically significant difference between these two strains.

Table 2

Synthesis of arylamine intermediates of purine biosynthesis by mutants amtA purE *and* amt$^+$ purE

Strain	Incubation mixture	Arylamines (μmol/ml)	Inhibition (%)
amtA32 purE801 (TK831)	Control	0.53	
	Aminotriazole, 10 mM	0.10	81
	Adenine, 0.2 mM	0.03	94
amt$^+$ *purE801* (TK833)	Control	0.36	
	Aminotriazole, 10 mM	0.12	67
	Adenine, 0.2 mM	0.05	84

In addition it has been shown that the activity of succinyl-AMP synthetase was practically the same in the wild-type strain LT-2 (*amt*$^+$) and mutant *amtA60* (Table 3). The strains were grown in minimal medium and in the medium supple-

Table 3

Effect of aminotriazole on the activity of succinyl-AMP synthetase in the wild-type and aminotriazole-resistant strains

In parentheses, number of assays of independently cultured bacteria are given.

Growth medium	Succinyl-AMP synthetase (units/mg protein)	
	LT-2	*amtA60*
Minimal	0.57 (3)	0.72 (2)
Minimal supplemented with aminotriazole and histidine	1.96 (1)	1.68 (2)

mented with 20 mM-aminotriazole and 0.1 mM-histidine. Both these compounds were able to derepress this enzymic activity to about the same extent when these strains were grown in the medium supplemented with aminotriazole and histidine. This indicates that *amtA* mutation had no effect upon synthesis of this enzyme.

Determination of catalase activity in the investigated mutants was also included since aminotriazole inhibits this enzyme of both mammalian (Margoliash *et al.*, 1960) and plant origin (Pyfrom, Appleman & Heim, 1957). As it is shown in Table 4, specific activity *k* value of catalase was four times higher in bacteria grown under aerobic conditions and was not affected by the *amtA* mutation. Preincubation of either strain with aminotriazole inhibited catalase activity by about 25%. The *amtA* mutation had thus no effect upon catalase sensitivity to aminotriazole.

Table 4

Catalase activity of the wild-type and amtA *strains grown aerobically and anaerobically*

Activity expressed in *k* values/mg dry weight. Preincubation with aminotriazole (50 mM) for 15 min. Incubation 10 min in Experiment 1 and 4 min in Experiment 2.

Strain	Addition	Aerobic cells	Anaerobic cells
Experiment 1			
LT-2	None	4.4	1.1
	Aminotriazole	3.5	0.8
amtA1	None	6.3	1.5
	Aminotriazole	4.7	1.1
Experiment 2			
LT-2	None		1.2
	Aminotriazole		0.8
amtA1	None		1.3
	Aminotriazole		1.0

Genetic mapping of amt *mutants.* For rough localization of *amt* mutations, mutant *HfrK5 serA13 amt-54* was used. It was crossed with female auxotrophs and the prototrophic recombinants were tested for *amt* character. It appeared that when mutants with lesions in tryptophan operon were used as recipients, about 95% of recombinants were *amt*$^-$. The same result was obtained when mutants in the nearby gene *pyrF* were used as recipients.

This finding indicating close linkage of *amt-54* mutation with *trp* and *pyrF* was confirmed in transduction experiments. Most of *amt* mutants were linked with these two markers. The respective locus was named *amtA*.

Gene order in the *amtA* region was established by the three-point tests presented in Tables 5 and 6. In the cross in which phage grown on strain *amtA80 pyrF146* and cells of strain *trpC109* were used, the rarest recombinant class was *trpC*$^+$ *amtA*$^+$ *pyrF*$^-$. This result pointed to the following gene order: *trpC amtA pyrF*. The linkage values with *trpC* calculated from the data given in Table 5 were: *amtA* 24.4% and

pyrF 11.9%, and they are consistent with the gene order deduced from the rarest recombinant class.

In the cross in which the same donor was used to transduce mutant *cysB403* the rarest recombinant class was *cysB*$^+$ *amtA*$^+$ *pyrF*$^-$. The results indicated the gene order: *cysB amtA pyrF*. The linkage values with *cysB* calculated from the data

Table 5

Three-point test by transduction

A. Donor: *amtA80 pyrF146* Recipient: *trpC109* Selected marker: *trp*$^+$					Possible gene orders: a — *amtA trp pyrF* b — *trp amtA pyrF* c — *trp pyrF amtA*	
Unselected recombinant types		Number of cross-overs required for the indicated gene order			Unselected types found	
amtA	*pyrF*	a	b	c	Number	%
+	+	2	2	2	427	75.3
+	146	2	4	2	2	0.3
80	+	2	2	4	77	12.8
80	146	2	2	2	65	11.6
				Total	571	100.0
Conclusion: Low frequency of type *trp*$^+$ *amtA*$^+$ *pyrF*$^-$ indicates the gene order (b): *trp amtA pyrF*						

Table 6

Three-point test by transduction

B. Donor: *amtA80 pyrF146* Recipient: *cysB403* Selected marker: *cysB*$^+$					Possible gene orders: a — *amtA cysB pyrF* b — *amtA pyrF cysB* c — *cysB amtA pyrF*	
Unselected recombinant types		Number of cross-overs required for the indicated gene order			Unselected types found	
amtA	*pyrF*	a	b	c	Number	%
+	+	2	2	2	220	50.0
+	146	2	2	4	8	1.5
80	+	2	4	2	107	26.2
80	146	2	2	2	114	22.3
				Total	449	100.0
Conclusion: Low frequency of type *cysB*$^+$ *amtA*$^+$ *pyrF*$^-$ indicates the gene order (c): *cysB amtA pyrF*						

given in Table 6 were: *amtA* 48.5% and *pyrF* 23.8%. These values are in agreement with the gene order deduced from the rarest recombinant class.

As the gene order of this chromosomal region is *trp cysB pyrF*, the locus *amtA* appears to be located between *cysB* and *pyrF*. Figure 2 presents a schematic map

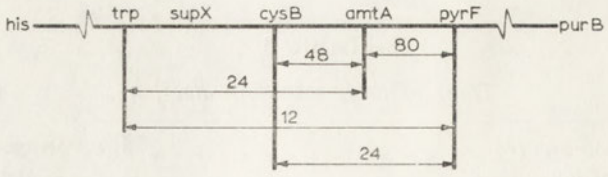

Fig. 2. Localization of genes in the *amtA* region of *S. typhimurium* chromosome. Linkage values expressed in percentage of contransducibility.

of this region based on the three-point tests and some other crosses. The linkage values of *amtA* with closest known neighbours indicate that it is separated from *cysB* and *pyrF* by at least several gene lengths.

Three of our *amt* mutants, mutants *amtB*, do not map in the *amtA* locus, and strain *HfrK5 serA13 amtB58* was used to localize the *amtB* locus. Linkage of *amtB58* with *trp* was only 20%. The highest linkage, 55% was found with *proA* recipients. However, no linkage was found by transduction with *proA* or *purE* loci.

DISCUSSION

Two classes of mutants: *amtA* and *amtB* have been isolated. The former was mapped in a locus between genes *cysB* and *pyrF*; it was contransducible with them and with the nearby tryptophan operon. This enabled to construct a strain *amtA purE* requiring purine for growth and accumulating the purine precursor, aminoimidazole ribotide. Accumulation of this compound was shown by Hulanicka *et al.* (1969) to be decreased in the presence of aminotriazole.

It appeared that *amtA* mutation had no effect upon synthesis of aminoimidazole ribotide. This indicates that *amtA* mutation does not change sensitivity of purine biosynthesis to aminotriazole.

As aminotriazole inhibits catalase of plant (Pyfrom *et al.*, 1957) and mammalian (Margoliash *et al.*, 1960) origin, the *S. typhimurium* enzyme from *amtA* mutant was compared with that of control *amt*$^+$ strain. Again, no significant differences could be detected.

Thus, it should be concluded that biochemical mechanism for aminotriazole resistance in *S. typhimurium* mutants remains unknown.

This study was partially supported by grant FG-Po-191 from the U.S. Department of Agriculture.

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MUTACJE POWODUJĄCE OPORNOŚĆ NA AMINOTRIAZOL W OBECNOŚCI HISTYDYNY U *S. TYPHIMURIUM*

Streszczenie

Wyizolowano mutanty *S. typhimurium amtA* i *amtB* oporne na aminotriazol w obecności histydyny. Cecha *amtA* jest zlokalizowana między genami *cysB* i *pyrF*. Mutanty *amtA* nie różnią się w zasadniczy sposób od szczepu dzikiego zdolnością do wytwarzania arylamin, prekursorów pierścienia purynowego i aktywnością syntetazy bursztynylo-AMP. Nie stwierdzono również różnic między mutantami a dzikim szczepem w aktywności katalazy i jej wrażliwości na aminotriazol.

Received 26 January, 1972.

ACTA BIOCHIMICA POLONICA

Vol. 19 1972 No. 3

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DNA METABOLISM IN GERMINATING EMBRYOS OF RYE (*SECALE CEREALE* L.)

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1. In germinated rye embryos [6-^{3}H]thymidine is incorporated preferentially into DNA of extranuclear subcellular fractions both *in vivo* and *in vitro*, whereas the fractions isolated from ungerminated embryos did not incorporate the precursor *in vitro*. 2. DNA turnover is discussed in relation to developmental processes of the cell organelles during plant embryo germination.

The existence of extranuclear DNA pool in plant and animal cells is firmly established (cf. Rabinowitz & Swift, 1970), and the metabolic semiautonomy of the cell compartments as plastids and mitochondria, has been discussed long ago (cf. Gibor & Granick, 1964).

Germinating seeds, or embryos isolated thereof, seem to be a suitable material for studies on nucleic acid metabolism in the cell compartments in view of the fact that during germination rapid organellogenesis takes place (Nawa & Asahi, 1971).

In this paper we present some data concerning DNA metabolism in various cell compartments of germinating rye embryos.

MATERIALS AND METHODS

Special reagents. [6-^{3}H]Thymidine, 21.27 Ci/mmol (Institute for Research, Production and Utilization of Radioisotopes, Prague, Czechoslovakia); calf thymus DNA, crystalline bovine pancreas ribonuclease I (Sigma Chemical Company, St. Louis, Mo., U.S.A.); sodium dodecyl sulphate (Light & Co. Ltd, Colnbrook, Bucks., England); diphenylamine (F.O.Ch., Gliwice, Poland); thymidine, unlabelled (Merck, Darmstadt, West Germany); bovine blood serum albumin cryst. (Charles Druce Ltd., London, England); 2,5-diphenyloxazole (PPO) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) were from Packard Instrument Co. Inc. (Downers Grove, Ill., U.S.A.); other chemicals used were analytical grade products of Polish origin.

Material. Embryos were separated from grains of rye (*Secale cereale*, var. "Włoszanowskie") according to Johnston & Stern (1957), using the flotation technique in 2 M-sucrose solution. In some experiments rye embryos purchased from Zakłady Zbożowo-Młynarskie (Świebodzin, Poland), were used. The embryos were germinated for 24 h on sterile gauze moistened with sterilized bidistilled water, at room temperature under normal light conditions. In all experiments the embryos before use were surface-sterilized with 0.1% (w/v) $HgCl_2$ solution in water and then rinsed with water.

Isolation of subcellular fractions. Ungerminated (10 g dry wt.) or germinated (about 15 g dry wt.) embryos were ground by hand in a chilled mortar with 0.25 M-sucrose - 10 mM-$MgCl_2$ - 13 mM-phosphate buffer, pH 7.3. The homogenate (about 60 ml) was squeezed through three layers of nylon tissue and subjected to differential centrifugation in the cold room. Three subcellular fractions were subsequently spun down: the nuclear fraction at 600 *g* for 3 min, the plastidial fraction at 1000 *g* for 14 min and the mitochondrial fraction at 12 000 *g* for 15 min.

Labelling experiments. In the *in vivo* experiments, about 15 g (wet wt.) of germinated rye embryos were incubated in 15 ml of bidistilled sterilized water with 8.8 nmol of [6-^{3}H]thymidine (12.5 μCi/ml) for 15 min at 37°C with constant aeration in the dark. The incorporation was stopped by rapid cooling to 2°C. The incubation medium was drained off on the Buechner funnel and the embryos were rinsed with five 6-ml portions of cold water containing an excess of unlabelled thymidine (90 μg/ml), and then with water. The embryos were homogenized, the subcellular fractions were isolated and DNA extracted.

In the *in vitro* experiments, the freshly isolated subcellular fractions (30 mg of protein) were suspended in 2 ml of 0.25 M-sucrose, using a glass homogenizer equipped with a loosely fitting teflon pestle, and 1.32 nmol of [6-^{3}H]thymidine (28 μCi) was added. The incubation was carried out at 37°C with occasional shaking. After 15 min the mixture was cooled to 2°C and three volumes of unlabelled thymidine solution with ethanol were added. This solution consisted of 1 vol. of thymidine dissolved in water (90 μg/ml) and 3 vol. of 96% ethanol. The mixture was left overnight in the cold, then the pellets were sedimented by centrifugation, washed with five 6-ml portions of the above thymidine-ethanol solution, and finally with cold 0.25 M-sucrose solution. From the pellets, DNA was extracted.

Isolation of DNA. The phenol method described by Saito & Miura (1963) was applied, the last ribonuclease T_1 treatment step being omitted. The pellets of isolated subcellular fractions were suspended in 0.1 M-tris-HCl buffer, pH 9.0, containing 1% sodium dodecyl sulphate and 0.1 M-NaCl. Then 1 vol. of freshly distilled phenol was added and the mixture was shaken in the cold for 15 min. After centrifugation, to the water phase 3 vol. of cold ethanol were added. The precipitate was collected by centrifugation and dissolved in a small volume of a solution consisting of 15 mM-NaCl - 1.5 mM-trisodium citrate, pH 7.0 (SSC solution). Then 100 μg/ml of pancreatic RNase I (preheated at 80°C for 10 min to remove the contaminating DNase) were added and the mixture was incubated at 37°C for 1 hour. After cooling, 3 vo of cold ethanol were added, the precipitate was collected and redissolved in a smal.

volume of SSC solution. An equal volume of chloroform - isoamyl alcohol mixture (24:1, v/v) was then added (Sevag, Lackman & Smolens, 1938) and, after vigorous shaking for 10 min, the mixture was centrifuged and the water phase collected. DNA was precipitated by adding 3 vol. of cold 96% ethanol.

Hydrolysis of DNA and chromatography of hydrolysis products. From subcellular fractions, after complete removal of RNA, DNA was extracted with 0.5 N-$HClO_3$ at 70°C for 15 min according to Ogur & Rosen (1950). Perchloric acid was removed with KOH, the supernatant evaporated to dryness, and complete DNA hydrolysis (0.1 ml of 70% $HClO_3$/mg of DNA) and chromatography were carried out as described by Seemayer (1970). The clear supernatant containing the hydrolysis products was evaporated to a small volume and subjected to descending chromatography on Whatman paper no. 1 in the isopropanol - concn. HCl - water (67:17:18, by vol.) system. The bases were located under ultraviolet light, the spots were eluted with 3 ml of 0.1 N-HCl at 37°C for 2 h with shaking. In the eluates the u.v. spectra were examined with Unicam SP 500 spectrophotometer. The extinction measured at 249, 263, 265 and 276 nm was converted into μmol of, respectively, guanine, adenine, thymine and cytosine according to Bendich (1957). The eluates were evaporated to dryness, the residue dissolved in 0.2 ml of 0.1 N-HCl and used for radioactivity counting.

Radioactivity measurement. This was done using an automatic liquid-scintillation spectrometer (Packard Tri-Carb model 3003, efficiency for ^3H, 50%). To 11 ml of the scintillation fluid (3 g of PPO and 0.3 g of POPOP per 1000 ml of toluene), 3.5 ml of absolute ethanol and 0.2 ml of DNA solution in water (50 - 70 μg of DNA) or nucleic bases dissolved in 0.2 ml of 0.1 N-HCl, were added.

Estimation of DNA. The diphenylamine procedure of Dische (1955) was employed, with calf thymus DNA as standard.

Protein determination. The method of Lowry *et al.* (1951) with the procedure for insoluble proteins, was applied; bovine blood serum albumin was used as standard.

RESULTS AND DISCUSSION

Incorporation *in vitro* of [6-^3H]thymidine into DNA of subcellular fractions isolated from ungerminated and germinated rye embryos, is presented in Table 1. With ungerminated embryos, there was practically no incorporation of thymidine. Of the subcellular fractions from germinated embryos, each incorporated thymidine to a somewhat different extent, as shown by differences in specific activities of DNA. The highest incorporation was observed with the 12 000 *g* pellet, and the lowest with the 600 *g* pellet.

In the experiments *in vivo*, when embryos germinated for 24 h were incubated for 15 min with [6-^3H]thymidine (Table 2), the specific activities of DNA of the isolated subcellular fractions were found to differ largely; the activity was the lowest for the 600 *g* pellet, four times as high in the 1000 *g* pellet, and about ten times as high in the 12 000 *g* pellet. The labelling of DNA of the 600 *g* pellet was practically

Table 1

In vitro *incorporation of* [6-^{3}H]*thymidine into DNA of subcellular fractions isolated from ungerminated and germinated for 24 h rye embroys*

Isolation of subcellular fractions and incubation conditions, DNA isolation and estimation, see Materials and Methods. The results of a typical experiment are given.

Subcellular fraction	Specific activities of DNA (counts/min/mg of DNA)	
	Ungerminated embryos	Germinated embryos
600 *g* pellet	200	2140
1 000 *g* pellet	0	2860
12 000 *g* pellet	0	4060

Table 2

Incorporation of [6-^{3}H]*thymidine* in vivo *into DNA of subcellular fractions of germinated rye embryos*

Conditions of thymidine incorporation, separation of subcellular fractions, hydrolysis of DNA and radioactivity determinations were as described in Materials and Methods. The results of a typical experiment are given.

Subcellular fraction	Specific activity of	
	DNA (counts/min/mg)	thymine (counts/min/μmol)
600 *g* pellet	3 000	307
1 000 *g* pellet	12 708	1115
12 000 *g* pellet	32 383	1928

the same *in vivo* and *in vitro*, whereas in the two other subcellular fractions the incorporation of labelled thymidine was much higher *in vivo* than *in vitro*.

On hydrolysis of DNA labelled *in vivo*, it was found that thymine was the only radioactive product, the radioactivities of other bases being almost equal to that found for the blank eluate. The highest specific activity was found for thymine originating from DNA of the 12 000 *g* pellet, and the lowest for that of the 600 *g* pellet (Table 2).

Rapid development of mitochondria is known to occur during early stages of plant seed germination (Breidenbach, Castelfranco & Criddle, 1967; Nawa & Asahi, 1971). In our experiments, the incorporation of radioactive thymidine *in vivo* into DNA of rye embryos germinating for 24 hours was much greater in the plastidial fraction, and still greater in the mitochondrial one, than in the nuclear fraction. This indicates that extranuclear DNA may play an important role in organellogenesis. A similar phenomenon of preferential incorporation of labelled precursors into extranuclear DNA over that found for nuclear DNA, has been observed by

Rabinowitz, Getz, Casey & Swift (1968) in yeasts during respiratory adaptation of the cells, that is at the time when mitochondriogenesis occurs.

Our data do not permit to differentiate between the complete *de novo* synthesis of DNA molecules, their repair or completion of incomplete molecules. However, Gross & Rabinowitz (1969) have shown that the incorporation of labelled precursors into rat liver DNA represented replication and not repair processes of the polymer. Although some differences in rates of reutilization of the DNA precursors in the cell compartments may play a role in the differences observed by us in DNA turnover, our data suggest that in germinating rye embryos two separate metabolic DNA pools exist, i.e. nuclear DNA showing low turnover, and extranuclear DNA characterized by higher metabolic activity. This observation may indicate that extranuclear cell compartments possess some degree of metabolic autonomy even in the early stages of development.

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

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METABOLIZM DNA U KIEŁKUJĄCYCH ZARODKÓW ŻYTA (*SECALE CEREALE* L.)

Streszczenie

1. U kiełkujących zarodków żyta [6-^{3}H]tymidyna włącza się preferencyjnie do DNA pozajądrowych frakcji podkomórkowych zarówno *in vivo* jak i *in vitro*. Natomiast frakcje podkomórkowe z zarodków nie poddanych kiełkowaniu nie włączają znakowanej tymidyny *in vitro*.

2. W pracy dyskutowany jest metabolizm DNA w odniesieniu do procesów rozwoju organelli komórkowych u kiełkujących zarodków nasiennych.

Received 31 January, 1972.

ACTA BIOCHIMICA POLONICA
Vol. 19 1972 No. 3

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5,6-DIHYDRO-5,6-CYCLOBUTANYLURACILS AND THEIR NUCLEOSIDES: INTERMEDIATES IN THE PHOTOCHEMICAL DEALKYLATION OF 5-PROPYL- AND 5-ISOPROPYL URACILS AND THEIR NUCLEOSIDES

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1. Irradiation at 254 nm in neutral aqueous medium of 5-propyluridine and 5-isopropyluridine — prepared enzymically from the corresponding bases — results in photochemical dealkylation, with formation in high yield in both cases of uridine and propylene. 2. The reaction involves, initially, intramolecular cyclization of the 5-alkyl groups about the 5,6 bond to form a cyclobutane ring, the resulting products being several stereoisomeric forms of 5,6-dihydro-5,6-cyclobutanyluridine derivatives. These intermediates then undergo photodissociation in high quantum yield, approximately one-third to one-half that for a pyrimidine photodimer (0.18 - 0.25), to eliminate propylene and yield uridine. 3. This reaction sequence is unaffected by the presence of oxygen. It also proceeds, but in much lower yield, at wavelengths to the red of 265 nm. No photodimerization was observed, even at the longer wavelengths in the absence of oxygen, in contrast to 5-ethyluridine which photodimerizes readily on irradiation at longer wavelengths. 4. The 5,6-dihydro-5,6-cyclobutanyluracil intermediates, including a series of new analogues of 5,6-dihydropyrimidine nucleosides of possible biological significance, can be obtained on a larger scale by photochemical addition of propylene to 1-methyluracil or uracil nucleosides. Several of these have been isolated and some of their properties characterized. 5. Photochemical addition of propylene to thymidine has also been demonstrated, suggesting that this phenomenon is quite general. 6. The mechanisms of the photochemical reactions, and the potential biological applications of the photoadducts, which are also the simplest possible analogues of pyrimidine photodimers, are discussed.

It was previously shown that 5-ethyldeoxyuridine, EtUdR[1], a thymidine analogue which exhibits anti-viral activity (Świerkowski & Shugar, 1969a), undergoes photo-

[1] Abbreviations: UR, uridine; TdR, thymidine; UdR, 2′-deoxyuridine; EtUdR, 5-ethyl-2′-deoxyuridine; PUdR, 5-propyl-2′-deoxyuridine; 1MeU, 1-methyluracil; PUR, 5-propyluridine; iPUR, 5-isopropyluridine; UR·H_2O, hydrated uridine, 5,6-dihydro-6-hydroxyuridine; UR·P, TdR·P, 1MeU·P, 1:1 adducts of UR, TdR and 1MeU with propylene.

dealkylation on irradiation at 254 nm and predominantly photodimerization at wavelengths to the red of 265 nm (Pietrzykowska & Shugar, 1968, 1970). Subsequent examination of two higher 5-alkyl analogues, 5-propyluridine and 5-isopropyluridine, demonstrated quite different behaviour on irradiation at wavelengths to the red of 265 nm, but similar photodealkylation reactions at 254 nm, the nature and mechanism of which have already been described in preliminary form (Krajewska & Shugar, 1971). The present paper deals in fuller detail with these interesting reactions and their extension to other derivatives, the properties of the intermediates involved and the potential biological significance of the latter as new analogues of both 5,6-dihydropyrimidine nucleosides and pyrimidine photodimers.

MATERIALS AND METHODS

Synthesis of 5-propyluridine and 5-isopropyluridine. Both of these nucleosides were prepared on a small scale, enzymically, from the corresponding bases (kindly supplied by Dr. B. Fiszer) and α-D-ribose-1-phosphate (Calbiochem, Los Angeles, Calif., U.S.A.) with the aid of the uridine phosphorylase system (uridine:orthophosphate ribosyltransferase, EC 2.4.2.4) from rat liver. The ribosides were isolated from the reaction mixture on silica gel H plates (Merck, Darmstadt, West Germany) with the chloroform - methanol (85:15, v/v) solvent system, and eluted with chloroform - methanol (70:30, v/v). The identity of the ribosides was established (a) enzymically, by their ability to undergo phosphorolysis with the pyrimidine nucleoside phosphorylase system; (b) by the similarity of their U.V. spectra at various pH values with that for thymine riboside. The spectra for the two nucleosides differed from that for thymine riboside only in that their long-wavelength maxima were shifted slightly to the red, so that λ_{max} was at 268 - 269 nm in acid and neutral medium, and at 270 nm for the anionic forms in alkaline medium. The small quantities of material synthesized made it difficult to determine their extinction coefficients, and it was assumed that they were the same as for thymine riboside. This is a reasonable assumption, since the extinction coefficient of 5-ethyluridine is practically identical with that for 5-methyluridine (thymine riboside), (Świerkowski & Shugar, 1969b).

Thymidine and uridine were Sigma (St. Louis, Mo., U.S.A.) products, while 1-methyluracil was prepared as elsewhere described (Shugar & Fox, 1952). Analytical grade buffer materials and glass double-distilled water were used throughout.

pH measurements made use of a Radiometer PHM22 compensating meter with a glass electrode. Melting points (uncorrected) were measured on a Boetius microscope hot stage.

Elementary analyses were carried out with a Hewlett-Packard Model 185 CHM analyser (through the kindness of Dr. St. J. Kubacki).

Gas chromatography was performed either with a 9.2 m column of 20% Ucon LB 500 X on diatomite 0.2 - 0.3 mm at 35°C and a nitrogen-flow of 40 ml/min, or as described elsewhere (Pietrzykowska & Shugar, 1970). Alcohols, aldehydes, etc. were determined with a 2.7 m column of Carbowax 20M on 100 - 120 mesh Celite or a 6 m column with 10% Carbowax 1000 on diatomite C "Q" 100 - 120 mesh

at 87°C and a nitrogen flow of 35 ml/min. A PYE type 104 instrument was employed for these measurements, for the use of which we are indebted to Dr. P. Krasnodębski.

Irradiation sources employed were: (A) a Phillips 6-watt germicidal lamp (254 nm) surrounded by a 2-mm layer of saturated sodium acetate to remove trace of radiation below 230 nm; the intensity at the filter surface was about 1.7×10^{-7} Einstein/cm^2/min; (B) a Phillips 40-watt germicidal lamp with a 2-mm layer of sat. sodium acetate, and an intensity 1 cm from the filter surface of about 2.6×10^{-7} E/cm^2/min; (C) a 700-watt Q-700 medium pressure mercury lamp with Schott WG7 and UG11 filters to cut off in the neighbourhood of 265 - 270 nm. Intensity measurements for the 254 nm sources made use of a 10^{-4} M aqueous solution of uridine, for which the quantum yield for photohydration is 0.021 (McLaren & Shugar, 1964, p. 180).

Spectral measurements made use of a Unicam SP-500 manual instrument and a SP-800 recording spectrophotometer. For most purposes 10-mm pathlength semi-micro cuvettes fitted with teflon stoppers were employed.

ORD spectra were run on a Jasco ORD-UV/CD5 spectropolarimeter, using 10-mm cuvettes. We are indebted to Dr Andrzej Rabczenko for recording these spectra.

NMR spectroscopy was carried out through the kindness of Dr. S. Brownstein of the National Research Council, Ottawa, Canada, with the aid of a Varian model 100 instrument, using [$^2H_6$]dimethyl sulphoxide as solvent.

Chromatography. Details are given in the text and Tables. Eastman (Rochester, N.Y., USA) cellulose 6065 and silica gel 6060 TLC sheets were used for analytical purposes. Ascending technique on Whatman no. 3 and 3 *MM* papers was employed for preparative work. The following solvent systems (v/v) were used: *A*. Water - *n*-butanol (14:86); *B*. Isopropanol - water - conc. NH_4OH (7:2:1); *C*. Isopropanol - 1 M-CH_3COONa - satur. $(NH_4)_2SO_4$ (1:9:20); *D*. Chloroform - ethanol (9:1); *E*. Chloroform - ethanol (8:2); *F*. Water - formic acid - *n*-butanol (1:1:8); *G*. Isopropanol - 1% $(NH_4)_2SO_4$ (4:1).

RESULTS

Photolysis of PUR and iPUR

Irradiation of 10^{-4} M neutral aqueous solutions of PUR or iPUR at wavelengths to the red of 265 nm (light source C) led to a very slow disappearance of the absorption maximum, 14% for PUR after 9 h irradiation, and 10% for iPUR after 11 h. The irradiated solutions were chromatographed on cellulose and silica gel TLC plates with solvent systems *A* and *E* to reveal immediately, under a dark U.V. lamp, only the initial substances. However, further irradiation of the chromatograms at 254 nm placed in evidence faint spots with R_F values lower than those for the starting compounds.

When the solutions of PUR and iPUR, following irradiation at wavelengths to the red of 265 nm, were alkalized by addition of concentrated NH_4OH, and

further irradiated at 254 nm (light source A) and chromatographed as above, new distinct spots were revealed with R_F values corresponding to uridine (both PUR and iPUR themselves are resistant to irradiation in alkaline medium at 254 nm). Analogous results were obtained when irradiation was carried out in the absence of oxygen. Unfortunately, the quantities isolated in this way were too small to be used for further investigation. It should be noted that, under fully analogous conditions, 5-ethyluracil and its glycosides undergo photodimerization in reasonably good yield (Pietrzykowska & Shugar, 1970).

The situation was quite different when irradiation was conducted at 254 nm, as shown in Fig. 1. Calculation showed that the quantum yields for disappearance of PUR and iPUR, at concentrations of 10^{-4} M, were 9×10^{-3} and 4.7×10^{-3},

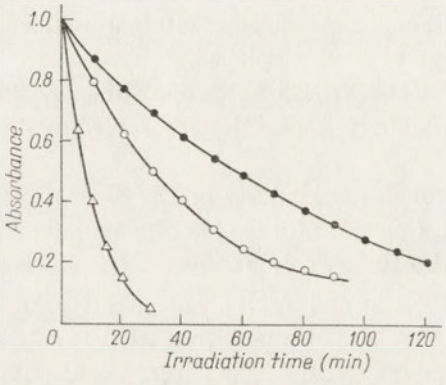


Fig. 1

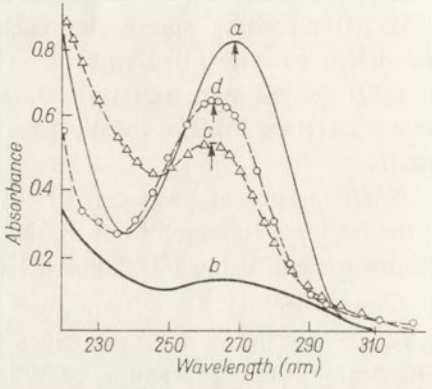


Fig. 2

Fig. 1. Course of photolysis in neutral aqueous medium, on irradiation at 254 nm (light source B) of UR (△), PUR (○) and iPUR (●) at 10^{-4} M concentration. The decrease in absorption was measured at 262 nm for UR and at 268 nm for PUR and iPUR.

Fig. 2. Changes in absorption spectrum of iPUR (10^{-4} M) due to photochemical transformation in neutral aqueous medium. (*a*), Spectrum prior to irradiation; (*b*), following 120 min irradiation (light source B); (*c*), following addition to the irradiated solution of concn. NH_4OH to pH 11.6; (*d*), following neutralization of the alkaline solution to pH 7. Arrows indicate locations of absorption maxima. *Note*: It will be seen that curves *c* and *d* correspond to the absorption spectra of UR in alkaline and neutral media, respectively. Analogous results were obtained with PUR.

respectively, as compared to 21.6×10^{-3} for hydration of uridine. Furthermore the rates of photochemical transformation of both alkylated nucleosides were unaltered when the concentrations were increased 10-fold (to 10^{-3} M), and when air (oxygen) was excluded from the solutions by flushing nitrogen through during irradiation.

Alkalization of the irradiated solutions led to the appearance of the characteristic absorption maxima (Fig. 2), this time at 262 nm for both nucleosides, as compared to the 268 nm maxima for the initial compounds. Chromatography of the solutions with several different solvent systems (Table 1), and absorption spectra of the eluates at different pH values, demonstrated unequivocally that the

alkali-generated substance was uridine in both cases. Consequently irradiation of both PUR and iPUR at 254 nm leads to dealkylation in fairly high yield as previously observed for 5-ethyluridine and 5-ethyldeoxyuridine (Pietrzykowska& Shugar, 1968, 1970).

Closer examination demonstrated that the alkali-induced dark regeneration of the absorption spectra occurred *via* an intermediate with an absorption maximum at 290 nm (Fig. 3), a process strikingly similar to that described for alkaline trans-

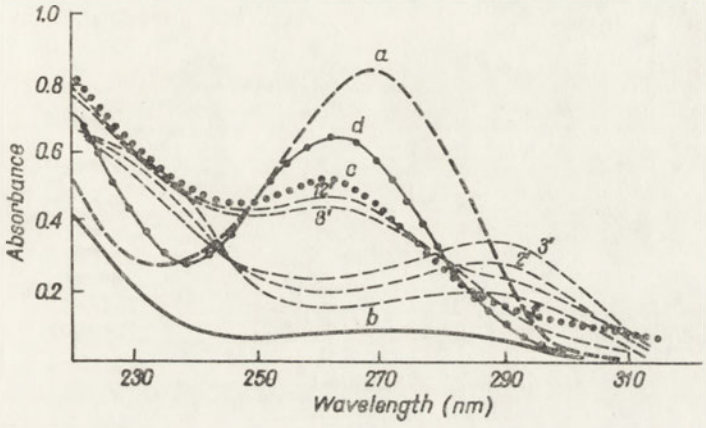


Fig. 3. Course of dark transformation in alkaline medium of PUR photoproduct(s); (*a*), initial PUR absorption spectrum at pH 7; (*b*), spectrum following irradiation at 254 nm for 90 min with light source B; 1′ - 12′ dashed lines show changes in spectrum with time (min) following alkalization of irradiated solution to pH about 11.6 with conc. NH_4OH; (*c*), final stabilized spectrum in alkaline medium; (*d*), spectrum following neutralization of alkaline solution. *Note*: Curves *c* and *d* correspond to absorption spectrum of UR in alkaline and neutral media, respectively. A completely analogous picture was observed with irradiated iPUR.

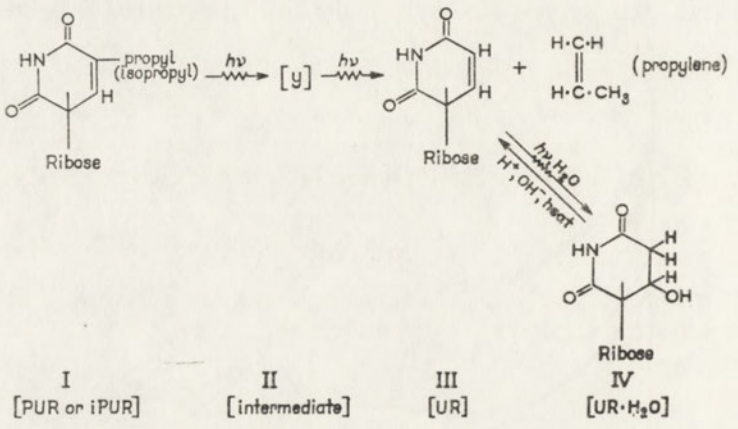
formation of the uridine photohydrate monoanion to uridine monoanion (Fikus & Shugar, 1966). This constitutes additional evidence that the products of photodealkylation of PUR and iPUR are uridine in both instances.

Following 40 - 50% photolysis of PUR and iPUR (as measured by the decrease in optical density at the absorption maxima), the photoproducts were found to be uridine and uridine hydrate ($UR \cdot H_2O$), in the ratio of about 1:1. On extensive photolysis (80 - 90%), UR and $UR \cdot H_2O$ were found in a ratio of about 1:3. Bearing in mind that $UR \cdot H_2O$ is stable to irradiation, it follows that the product of photodealkylation must be UR, which subsequently undergoes photohydration. This behaviour is again similar to that previously observed for the photochemical transformation of EtUdR at 254 nm (Pietrzykowska & Shugar, 1970).

Gas chromatography was now applied to determine the fate of the 5-propyl and 5-isopropyl side chains. Examination of *freshly* irradiated solutions revealed propylene as the only volatile product of irradiation for both PUR and iPUR, as well as of 5-propyluracil and 5-isopropyluracil. Storage of the irradiated solutions

prior to gas chromatography led to gradual disappearance of propylene and to the appearance, in the presence of oxygen, mainly of aldehydes (acetic, formic); and, in the absence of oxygen, of acetone and alcohols (ethyl, isopropyl).

The foregoing results suggested the following pathway for the photochemical transformation of both PUR and iPUR to UR, illustrated in Scheme 1.



Scheme 1

The problem now was to identify the nature of the presumed intermediate indicated in Scheme 1. In the case of EtUdR, the amount of starting material available made it possible to isolate such an intermediate and to show that it was a 5,6-di-

Table 1

R_F *values of products identified chromatographically following more than 80% photolysis of* 10^{-4} M *neutral aqueous solutions of PUR and iPUR at 254 nm*

Data in parentheses are for standard compounds.

Solvent system	R_F values					
	PUR	iPUR	Photoproducts			
			UR	UR·H_2O[a]	X[b]	Y[c]
	Eastman cellulose 6065 sheets					
A	0.70 (0.71)	—	0.28 (0.27)	0.17	0.37	0.59
	—	0.74 (0.72)	0.27 (0.27)	0.17	0.38	0.60
B	0.80 (0.78)	—	0.37 (0.37)	—	0.49	0.74
	—	0.82 (0.81)	0.38 (0.37)	—	0.50	0.72
	Eastman silica gel 6060 sheets					
E	—	0.51 (0.52)	0.27 (0.27)	0.15	0.36	0.45

[a] Spots revealed following exposure of the developed chromatograms to ammonia vapour.
[b] Present in only trace quantites.
[c] Spots revealed following irradiation at 254 nm of the developed chromatograms.

hydropyrimidine nucleoside analogue which retained the 5-ethyl substituent, and eliminated ethylene photochemically to give UdR (Pietrzykowska & Shugar, 1970).

The quantities of PUR and iPUR at our disposal were insufficient for this purpose. It did, however, prove possible to demonstrate chromatographically in irradiated PUR and iPUR aqueous solutions the presence, apart from UR and UR·H_2O, of two additional photoproducts, which we refer to as X and Y (Table 1). Photoproduct X appeared in only trace amounts, and was not further examined. But Y, which made its appearance from the start of irradiation, appeared to satisfy the requirements of the sought-for intermediate. The product eluted from chromatograms exhibited only end absorption in neutral medium in the quartz ultraviolet, an absorption maximum at about 240 nm in alkaline medium, while irradiation at 254 nm led to its conversion to a product with a λ_{max} at 262 nm at neutral pH, indicative of the identity of this product with uridine.

Preparation of intermediates (propylene adducts)

From the foregoing, it appeared most likely that the intermediate in Scheme 1 was a propylene adduct of uridine. Since ethylene adduct of deoxyuridine had already been prepared on a larger scale by the photochemical addition of ethylene to UdR (Pietrzykowska & Shugar, 1970), it was decided to attempt the preparation of the presumed intermediate by photochemical addition of propylene to UR, with a view to comparing its properties with the presumed intermediate Y indicated in Scheme 1. The results are presented in Table 2.

Table 2

R_F *values of photoproducts detected chromatographically following irradiation at 254 nm of* 10^{-4} - 10^{-3} M-*uridine solutions in propylene-saturated water*, pH *7*

Solvent system	R_F values			
	UR	UR·H_2O[a]	photoproduct X[b]	photoproduct Y[c]
	Eastman cellulose 6065 sheets			
A	0.27	0.17	0.40	0.58
B	0.38	—	0.60	0.75
	Eastman silica gel 6060 sheets			
E	0.26	0.13	0.37	0.46

[a] Spots revealed following exposure of the developed chromatograms to ammonia vapours.
[b] Present only in trace amounts.
[c] Spots revealed following irradiation of the developed chromatograms at 254 nm.

Initial trials involved irradiation with light sources A or B of uridine, 1-methyluracil or thymidine (10^{-4} to 10^{-3} M) aqueous solutions saturated with propylene in 10-mm or 1-mm cuvettes. It was immediately noted that, under these conditions, the starting compounds disappeared at a faster rate than if irradiation were con-

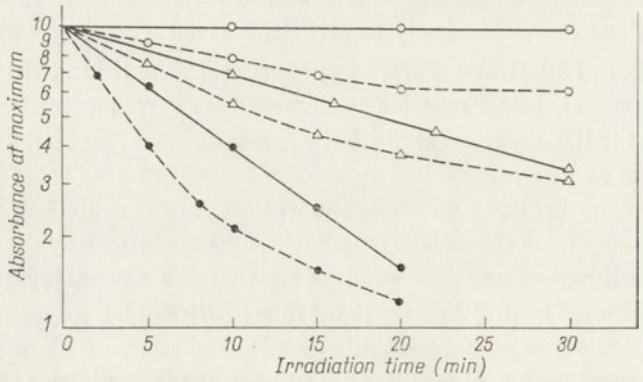


Fig. 4. Course of photochemical transformation at 254 nm (light source B) in neutral aqueous medium (——), and in aqueous medium saturated with propylene (— — —) of 10^{-4} M solutions of UR (●), TdR (○) and 1MeU (△).

ducted in the absence of propylene (Fig. 4). If we assume, as seems reasonable, that in the presence of the latter we are dealing with both hydration and propylene addition, then the quantum yield for the latter may be calculated as follows:

$$\Phi \text{ (addition)} = \Phi \text{ (hydration+addition)} - \Phi \text{ (hydration)}$$

Table 3 presents values for the addition reaction calculated in this way; the quantum yield for the addition reaction to thymidine, which does not undergo hydration, is also included in the Table.

Table 3

Initial quantum yields for propylene photoaddition at 254 nm as compared with quantum yields for photohydration

See text for details.

Compound	$\Phi_{addition}$ ($\times 10^3$)	$\Phi_{hydration}$[a] ($\times 10^3$)
Uridine	26.1	21.6
Thymidine	8.8	0.6[b]
1-Methyluracil	8.2	12.2

[a] Values from McLaren & Shugar (1964).

[b] This is for degradation; thymidine does not form stable photohydrates under these conditions.

For preparative purposes several hundred mg (10^{-3} M solutions) of uridine, 1-methyluracil and thymidine were irradiated at 254 nm in a specially constructed reactor (Fikus, Wierzchowski & Shugar, 1965), with continuous bubbling of propylene through the solution. The course of the reaction was followed by removal of aliquots at various time intervals and determination of the optical density following 10-fold dilution. The reactions were terminated after an approximately 60% de-

crease in absorption for uridine, 40% for 1-methyluracil and 30% for thymidine. In the latter case, the reaction appeared to come to an equilibrium at this point, analogous to that found on irradiation of thymidine in the presence of ethylene at about 35% photolysis (Z. Zarębska, personal information) and in some respects analogous to the equilibrium normally attained between photodimerization and photodissociation of pyrimidines.

The reaction mixtures were evaporated to dryness under reduced pressure at 40°C and the residues taken up in hot water. The uridine-propylene adduct, poorly soluble in water, was isolated by stepwise crystallization from this medium. In this manner several different preparations in crystalline form were obtained, which exhibited clear differences in melting points (see Table 5), but showed the same elementary analysis, and similar U.V. spectra and photochemical behaviour (photodissociation, see below). There is little doubt but that the crude preparations include a mixture of stereoisomers.

Thymidine and 1-methyluracil adducts, which proved to be more soluble in water and ethanol than the starting compounds were isolated by ascending paper chromatography. Whatman 3*MM* paper, pre-washed with the solvent system and then with water, was employed with the systems *A* or *B*. The spots were eluted with water, the eluates brought to dryness under reduced pressure and the residues dissolved in a minimal quantity of hot ethanol. Addition of hexane in the cold led to slow crystallization of 1MeU·P. Attempts to induce crystallization of the thymidine adduct were unsuccessful.

Properties of propylene adducts

Elementary analysis. The propylene adduct of uridine, probably a mixture of several isomers, gave the following: Experimental: C, 49.54%; H, 6.46%; N, 9.60%. Calculated (for 1:1 adduct): C, 50.3%; H, 6.34%; N, 9.79%.

Chromatographic mobility. On silica gel and cellulose the propylene adducts exhibited a higher mobility than the parent compounds with polar solvent systems (Table 4); under the same conditions the corresponding UR and 1MeU hydrates

Table 4

R_F *values of propylene adducts as compared to* R_F *values of UR, TdR and 1MeU (in parentheses) in several solvent systems*

Solvents *A*, *B* and *C* were employed with Eastman cellulose 6065 TLC sheets, and solvents *D*, *E*, *F*, *G* with Eastman silica gel 6060 TLC sheets.

Compound	R_F values						
	A	*B*	*C*	*D*	*E*	*F*	*G*
UR·P	0.57 (0.27)	0.73 (0.38)	0.68 0.55 (0.80)	0.36 (0.25)	0.45 (0.28)	0.56 (0.48)	—
TdR·P	0.83 (0.63)	0.88 (0.70)	0.55 (0.60)	0.52 (0.46)	0.63 (0.54)	0.74 (0.66)	—
1MeU·P	0.80 (0.48)	—	0.50 (0.63)	0.74 (0.59)	0.78 (0.70)	0.68 (0.57)	0.74 (0.66)

possess the same or somewhat lower R_F values than the parent compounds. As regards the UR·P preparations crystallized from water, despite their different melting points, they all exhibited identical mobilities on silica gel and cellulose TLC plates with a variety of different solvent systems employed, both polar and non-polar, commonly used for photodimer separations (Zarębska & Shugar, 1971).

Stability in acid. All the adducts were reasonably stable in acid even at elevated temperatures. Exposure to 1 N-HCl for several days at room temperature led to a 10% decrease in absorption, at 45°C the decrease was about 15 - 18%. At 100°C in 1 N-HCl, no significant cleavage of the glycosidic linkage was observed after 1 hour.

U.V. spectra and pK values. The absorption spectra of all three adducts, constituting mixtures of isomers, are shown in Fig. 5, and the appropriate spectral

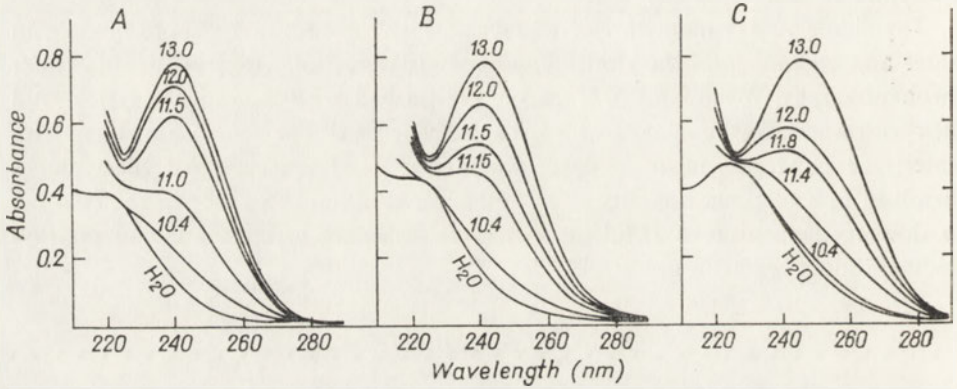


Fig. 5. Absorption spectra of propylene adducts at various pH values, as indicated beside each curve: (*A*), UR·P; (*B*), TdR·P; (*C*), 1MeU·P. Curves for pH 13 were essentially unaltered on raising pH to 14.

constants in Table 5. It will be seen that in neutral medium they all exhibit end absorption in the quartz ultraviolet, and maxima in alkaline media at about 240 nm, which become well-defined with increasing pH, as prevails for 2,4-diketopyrimidines and their glycosides following saturation of the 5,6 bond (Janion & Shugar, 1960). The change in absorbance with pH is due to dissociation of the ring $N_{(3)}$ hydrogen, and spectral titrations (Fig. 6) gave the appropriate p*K* values, listed in Table 5. It should be observed that these values, which fall in the pH range 11 - 12, are close to those for the corresponding 2,4-diketo-5,6-dihydropyrimidine derivatives (Janion & Shugar, 1960) as in the case of 1MeU·P. Somewhat lower values were found for the UR·P and TdR·P adducts, as observed for the corresponding 5,6-dihydropyrimidines (Pojarlieff, Blagoeva & Kurtev, 1969), and in accordance with what might have been anticipated for nucleosides (Fox & Shugar, 1952).

Alkaline lability. In alkaline medium, all the propylene adducts exhibited lability which was dependent on the OH^- concentration and varied for the different adducts (Table 5). There is little doubt but that, as in the case of dihydropyrimidines,

Table 5

Some properties of propylene adducts

Compound	Spectral data				M.p. (°C)	pK_a	$t_{1/2}$ for decomposition in alkali (KOH) at 20°C			Colour reaction with *p*-dimethylaminobenzaldehyde at room temp.		Ring closure in 0.1 - 1 N-HCl (following alkaline hydrolysis)
	H_2O		0.1 N-KOH							before	after	
	λ_{max}	ε_{max} ($\times 10^{-3}$)	λ_{max}	ε_{max} ($\times 10^{-3}$)			pH 14	pH 13	pH 12	alkali treatment (1 N-NaOH)		
UR·P	220 (inflexion)	4.0	240-242	8.0	259-261 255-257 249-252 245-247 232-234	11.2 11.1	~ 9 min[a]	~40 min[a]	5.5 h	no	yellow (after 10-15 min)	80% (after 2 h at room temp.)
TdR·P	215-220 (shoulder)	[b]	240	[b]	[b]	11.4	~14 h	~67 h	—	no	yellow (after several hours)	80% (after 10 min at room temp.)
1MeU·P	223-225	3.0	245-246	5.0	132-4	11.8	~30 min	~ 7.5 h	178 h	no	yellow (after several hours)	100% (after 15-20 min at room temp.)

[a] Data for the labile fraction, details in text.

[b] Not determined because product not crystalline.

this lability is due to opening of the 3,4 bond of the pyrimidine ring to give the corresponding β-ureido derivatives, as shown by the following observations: (a) all the adducts, following treatment with alkali, gave a positive yellow reaction with *p*-

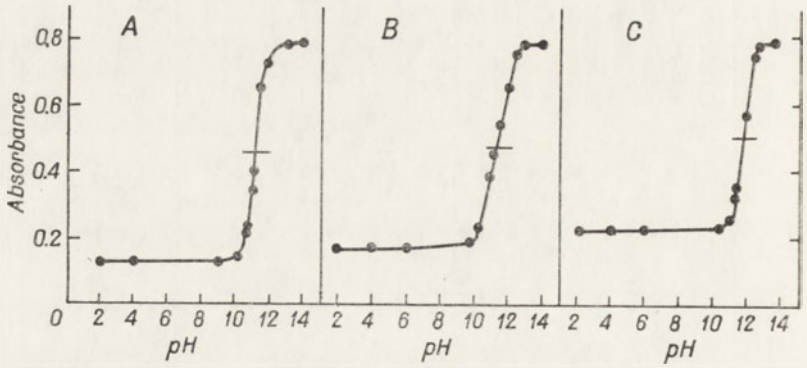


Fig. 6. Spectrophotometric titration of propylene adducts: (*A*), UR·P; (*B*), TdR·P; (*C*), 1MeU·P. Absorbance as function of pH measured at λ_{max} in each case, at 240 nm for *A* and *B*, and at 245 nm for *C*. p*K* values were: 11.2, 11.4 and 11.8, respectively.

-dimethylaminobenzaldehyde (Fink, Cline, Mc Gaughey & Fink, 1956), (b) following alkaline "decomposition", regeneration of the adducts resulted from subsequent treatment with acid, revealed both by regeneration of the absorption spectra, as well as the ability to undergo photodissociation with elimination of propylene and regeneration of the parent pyrimidine or nucleoside (see below). Attention is drawn to the wide variation in alkaline stabilities of the various adducts (Table 5). In particular the much higher stability of the TdR·P, as compared to UR·P, adduct is in agreement with the observed stabilizing effect of a 5-methyl substituent on 5,6-dihydropyrimidines and their nucleosides (Janion & Shugar, 1960; Janion, 1964; Pojarlieff *et al.*, 1969).

It was further noted that all UR·P preparations underwent only partial degradation at pH 13 - 14, approximately 40 - 60%, as measured by the decrease in the U.V. absorption maximum. For a given crystalline preparation, the degree of alkaline lability was constant. By contrast, the rate constants for alkaline degradation of the remaining 60 - 40% of a given preparation were at least two orders of magnitude lower. It is most likely that this partial lability in alkali further reflects the presence of stereoisomers with differing alkaline stabilities and, in retrospect, it is, perhaps, unfortunate that this property was not made use of either for the separation or additional characterization of such isomers. This is being taken into account in further studies. One additional finding was the observation that, e.g., a sample, which underwent 45% hydrolysis at pH 13 - 14, was hydrolysed to the extent of more than 80% at pH 12, as shown in Fig. 7, but this is possibly not too surprising in the light of the reports of Pojarlieff *et al.* (1969) and Sander (1969) on the complex multistep character of the alkaline hydrolysis of some dihydropyrimidines.

In contrast to UR·P, the 1:1 adduct of propylene and 1-methyluracil, i.e. 1MeU·P, readily underwent degradation in alkaline medium to the extent of almost 100%, a reaction which was fully reversible in acid medium (see below).

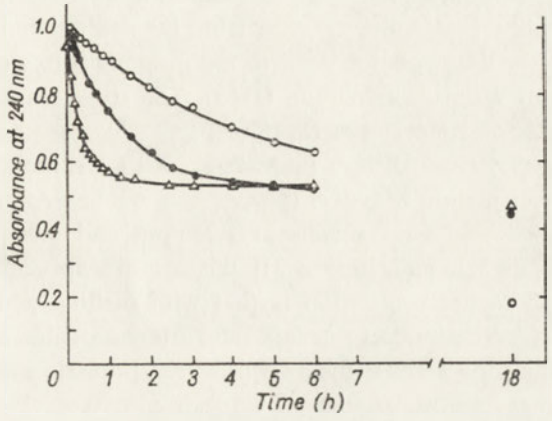


Fig. 7. Course of alkaline hydrolysis of UR·P as function of hydroxyl ion concentration, followed by decrease in absorbance at 240 nm; (○), 0.01 N-KOH (pH 12); (●), 0.1 N-KOH (pH 13); (△) 1.0 N-KOH (pH 14).

ORD spectra. All of the preparations of 1:1 adducts of uridine and propylene, with the exception of one, exhibited the optical rotatory dispersion spectrum *B* in Fig. 8, showing a positive Cotton effect with a broad band over the range 258 -

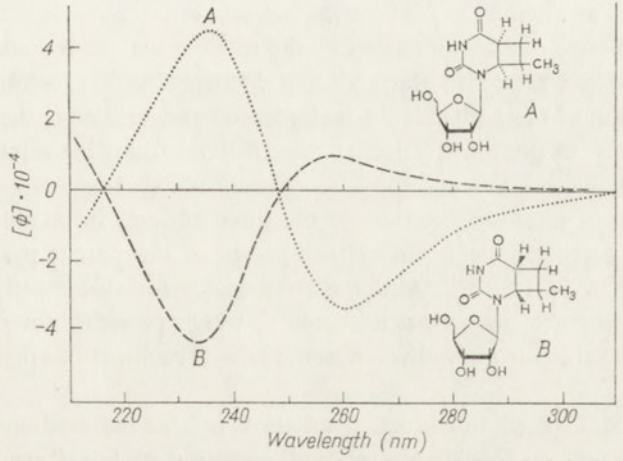


Fig. 8. ORD spectra of 1:1 adducts of uridine and propylene in neutral aqueous medium at room temperature. Curve *B* represents the spectrum obtained with all sample preparations but one, and corresponds to the spectrum of the β-form of 5,6-cyclopropyluridine reported by Kunieda & Witkop (1969). Curve *A* is the spectrum for only one sample adduct, and probably corresponds to the α-form of 5,6-cyclopropyluridine, but it was thermally unstable.

- 260 nm and a pronounced, relatively symmetrical, trough at 232 - 233 nm. The molecular rotation of the positive peak at about 259 nm is about 20% that of the trough at 232 nm. One of the preparations (*A*, Fig. 8) exhibited a negative Cotton effect with a trough at 259 - 260 nm and a pronounced positive peak at 232 - 233 nm; however, this preparation, which is undoubtedly an isomeric form of the other, proved to be unstable. It is, however, clear from the spectra that the two principal isomeric forms of the adduct correspond to the isomeric forms of 5,6-cyclopropyluridines reported by Kunieda & Witkop (1969). The spectrum of *B* would correspond to the *β*-form of 5,6-cyclopropyluridine.

NMR spectroscopy. One fraction (m.p. 259° - 261°C) exhibited a CH_3 doublet at 1.23 ppm due to coupling with H-8 ($J_{CH3,\ H-8}$ = 6.8 hz). Another fraction, with m.p. 249 - 252°C, showed CH_3 signals at 1.23 ppm and 1.06 ppm with relative ntegral intensities of 3:1, indicating a 3:1 mixture of two isomers, of which the one present in the higher proportion is that with melting point at 259 - 261°C. It would obviously be desirable to isolate the various possible isomeric forms for further NMR studies, or at least the two principal isomeric forms corresponding to the *α*- and *β*-5,6-cyclopropyluridines of Kunieda & Witkop (1969). Such attempts are under way in this laboratory.

Photodissociation of propylene adducts. As in the case of pyrimidine photodimers, and as previously observed for the ethylene adducts of deoxyuridine (Pietrzykowska & Shugar, 1968, 1970), irradiation of the propylene adducts in aqueous medium at 254 nm leads to photodissociation with concomitant liberation of propylene as the only volatile product (demonstrated by gas chromatography), and the parent pyrimidine (or pyrimidine nucleoside), the identity of which was verified by paper and/or TLC chromatography and by spectral methods.

Adduct photodissociation occurs in acid, neutral and alkaline media, the rate being maximal at alkaline pH where the absorption at 254 nm is considerably increased (see Fig. 5) due to formation of the monoanions of the adducts. An additional contributing factor to the apparent increased rate of photodissociation in alkaline medium of the adducts of 1-methyluracil and uridine is the absence, under these conditions, of photohydration at the 5,6 bond following removal of the propylene moiety.

The course of photodissociation of the three adducts in alkaline medium, followed by the rate of appearance of the spectra of the parent pyrimidine ring, is exhibited in Fig. 9. The estimated quantum yields for this reaction are virtually identical for all three, as shown in Table 6, which presents, for comparison, the corresponding value for photodissociation of the 1:1 adduct of ethylene and deoxyuridine.

For the 1MeU·P photoadduct, irradiation in alkaline medium led to quantitative regeneration of 1MeU. The photodissociation of UR·P was somewhat less quantitative, varying in different experiments from 85 to 90%, due in part to the formation of a minor unidentified photoproduct, as well as to slight degradation of UR·P itself at alkaline pH during irradiation.

For all three adducts embraced in this study, it was found that alkaline degrada-

tion led to a decrease in the irradiation-induced regeneration of the parent pyrimidine ring. This decrease was approximately equal to the amount of prior alkali-

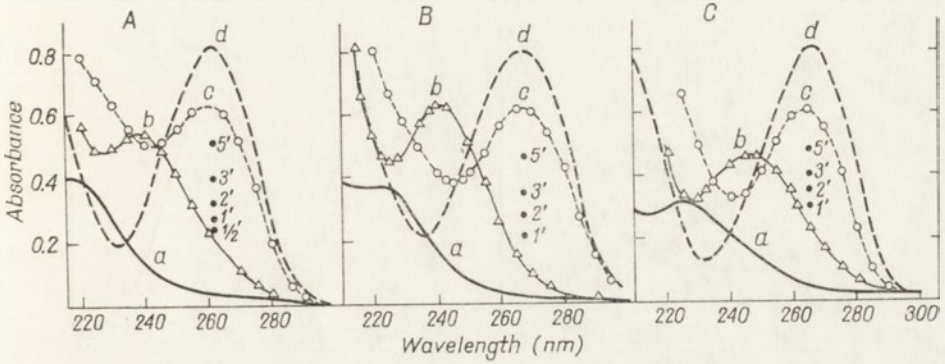


Fig. 9. Photochemical dissociation of propylene adducts: (A), UR·P; (B), TdR·P; (C), 1MeU·P, to parent compounds. Absorption spectra of adducts; (*a*), at neutral pH; (*b*), at alkaline pH; (*c*), spectra following irradiation of alkaline adduct solutions at 254 nm (light source A); figures refer to the appearance of these maxima with time of irradiation (min); (*d*), spectra resulting from neutralization of irradiated alkaline solutions. *Note*: In each case curves *c* and *d* correspond to absorption spectra at alkaline and neutral pH of the parent compounds.

Table 6

Initial quantum yields, Φ, for photodissociation of propylene adducts at 254 nm in neutral and alkaline media

Initial absorbancies of neutral solutions at λ_{max} for the adducts were about 1; and for alkaline solutions about 0.5. Measurements were based on the appearance of the λ_{max} of the parent pyrimidine or pyrimidine nucleoside after about 1 min irradiation with source A. Values are averages of 4 - 5 measurements; those measured at pH 7 are subject to about ±10% error, due to low absorption at 254 nm.

Adduct	$\Phi_{dissociation}$	
	pH 7	pH 12
UR·P	0.20[a]	0.20[a]
TdR·P	0.19	0.20
1MeU·P	0.23	0.23
Deoxyuridine : ethylene	—	0.30[b]

[a] Values ranged from 0.18 to 0.22 for different preparations.
[b] Taken from Pietrzykowska & Shugar (1970).

-induced ring opening, and is illustrated for UR·P in Fig. 10. This was studied somewhat more extensively in the case of 1MeU·P where, e.g., following 10% alkaline hydrolysis of the adduct, the extent of photodissociation was 92%, whereas for 47% ring opening, 54% regeneration of 1MeU was observed. Furthermore, as pointed out above, the alkali-catalysed ring opening could be reversed in acid me-

dium; for 1MeU·P, ring closure in 0.1 N-HCl at room temperature proceeded rapidly, being complete in 15 - 20 min, following which irradiation in alkaline medium again led to quantitative regeneration of 1MeU (See Fig. 11 for details).

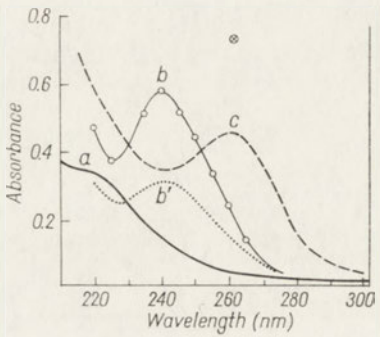


Fig. 10

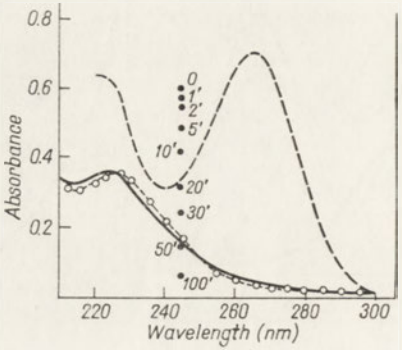


Fig. 11

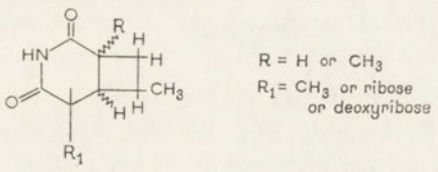
Fig. 10. Photochemical conversion of partially alkali-degraded UR·P to UR. Absorption spectrum of UR·P: (*a*), at neutral pH; (*b*), immediately after addition of KOH to pH 13; (*b'*), after exposure to pH 13 for 26 h at room temperature; note that absorbance at λ_{max} has decreased about 50%; (*c*), UR spectrum following irradiation at 254 nm (light source A) of partially alkali-degraded UR·P; ⊗ indicates the maximal absorbance attained on irradiation of UR·P solution immediately following addition of KOH, hence prior to alkaline degradation.

Fig. 11. Photochemical conversion of 1MeU·P to 1MeU following alkali degradation of the former, followed by acid-catalysed ring closure: (——), Absorption spectrum of 1MeU·P at neutral pH. Points 0 - 100′ (min): KOH was added to attain pH about 14, leading to the appearance of a new maximum at 245 nm; absorbance at this wavelength decreased with time, as indicated. When, at 100 min, the absorbance had decreased to about 10%, HCl was added to a pH about 1, and the solution was left at this pH for 20 min at room temperature. (o - o - o), Absorption spectrum of acidified alkali-degraded solution; note that it is almost identical with the initial spectrum. (— — —), Absorption spectrum of the acid-treated solution following alkalization to pH 12 and irradiation at 254 nm, leading to the appearance of the alkaline absorption spectrum of 1MeU, practically identical with that resulting from irradiation of initial, untreated, 1MeU·P.

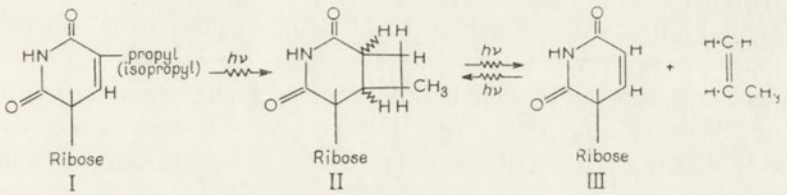
DISCUSSION

The foregoing data are all in accord with the structure presented in Scheme 2 for the various propylene adducts, as well as for the intermediates in the photochemical dealkylation of the 5-alkyluracil glycosides, i.e. photochemical dealkylation of 5-propyl and 5-isopropyl uridines involves two steps: formation of the intramolecular cycloaddition product, followed by photodissociation of the latter to propylene and uridine, as illustrated in Scheme 3. The overall results are fully analogous to those previously reported for 5-ethyluracil and its nucleosides irradiated at 254 nm (Pietrzykowska & Shugar, 1970), and demonstrated that the photochemical dealkylation of 5-alkyluracils, and their glycosides, is a fairly general reaction. This is further supported by the observation that 5-hexyluridine undergoes

dealkylation, with simultaneous elimination of 1-hexene (E Sztumpf-Kulikowska & D. Shugar, in preparation).



Scheme 2



Scheme 3

The ability of thymidine to form a 1:1 adduct with propylene, as well as with ethylene (Z. Zarębska, in preparation) suggests furthermore that a wide variety of such adducts may probably be obtained in this way. The fact that they are all analogues of 2,4-diketo-5,6-dihydropyrimidines (and dihydropyrimidine nucleosides) also suggests that they may well be of biological interest; it is hoped to prepare some of these on a sufficient scale to study their potential antimetabolic properties.

The differing melting points of the various preparations of the propylene adducts; the NMR spectra; and the ORD results, when compared to those of Kunieda &Wikop (1969) for the corresponding 5,6-cyclopropyluridines, all testify to the formation of several stereoisomers of the adducts. It is obvious from Scheme 2 that the propylene adducts of UR may include a mixture of as many as 8 stereoisomers; although it is likely that fewer are actually formed, due to stereospecificity of the addition reaction. It would obviously be even more desirable to isolate the intermediate formed during the photochemical dealkylation of the 5-alkyluracils, since in these cases the number of isomers formed, when the 5-alkyl substituent is higher than ethyl (e.g. propyl), should be only one-half the possible number formed in the case of adduct formation. However, the relatively low quantum yields for photodealkylation suggest that this may prove too formidable an undertaking.

Wavelength dependence of photoproduct formation. There are numerous illustrations in the literature of formation of different photoproducts on varying the irradiation wavelength. In some instances this has been ascribed to the formation of different excited states at different wavelengths, a subject extensively reviewed by Ullman (1968). We have previously discussed this effect in relation to the photochemical transformation of 5-ethyldeoxyuridine which, like the higher 5-alkyl nucleosides embraced in this study, undergoes dealkylation on irradiation at 254 nm,

but predominantly photodimerization at wavelengths to the red of 265 nm. It was shown, however, that the differences in photoproduct formation were due, not necessarily to differences in excited states at the two wavelengths, but to differences in behaviour of the primary photoproducts at the different wavelengths (Pietrzykowska & Shugar, 1970).

The 5-propyl and 5-isopropyl uracil nucleosides examined in this investigation differ from the 5-ethyl nucleosides in that, under these conditions, they apparently do not photodimerize at the longer wavelengths, even in the absence of oxygen. Instead they undergo only dealkylation, but in much lower yield. The failure to detect photodimerization at the longer wavelengths may, conceivably, be due to steric hindrance of the bulkier 5-propyl and 5-isopropyl groups. Attempts to isolate photodimers of 1-methyl-5-propyluracil and 5-isopropyldeoxyuridine irradiated in an ice matrix have provided contradictory results (see below).

It was previously shown that EtUdR undergoes photodimerization in an ice matrix at about the same rate as TdR, if the extent of photolysis does not exceed about 35%. Additional irradiation led to formation in both instances of unidentified products, other than photodimers, since they were not susceptible to photodissociation to monomers following thawing of the medium (Pietrzykowska & Shugar, 1970). Photodimerization of EtUdR in frozen medium has also been observed by Gauri, Pflughaupt & Müller (1969).

It was initially reported by Gauri (1967) that 1-methyl-5-propyluracil readily dimerizes in frozen medium, although no data were furnished as regards dimer photodissociation following thawing of the medium (Gauri *et al.*, 1969). Subsequently Gauri, Rüger & Wacker (1971) found that the U.V. absorption of PUdR, irradiated in an ice matrix, decreased at the same rate as for EtUdR. However, subsequent irradiation of the thawed medium showed no photodissociation of the presumed PUdR photoproduct, indicating that some reactions other than photodimerization had occurred in frozen medium.

The photochemical transformations of the 5-alkyluracil glycosides in aqueous medium do not necessarily reflect their behaviour in polynucleotide chains, where the mutual orientation of neighbouring residues is involved.

Photodissociation of adducts. It is of interest that the quantum yields for adduct photodissociation are similar in neutral and alkaline media (Table 6), although it must be conceded that the values obtained in neutral medium are somewhat less accurate due to the low end absorption of the adducts in the neutral form (see Fig. 5). A similar result prevails for photodimer photodissociation (Sztumpf & Shugar, 1962). It should likewise be noted that the photodissociation quantum yields for the 1:1 adducts (0.2-0.3) are approximately one-third to one-half those for the corresponding photodimers (Sztumpf & Shugar, 1962) which contain two pyrimidine rings, a result which might have been anticipated. Furthermore, the difficulty of isolating the 1:1 adducts on a large scale from irradiated solutions of 5-alkyluracils can be seen to be due to the fact that the quantum yield for photodissociation of the adducts is 10 - 20 fold greater than for their formation, at least at 254 nm (Table 3).

The propylene adducts of the uracil (and thymine) nucleosides described in this investigation, as well as in a previous one (Pietrzykowska & Shugar, 1970), are clearly analogues of pyrimidine and pyrimidine nucleoside photodimers. As such, they may well prove of value in further studies on the properties of these photodimers which are of such great interest in investigations on the genetic effects of U.V. radiation and, particularly, in relation to enzymic repair processes. It is also possible that such adducts may be formed by irradiation of synthetic or natural nucleic acids in the presence of propylene (or ethylene). Such polynucleotides containing propylene or ethylene adducts of pyrimidine residues may provide interesting new model systems for studies on the mechanisms of enzymic repair systems.

Finally, attention should be drawn to the fact that all the adducts described herein, as well as previously (Pietrzykowska & Shugar, 1970) are likewise analogues of the corresponding 5,6-dihydropyrimidines and their nucleosides. As such they may well exhibit properties of interest in some biological and enzymic systems.

We should like to thank Mrs. K. Myszkowska for technical assistance in some of the experiments. This investigation was supported by the Polish Academy of Sciences (Project 09.3.1) and profited also from the support of the World Health Organization, The Wellcome Trust, and the Agricultural Research Service, U.S. Dept. of Agriculture.

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5,6-DWUHYDRO-5,6-CYKLOBUTANYLOURACYL I JEGO NUKLEOZYDY: ZWIĄZKI POŚREDNIE PODCZAS FOTOCHEMICZNEJ DEALKILACJI 5-PROPYLO- I 5-IZOPROPYLOURACYLU ORAZ ICH NUKLEOZYDÓW

Streszczenie

1. Naświetlanie przy 254 nm obojętnych wodnych roztworów 5-propylourydyny i 5-izopropylourydyny — otrzymanych enzymatycznie z odpowiednich zasad — powoduje fotochemiczną dealkilację i wytwarzanie, z dużą wydajnością w obu przypadkach, urydyny i propylenu.

2. W pierwszym etapie zachodzi wewnątrzcząsteczkowa cyklizacja grupy 5-alkilowej wokół wiązania 5,6 z wytworzeniem pierścienia cyklobutanowego i utworzeniem szeregu form stereoizomerycznych 5,6-dwuhydro-5,6-cyklobutanylourydyny. Związki te ulegają następnie fotodysocjacji, w wyniku której powstaje urydyna i uwalniany jest propylen. Fotodysocjacja przebiega z dużą wydajnością (0.18 - 0.25), która stanowi 1/3 do 1/2 wydajności fotodysocjacji dimerów.

3. Obecność tlenu nie ma wpływu na badane przemiany fotochemiczne. Reakcja ta zachodzi również — lecz ze znacznie mniejszą wydajnością kwantową — pod wpływem promieniowania dłuższego niż 265 nm. W przeciwieństwie do 5-etylodezoksyurydyny, która tworzy dimery pod wpływem naświetlania w tych warunkach, 5-propylourydyna i 5-izopropylourydyna nie tworzą dimerów nawet w warunkach beztlenowych.

4. Pochodne 5,6-dwuhydro-5,6-cyklobutanylouracylu, stanowiące serię nowych analogów, w tym też nukleozydów 5,6-dwuhydropirymidyn, które mogą mieć znaczenie biologiczne, można otrzymać na większą skalę w wyniku fotochemicznej addycji propylenu do 1-metylouracylu lub nukleozydów uracylu. Otrzymano szereg takich związków i opisano pewne ich własności.

5. Stwierdzono również fotochemiczną addycję propylenu do tymidyny, co wskazuje, że zjawisko to ma charakter ogólny.

6. Przedyskutowano mechanizmy reakcji fotochemicznych i ewentualne zastosowania biologiczne fotoadduktów, które są możliwie najprostszymi analogami fotodimerów pirymidynowych.

Received 17 February, 1972.

ACTA BIOCHIMICA POLONICA
Vol. 19 1972 No. 3

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ASSAY FOR THE INTACTNESS OF THE OUTER MEMBRANE IN ISOLATED MITOCHONDRIA

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The degree of damage of the outer membrane in isolated mitochondria can be estimated by measuring the rate of oxidation of external reduced cytochrome *c*. The assay is based on previous observations that the outer mitochondrial membrane is impermeable to cytochrome *c*. Experimental conditions of the assay are described and its application to various mitochondrial preparations is presented.

The role of the outer mitochondrial membrane is not yet fully understood. Due to its impermeability to compounds of high molecular weight (Pfaff, Klingenberg, Ritt & Vogell, 1968), one of its possible functions is to maintain certain enzymes, as adenylate kinase (Sottocasa, Kuylenstierna, Ernster & Bergstrand, 1967; Schnaitman & Greenawalt, 1968; Silva Lima, Nachbaur & Vignais, 1968) and nucleoside diphosphokinase (Silva Lima *et al.*, 1968), close to the inner membrane. In earlier biochemical investigations on isolated mitochondria rather little attention has been paid to the outer membrane, the main interest being focused on the inner membrane and the inner compartment. Recently, however, as more and more information is accumulating on the function of the outer membrane, the knowledge of its state in mitochondrial preparations becomes more important, e.g. in discussing the exchange of phospholipids (Blok, Wirtz & Scherphof, 1971; Wojtczak, Barańska, Zborowski & Drahota, 1971) and proteins (Kadenbach, 1967), the effect of antibodies (Racker, Burstein, Loyter & Christiansen, 1970) and proteolytic enzymes (Kuylenstierna, Nicholls, Movmöller & Ernster, 1970) etc. On the other hand, the outer mitochondrial membrane is often exposed to damage, e.g. during isolation of mitochondria or as result of their swelling (Wlodawer, Parsons, Williams & Wojtczak, 1966). Therefore, a test for the intactness of the outer membrane may be very useful in several experimental approaches.

No such quantitative test has been available so far. Electron microscopy is tedious and qualitative rather than quantitative. Besides, any rupture of the outer membrane beyond the sectioning plane is not visualized. Measurement of the release of adenylate kinase, used by Blok *et al.* (1971) and ourselves (Wojtczak & Załuska, 1969), reveals only the damage of the outer membrane that occurs during experi-

mentation, but does not reflect disruption of this membrane during the isolation procedure.

The present report describes a simple, rapid and quantitative or semi-quantitative assay for the intactness of the outer membrane in isolated mitochondria, based on previously found impermeability of this membrane to cytochrome *c* (Wojtczak & Załuska, 1969; Wojtczak, Załuska & Zborowski, 1969; Wojtczak & Sottocasa, 1972).

METHODS AND MATERIALS

Mitochondria from animal tissues were isolated by procedures referred to in Table 2. Oxidation of cytochrome *c* was measured either spectrophotometrically (Smith, 1955) or by using a Clark type oxygen electrode in a system containing cytochrome *c* plus ascorbate.

For electron microscopy, mitochondrial pellet was fixed by 1% osmium tetroxide in 0.1 M-cacodylate buffer, pH 7.4, embedded in Epon and post-stained with uranyl acetate and lead citrate. Thin sections were examined in JEM-7A electron microscope (Japan Electron Optics Laboratory Co., Tokyo) at 80 kV.

Sonication of mitochondria was performed in 0.2 M-tris-HCl buffer, pH 7.5, during 2 min by means of a 60 W sonifier (M.S.E., London, England).

The chemicals used throughout were of the following origin: sucrose and EDTA from Polskie Odczynniki Chemiczne (Gliwice, Poland); ethyleneglycol-bis-(β-aminomethyl ether) *N,N'*-tetraacetic acid (EGTA), Triton X-100 and bovine serum albumin V fraction from Sigma Chemical Comp. (St. Louis, Mo., U.S.A.); ATP from Papierwerke "Waldhof-Aschaffenberg" (G.F.R.); cytochrome *c* from Biomed (Kraków, Poland); ascorbic acid from Cefarm (Katowice, Poland); sodium deoxycholate from Polfa (Jelenia Góra, Poland); Lubrol WX from I.C.I. Organics (Providence, R.I., U.S.A.), oxyethylated *tert.*-octyl phenol formaldehyde polymer (WR--1339) from Winthrop Laboratories (New York, U.S.A.); polyoxyethylene sorbitan monostearate (Tween 60) and polyethylene sorbitan monooleate (Tween 80) from Schuchardt (Munich, G.F.R.); pronase from Koch-Light Lab. Ltd. (Colnbrook, Bucks., England); digitonin from British Drug Houses (Poole, England), or from Fisher Scientific Co. (Fair Lawn, N.J., U.S.A.); the former preparation was recrystallized from ethanol before use, the latter was used without any further purification. Other chemicals were of analytical grade.

For spectrophotometric determination of cytochrome oxidase, cytochrome *c* was reduced with ascorbate as described previously (Bogucka & Wojtczak, 1966)

Protein was determined by the biuret method (Gornall, Bardawill & David, 1949).

RESULTS AND DISCUSSION

As shown previously (Wojtczak & Załuska, 1969), the reactivity of freshly prepared rat liver mitochondria with external cytochrome *c* is very low as measured

by oxidation of ferrocytochrome *c* or by reduction of ferricytochrome *c* in the presence of succinate and cyanide; this reactivity increases with increasing concentration of digitonin, which is known to detach the outer mitochondrial membrane (Lévy, Toury & André, 1967; Schnaitman, Erwin & Greenawalt, 1967; Schnaitman & Greenawalt, 1968). This has been interpreted as an indication of the impermeability of the outer mitochondrial membrane to cytochrome *c* (Wojtczak & Załuska, 1969). The maximum activity of mitochondrial cytochrome oxidase with external cytochrome *c* was found at concentrations of digitonin equal to or higher than 0.16 mg/mg mitochondrial protein. Under these conditions mitochondria were completely stripped off their outer membrane (Wojtczak & Załuska, 1969; Wojtczak *et al.*, 1969). The same maximum activity was obtained by solubilizing mitochondria with the non-ionic detergent Lubrol WX, deoxycholate, Tween 60 and Triton X-100 (Table 1). Other agents such as Tween 80, WR-1339 and sonication resulted in lower activities, apparently due to incomplete exposure of the enzyme to external cytochrome *c*, since a subsequent addition of Lubrol WX produced the maximum activation (Table 1) (for the effect of sonication cf. also Muscatello & Carafoli, 1969). It can be thus concluded that either Lubrol XW, Tween 60, Triton X-100, deoxycholate or digitonin at proper concentrations can be used to unmask completely mitochondrial cytochrome oxidase. On comparing the activity of this enzyme under these conditions (100%) with the activity of untreated mitochondrial preparations one can estimate the degree of the exposure of the inner membrane to external cytochrome *c* and hence the degree of damage of the outer mitochondrial membrane.

The dependence of the rate of cytochrome *c* oxidation by mitochondria on the concentration of added cytochrome *c* is shown in Fig. 1. With solubilized mitochondria the reaction rate attains its maximum at about 100 μM cytochrome *c* (apparent K_m about 15 μM), whereas with untreated mitochondria the oxidation rate increases linearly with increasing concentration of cytochrome *c*. The percentage of the activity of untreated mitochondria with respect to solubilized mitochondria remains essentially independent of the concentration of cytochrome *c* up to 100 μM and then slowly increases with increasing cytochrome *c* concentration. The reason for this increase is not well understood. It is not likely to be due to an increased rupture of the outer membrane at higher cytochrome *c* concentrations, since mitochondria preincubated with high concentrations of cytochrome *c* and then diluted did not show any increase in the degree of the damage of the outer membrane. The enhanced rate of oxidation may be therefore due to the increased diffusion of cytochrome *c* through the ruptures already existing in mitochondrial membrane.

The conditions of the enzymic assay should fulfill certain requirements; first of all swelling of mitochondria should be minimized. Isotonic sucrose is known to retard and 0.75 M-sucrose to prevent completely swelling (Lehninger, Ray & Schneider, 1959). Sucrose solution is, however, not optimal for measuring cytochrome oxidase since the activity of this enzyme is low at low ionic strength (Wainio & McGuinness, 1963). Therefore, either isotonic 120 mM-KCl or a mixture of 120 mM-KCl and 250 mM-sucrose (1:1, v/v) buffered with 10 - 20 mM-tris-HCl, pH 7.4,

Table 1

Effect of solubilizing and dispersing agents on the activity of mitochondrial cytochrome oxidase with external cytochrome c

Mitochondria were solubilized by the use of detergents at indicated concentrations, and 0.1 ml of the preparation containing 0.4 mg mitochondrial protein was transferred to the polarograph vessel. Sonicates, obtained as described under Methods and Materials, contained the same amount of protein.

Treatment	Oxidation of cytochrome *c*	
	ng atoms oxygen/min/mg protein	%*
None	60	8
Lubrol WX 0.2%	699	
0.5%	733	100
1.5%	712	
Deoxycholate 0.3%	731	102
Triton X-100 0.2%	420	59
0.5%	587	82
1.0%	640	89
Tween 60 0.1%	694	97
Tween 80 0.2%	360	50
0.5%	353	49
Tween 80 0.25%+Lubrol WX 1%	648	90
WR-1339 0.2%	75	10
0.5%	66	9
WR-1339 0.25%+Lubrol WX 1%	655	91
Digitonin 0.16 mg/mg protein	574	80
0.3 mg/mg protein	644	90
Sonication	319	45
Sonication+Lubrol WX 1%	645	90

* The mean value of the activity of Lubrol WX-treated mitochondria was taken as 100.

were used. Respiratory control at the 3rd phosphorylation site is usually rather low. Nevertheless, in order to avoid any limitation of the reaction rate by the phosphorylation system an uncoupler [0.1 mM-2,4-dinitrophenol or 1 μM-carbonyl-cyanide *m*-chlorophenylhydrazone (CCCP)] was included in the assay mixture. Rotenone or antimycin A may also be added in order to exclude any interference by endogenous substrates.

A typical assay medium was as follows. (*a*), For spectrophotometric procedure: 120 mM-KCl, or 60 mM-KCl - 125 mM-sucrose, 10 mM-tris-HCl (pH 7.4), 1 μM-CCCP or 0.1 mM-2,4-dinitrophenol, 1 μM-rotenone, 30 μM-cytochrome *c* and about 0.1 mg mitochondrial protein; total volume, 3.0 ml. (*b*), For polarographic procedure: the same medium except that concentration of cytochrome *c* was 80 - 100 μM and the medium also contained 25 mM-sodium ascorbate. The content of mitochondrial protein was about 0.5 mg; total volume, 3.0 ml.

In order to obtain full activity of cytochrome oxidase in both procedures, mitochondria were solubilized prior to use in 0.2 - 1.0% Lubrol WX and the amount taken for analysis was small enough to keep the concentration of the detergent in the final assay system below 0.02%.

It is well known (Smith, 1955) that oxidation of cytochrome *c* by cytochrome oxidase proceeds according to the first order kinetics. Therefore, using spectrophotometric procedure (*a*) the degree of damage of the outer mitochondrial membrane can be calculated by comparing the velocity constants (Smith, 1955) of cytochrome *c* oxidation by solubilized and "intact" mitochondria. Somewhat less precise but simpler calculation can be made by comparing actual reaction rates drawn as tangentials to the spectrophotometric traces, at identical concentrations of cytochrome *c*. This is exemplified by Fig. 2. Trace *A* represents the oxidation of cytochrome *c* by a preparation of rat heart mitochondria solubilized in Lubrol WX. From this trace the velocity constant *k* of the first order reaction was calculated (Smith, 1955) to be 2.157 min^{-1}. The "intact" mitochondria from the same preparation added in the same amount (corresponding to 0.1 mg protein) gave the

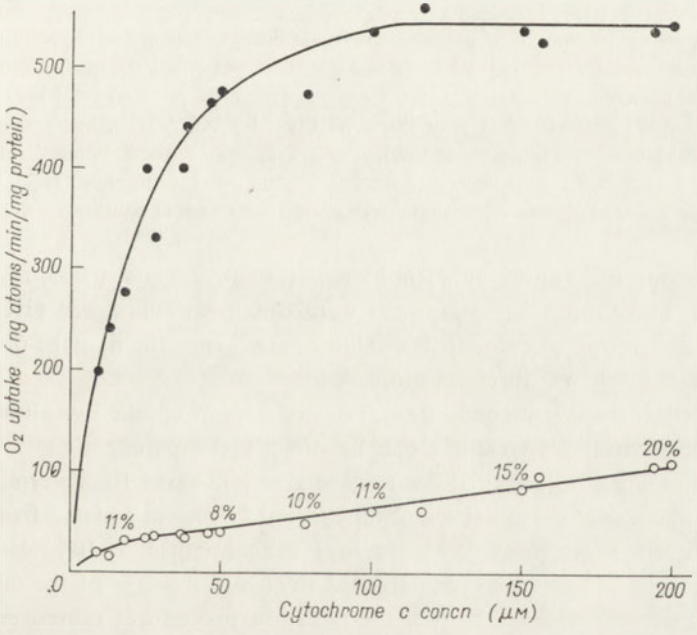

Fig. 1. Effect of concentration of cytochrome *c* on the rate of its oxidation by preparations of rat liver mitochondria. Incubation medium: 60 mM-KCl, 125 mM-sucrose, 10 mM-tris-HCl (pH 7.4), 0.1 mM-2,4-dinitrophenol, 1 μM-rotenone, 25 mM-ascorbate, 0.5 mg mitochondrial protein and cytochrome *c*; total volume, 3.0 ml; temp. 25°C. Oxygen uptake was measured polarographically. ○, "Intact" mitochondria; ●, mitochondria solubilized in 1% Lubrol WX. Figures besides the lower curve indicate the percentage of the activity with respect to solubilized mitochondria (upper curve).

velocity constant $k=0.672\ \text{min}^{-1}$ (trace *B*). This amounts to 31% of the value for solubilized mitochondria, indicating a rather high degree of damage of the outer membrane. When the initial reaction rates, as indicated by the dashed lines, are

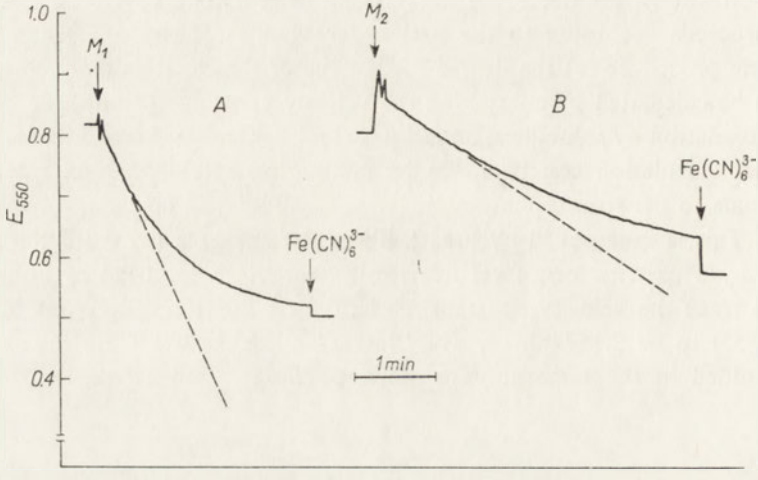

Fig. 2. Oxidation of cytochrome *c* by preparations of rat heart mitochondria. Spectrophotometric traces. Incubation medium: 120 mM-KCl, 10 mM-tris-HCl (pH 7.4), 0.1 mM-2,4-dinitrophenol, 1 μM-rotenone, 30 μM-ferrocytochrome *c* and 0.1 mg mitochondrial protein; total volume, 3.0 ml; temp. 23°C. Trace *A*: Mitochondria solubilized in Lubrol WX (M_1); velocity constant, $k=2.157\ \text{min}^{-1}$; initial reaction rate (dashed line), $v=0.275\ \Delta\text{E/min}$. Trace *B*: "intact" mitochondria (M_2); $k=0.672$; $v=0.085\ \Delta\text{E/min}$; degree of damage of the outer membrane, 31%. $Fe(CN)_6^{3-}$, a few microliters of saturated potassium ferricyanide solution.

compared instead, the values of ΔE/min equal to 0.275 and 0.085, respectively, for solubilized and "intact" preparations were obtained. This again gives a value of 31% for the exposure of cytochrome oxidase, and hence the damage of the outer membrane, in the "intact" preparation.

With the polarographic procedure (*b*), rates of oxygen uptake by solubilized and "intact" mitochondrial preparations can be directly compared.

Table 2 illustrates the use of the suggested enzymic assay for determination of the degree of damage of the outer membrane in mitochondria isolated from various tissues by different procedures. With rat liver mitochondria 5 - 10% damage was routinely obtained. In heart mitochondria the degree of damage of the outer membrane was higher and depended upon the isolation procedure; moreover, it often varied from one preparation to another even when the same procedure was employed.

Electron micrographs of two preparations of rat heart mitochondria obtained by various procedures (Fig. 3) clearly show that the degree of damage of the outer membrane as determined by the enzymic assay reflects the state of the preparation as revealed by electron microscopy.

The accuracy of the test is limited by the fact that inner membrane particles with the reverse sidedness of the membrane are less accessible to external cyto-

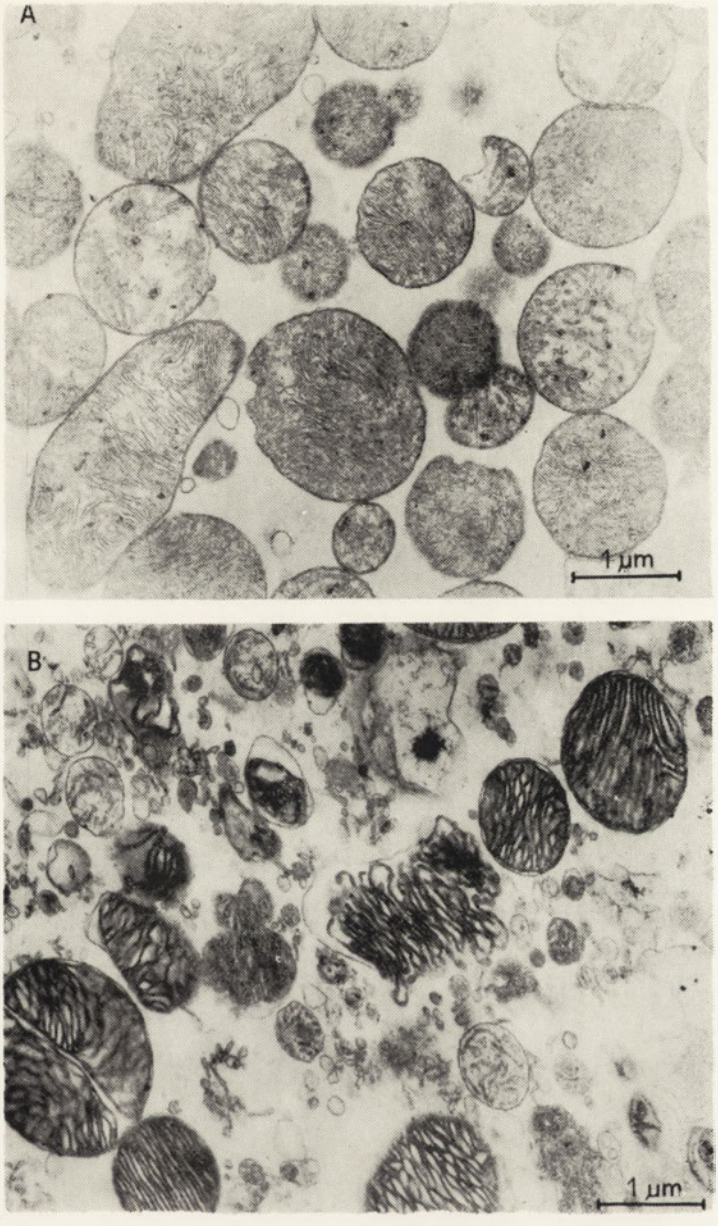

Fig. 3. Electron micrographs of two preparations of rat heart mitochondria. *A*, Mitochondria obtained in the medium containing 250 mM-sucrose - 1 mM-EDTA - 1 mM-ATP - pronase. Orthodox configuration; occasional discontinuity of the outer membrane, most likely due to digestion by pronase. The degree of damage of the outer membrane as determined by enzymic assay 20%. *B*, Mitochondria obtained in the medium containing 250 mM-sucrose - 1 mM-EDTA. Condensed configuration, highly damaged particles. The degree of damage of the outer membrane determined by the enzymic assay: 44%.

Table 2

The degree of damage of the outer membrane in mitochondria isolated from various tissues

Species and tissue	Isolation medium	Damage of the outer membrane (%)
Rat liver	250 mM-sucrose - 1 mM-tris-HCl, pH 7.4 (Hogeboom, 1955)	5 - 10
heart	250 mM-sucrose - 10 mM-EDTA or 2 mM-EGTA (Slater & Cleland, 1953)	20 - 44
heart	250 mM-sucrose - 1 mM-EDTA or 2 mM-EGTA - 1 mM-ATP - -pronase (Chance & Hagihara, 1963, modified)	16 - 20
heart	180 mM-KCl - 2 mM-EDTA - 1% serum albumin (von Korf, 1965)	25*
brain	250 mM-sucrose - 1 mM-EDTA - 10 mM-tris-HCl (Chappell & Hansford, 1969)	8 - 30
kidney	250 mM-sucrose - 2 mM-EDTA	22*
Rabbit heart	250 mM-sucrose - 1 mM-EGTA - 1 mM-ATP - pronase (Chance & Hagihara, 1963, modified)	16*
Beef heart	250 mM-sucrose - 1 mM-tris-HCl, pH 7.4 (Löw & Vallin, 1963)	16*
adrenal cortex	250 mM-sucrose - 2 mM-tris-HCl, pH 7.4 (Satre, Chambaz, Vignais & Idelman, 1971)	18*

* Single preparations.

chrome *c* than normally oriented inner membrane (Muscatello & Carafoli, 1969; Chance, Erecińska & Lee, 1970). If such particles are present in the preparation (see for example sonicated mitochondria in Table 1), the value for the damage of the outer membrane will be underestimated. Another inaccuracy of the method may be due to a limited rate of cytochrome *c* diffusion into the intermembrane and intracristal spaces. If the rupture of the outer membrane is too small, the rate of diffusion may be lower than the rate of oxidation. The fact that increasing the concentration of cytochrome *c* above 100 μM enhances the rate of its oxidation by "intact" mitochondria (Fig. 1) indicates that this may indeed be the case. Thus, it can be speculated that, in the case of a small damage, not the whole surface of the inner membrane is exposed to the access of external cytochrome *c*.

Because of these, and possibly also other, limitations the suggested enzymic assay cannot be regarded as strictly quantitative. Nevertheless, it can provide semi--quantitative data which may be informative in investigations on isolated mitochondria.

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TEST OKREŚLAJĄCY STOPIEŃ USZKODZENIA ZEWNĘTRZNEJ BŁONY IZOLOWANYCH MITOCHONDRIÓW

Streszczenie

Pomiar stopnia uszkodzenia zewnętrznej błony izolowanych mitochondriów oparto na oznaczaniu szybkości utleniania dodanego zredukowanego cytochromu *c*. Test ten opiera się na uprzednio stwierdzonej nieprzepuszczalności cytochromu *c* przez zewnętrzną błonę mitochondrialną. Opisano warunki przeprowadzania testu i podano przykłady zastosowania go do różnych preparatów mitochondrialnych.

Received 19 February, 1972.

ACTA BIOCHIMICA POLONICA
Vol. 19 1972 No. 3

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SOME FEATURES OF HIGHLY REITERATED DNA IN RAT GENOME

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1. Three arbitrary fractions, "fast", "intermediate" and "slow" were isolated from rat liver DNA, sheared down to 200 000 - 400 000 daltons, and submitted to reassociation and fractionation on hydroxyapatite. 2. The "fast" fraction amounted to 5 - 10% of the total DNA and showed a narrow thermal transition, a high hyperchromic effect, and T_m value close to that of unfractionated DNA. The G+C content of this fraction was 40 - 42 mol%. Strand separation either by alkaline CsCl gradient centrifugation or by chromatography on poly-I column was unsuccessful. Pyrimidine isostichs analysis showed no major differences in the oligonucleotide composition as compared to the native total DNA. 3. The "fast" fraction submitted to thermal chromatography showed biphasic transition due to the presence of molecules with various degree of precision in the reconstituted double helix. 4. The "fast" fraction submitted to a second denaturation and reassociation revealed the presence of three components. The first component amounting to 40% of the "fast" fraction reassociated at $C_0t < 10^{-5}$, the second amounting to 20% reassociated at $C_0t_{1/2} = 5 \times 10^{-4}$, and the remaining, third component reassociated at $C_0t_{1/2} = 5$, i.e. with a rate corresponding to that for the "intermediate" fraction. 5. The precise calculation of reiteration rate on the basis of reassociation kinetics should be taken with caution, as fractions isolated by hydroxyapatite chromatography are still heterogeneous.

Britten & Kohne (1968) showed that during the reassociation of DNA isolated from higher organisms (Eucaryotes) a portion of nuclear DNA reassociates more rapidly than expected, indicating that this portion has repetitive nucleotide sequences. Subsequent observations (for review see: Britten, 1969; Britten, 1970) established that repeated DNA sequences exist in all species examined, above the Fungi. The amount of repeated sequences varies from 20 to 80% of the total nuclear DNA. It was also observed that repetition frequency fluctuates from 50 to 10^7 related sequences per family. It seems that the bulk of these sequences are scattered throughout the genome with the exception of some well defined clases, e.g. DNA coding rRNA which may exist as a cluster of several tandemly repeated cistrons (Dawid, Brown & Reeder, 1970).

It is suggested that different sets of repeated sequences are transcribed in different tissues and stages of development. However, several properties of the repeated sequences remain unknown and the only way to extend out knowledge of repeated

DNA sequences and their biological role is to isolate biologically defined DNA fractions and to study their structure and function. The most easily obtainable DNA fractions are these with average base composition different from that of the bulk DNA, sufficient to give a distinct band during centrifugation in CsCl, and called "satellite" DNA (Kit, 1961). Mammalian satellites, especially mouse satellite DNA and guinea pig satellite DNA have been extensively studied (Flamm, Walker & McCallum, 1969a,b; Corneo, Ginelli, Soave & Bernardi, 1968; Southern, 1970).

The satellite fraction in DNA of the rat was not observed (Walker, 1968; Walker, Flamm & McLaren, 1969), therefore CsCl density gradient centrifugation cannot be used for isolation of a defined fraction of rat DNA. The only experimental approach to the study of repetitiveness of rat DNA at the moment remains fractionation of sheared rat DNA on hydroxyapatite according to reannealing rate after thermal denaturation (Britten & Kohne, 1968).

The aim of this work was to fractionate rat liver DNA, into fractions with unique and reiterated sequences and to study some properties of the latter.

MATERIALS AND METHODS

Preparation of DNA. DNA was prepared from livers of three-month-old Wistar rats according to the method of Savitsky & Stand (1966). Samples of DNA were purified further by digestion with pancreatic RNase (5×crystallized, Sigma Chemical Co., St. Louis, Mo., U.S.A.), and pronase B (Calbiochem, Los Angeles, Calif., U.S.A.), and in few instances were further purified by chromatography on hydroxyapatite column as previously described (Szala, Kilarski & Chorąży, 1970).

Fractionation of DNA on hydroxyapatite column. Denaturation, annealing and fractionation of DNA on hydroxyapatite after Britten & Kohne (1968) was performed as described earlier (Szala *et al.*, 1970). Hydroxyapatite was prepared according to Miyazawa & Thomas (1965).

Breakage of DNA. DNA preparations in 0.2 M-NaCl (0.5 mg/ml) were sheared mechanically as previously described (Szala *et al.*, 1970). Occasionally DNA preparations were degraded by limited depurination as described by McConaughy & McCarthy (1967). Controlled depurination was carried out for 3 to 5 hours at 70°C in 0.1 M-sodium acetate (pH 4.3) and was followed by alkaline hydrolysis (pH 12 for 10 min) to effect chain scission at the sites of depurination.

Measurements of DNA molecular weight. The molecular weight of sheared DNA was measured by the neutral sucrose density gradient sedimentation by the method of Burgi & Hershey (1963). DNA samples of 0.2 ml in 0.125 M-Na-phosphate buffer, pH 6.8, containing 4.6 $O.D._{260}$ units were layered on a 4.8 ml linear 5 - 20% (w/v) sucrose density gradient containing 0.1 M-NaCl-0.05 M-Na-phosphate (pH 6.8), and centrifuged for 3 h in a Spinco SW 39 swing-out rotor at 35 000 rev/min at 4°C. At the end of the run 3-drop fractions were collected from the bottom of the tube. Each fraction was diluted to 1 ml with water, the absorbance values measured at 260 nm were plotted against fraction number and the distance of the main band

from the meniscus was estimated. The approximate sedimentation coefficient can be the calculated from the position to which DNA has moved:

$$S^{\circ}_{20,w} = \frac{6.45 \times 10^{10} d}{\omega^2 t} \quad (1)$$

where d is the distance in cm of the DNA band from the meniscus, ω is the angle velocity expressed in r.p.m., and t is sedimentation time in hours. The approximate sedimentation constant, $S_{20,w}$, for sheared DNA varied from 6 to 10.

Melting profiles of DNA. The absorbance of DNA (20 - 30 μg/ml of 0.02 M--NaCl) during heating was measured in thermostatically controlled cell in the Beckman DU spectrophotometer equipped with a copper-constantan thermistor. Absorbance was corrected for thermal expansion of solution using indexes given by Mandel & Marmur (1968).

Ultraviolet absorption spectra of DNA in 0.02 M-NaCl or 0.002 M-Na-phosphate buffer (pH 6.8) at room temperature (native DNA) and at 100°C (denatured DNA) were analysed for A+T content and the amount of paired regions estimated according to the method of Felsenfeld (1968).

Preparative density gradient centrifugation. Centrifugation of DNA in neutral CsCl density gradient was conducted at 25°C in a Spinco L 40 angle rotor operated at 33 000 r.p.m. for 60 h. To the 3.3 ml solution of 0.1 M-NaCl - 0.01 M-tris-HCl, pH 7.5, containing DNA (50 - 100 μg/ml), 4.20 g of CsCl (Suprapur, E. Merck, Darmstadt, G.F.R.) was added to the initial density of 1.720 g/cm^3. At the end of the run 3-drop fractions were collected, and after dilution to 1 ml with water the absorbance was read at 260 nm. The densities of particular fractions were calculated from the refractive index measured with Abbe refractometer using formula:

$$\rho^{\circ,25^{\circ}} = 10.8601 \times n_D^{25^{\circ}} - 13.4974 \quad (2)$$

given by Vinograd (1963).

Centrifugation in alkaline CsCl density gradient was performed according to Flamm *et al.* (1967, 1969b). To 3.0 ml of 0.01 M-tris (pH 8.4) solution containing DNA (100 μg/ml) and 4.62 g CsCl were added: 0.1 ml portions of 1 N-NaOH, 0.1 M-EDTA and 1% sodium lauryl sulfate (SLS). Initial density of this solution was in the range of 1.750 - 1.760 g/cm^3, and final pH of about 12.4. Centrifugation was conducted at 13°C, in the Spinco L 50 angle rotor at 34 000 r.p.m. for 24 h using polypropylene tubes. The 7-drop fractions were collected and after dilution to 1 ml, the absorbance was measured at 260 nm.

Chromatography on kieselguhr coated with poly-I. In an attempt to separate G-rich strands, chromatographic columns containing kieselguhr coated with poly-I were prepared according to Lin (1970). To 100 ml of 0.01 M-tris-HCl (pH 9.8) containing 1 mg of poly-I were added 100 ml of 0.007 M-hexamine cobalt chloride [$Co(NH_3)_6Cl_3$], and 5 000 mg of kieselguhr. The suspension was stirred for 15 min, the particles were collected, washed with ethanol and acetone and finally dried. One gram of kieselguhr coated with poly-I was then suspended in 45% dioxane - - 55% 0.001 M-tris-HCl, pH 7.2, packed in a column (1×5 cm) and the column

was washed with the same solution. The solution of 50 ml of heat-denatured "fast" DNA (10 μg/ml in dioxane - 0.001 M-phosphate buffer, pH 7.2, 1:1, v/v) was introduced to the column at the rate of about 50 ml/h.

The column was then eluted successively with: (a) 20 ml of dioxane - 0.001 M-phosphate buffer, pH 7.5; (b) gradient formed by 20 ml of (a) and 20 ml of 0.001 M-phosphate buffer; (c) 30 ml of 0.001 M-phosphate buffer, pH 7.5; and (d) 30 ml of 0.1 M-NaCl solution. Fractions of 3 ml were collected; all operations were carried at 4°C, and the absorbance of the fractions was monitored at 260 nm.

Degradation of DNA to pyrimidine oligonucleotides. Degradation of DNA was performed according to Burton (1967). To 1 - 5 mg of dry DNA were added 2 ml of 3% diphenylamine - 66% HCOOH. The DNA samples were then incubated for 17 h at 30°C. Approximately 10 ml of water was then added to the reaction mixture, and diphenylamine and most of the formic acid removed by 4 - 5 extractions with 100 ml of ether. The aqueous phase was then neutralized with ammonia to pH 5.0.

Separation of pyrimidine oligonucleotides on DEAE-cellulose. Chromatographic separation of the pyrimidine oligonucleotides according to chain length was performed on a 1×10 cm DEAE-cellulose column (Černy, Mushynski & Spencer, 1968). After adsorption of the oligonucleotides, the column was extensively washed with 0.01 M-Li-acetate (pH 5.0) to remove free purines; the nucleotides were then eluted with an increasing linear gradient of LiCl up to 0.35 M in the same lithium acetate buffer. The total volume of the eluent was 500 ml, and 35-drop fractions were collected. After gradient elution the column was washed with 50 ml of 1 M-LiCl. Absorbances of fractions were read at 270 nm. The pooled pyrimidine isostich fractions were diluted with 3 - 5 volumes of water and each of them was then applied to a DEAE-cellulose column (1×5 cm) and subsequently eluted with a linear gradient of 0.01 M - 0.3 M-LiCl (pH 3.0) in the same Li-acetate buffer. Total volume of eluent was 200 ml; 35-drop fractions were collected and their absorbance was measured at 267.5 nm.

RESULTS

Kinetics of reassociation of rat liver DNA. The reassociation curve for rat DNA (Fig. 1) reveals the presence of three components: "fast", "intermediate" and "slow", which correspond to the components of rat DNA described by Melli & Bishop (1970). The "fast" component amounts to 5 - 10% of total DNA and is half-renatured at a C_0t value of 5×10^{-4}. The "intermediate" component amounts to 15 - 20% of total DNA and has $C_0t_{1/2} = 3 \times 10^{-2}$. The "slow" component constitutes 75% of the total DNA and is half-renatured at $C_0t = 3 \times 10^3$; it corresponds, as may be judged from analytical and kinetic complexity (Britten & Kohne, 1968), to non-repetitive nucleotide sequences of rat genome. From the relation between reassociation rate and repetition frequency (Britten & Kohne, 1968) it can be estimated that frequency of repetition of nucleotide sequences of the "fast" and "intermediate" fractions are equal to 6×10^6 and 10^3 times, respectively.

Melting profiles of DNA reassociated to $C_0t=10^{-3}$. The melting profile of DNA reassociated to $C_0t=10^{-3}$ (Fig. 2) shows a sigmoid profile, similar to that of total, sheared, unfractionated native DNA and to DNA renatured at high value of C_0t

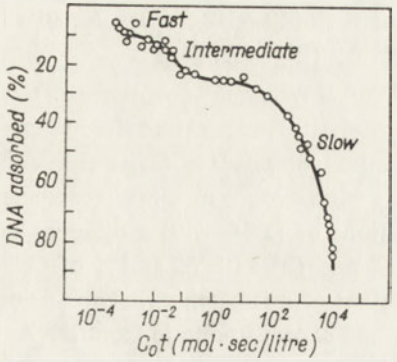

Fig. 1. Reassociation kinetics of rat liver DNA. DNA was sheared to $S_{20,w}=6-10$, denatured at 100°C for 10 min, and reassociated in 0.125 M-Na-phosphate buffer, pH 6.8, at 60°C, then adsorbed on hydroxyapatite. C_0t is the product of initial DNA concentration (mol of nucleotide/litre) and time of incubation in seconds; approximately $C_0t=1$ when 1.0 O.D.$_{260}$ unit of denatured DNA is reassociated during 1 hour.

(i.e. $C_0t\cong10^4$). However, upon close examination the fraction reassociated to $C_0t=10^{-3}$ reveals a biphasic melting curve. This is clearly visible when the curve is plotted differentially according to Bernardi, Faures, Piperno & Slonimski (1970)

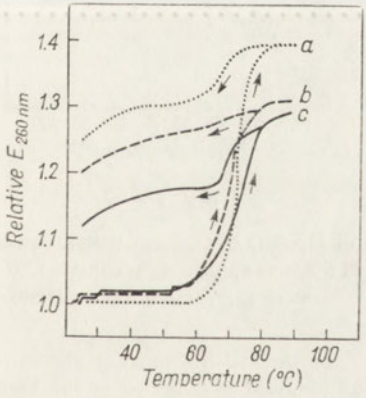

Fig. 2

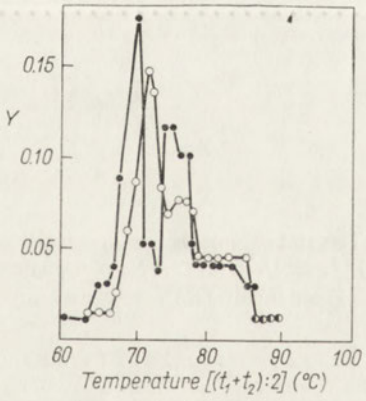

Fig. 3

Fig. 2. Melting profiles in 0.02 M-NaCl of total, native sheared DNA (*a*) and fractions of DNA reassociated to a C_0t value of 8.4×10^3 (*b*) or 10^{-3} (*c*).

Fig. 3. Differential melting profiles of unfractionated, native DNA (○), and DNA reassociated to $C_0t=10^{-3}$ (●). *Y* indicates the increment in relative absorbance per degree:

$$Y=\frac{E_{t1}-E_{t2}}{E_{100}-E_{25}}(t_1-t_2),$$

where E_{t1}, E_{t2}, E_{100}, E_{25} are absorbances measured at temperatures t_1, t_2, 100°C, 25°C, respectively.

(Fig. 3); in 0.02 M-NaCl solution one can see two separate regions of melting: (a) $T_m = 70°C$, and (b) $T_m = 74.5°C$. Sheared, native unfractionated DNA shows under the same conditions a monophasic curve with $T_m = 72.5°$. Upon cooling at a rate of 1°C/min native total DNA showed very little reassociation of strands, while the fraction renatured to $C_0t = 10^{-3}$ reassociated with a recovery of approximately 70% hyperchromicity (Fig. 2).

Thermal fractionation of DNA on hydroxyapatite. Columns of hydroxyapatite eluted at continuously increasing temperature fractionate DNA according to its melting point and nucleotide composition (Miyazawa & Thomas, 1965). One may assume that the biphasic melting curve of DNA reassociated to $C_0t = 10^{-3}$ reflects the presence of two fractions of DNA with a different base composition: (a) with about 41 mol% of G+C, and (b) with 52 mol% of G+C (calculated from their T_m's) or with different extent of mismatching. Thus using the method of Miyazawa & Thomas (1965) one can expect to obtain from DNA reasociated to $C_0t = 10^{-3}$ two components differing in base composition. However, the thermal chromatogram shows a unimodal distribution pattern (Fig. 4), and the fractions eluted at different

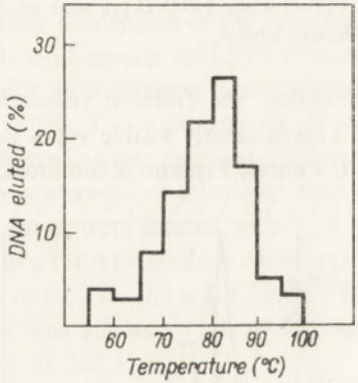

Fig. 4

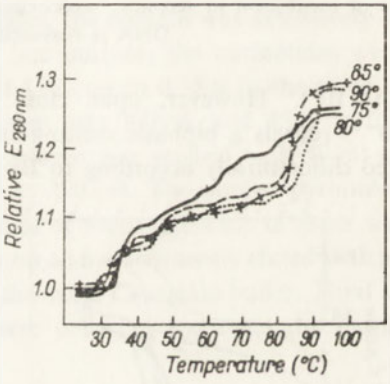

Fig. 5

Fig. 4. Thermal chromatography on hydroxyapatite of DNA (29.7 O.D.$_{260}$ units) reassociated to $C_0t = 10^{-3}$. DNA in 0.125 M-Na-phosphate buffer, pH 6.8, was applied on a column (2.0×2.0 cm) at 60°C. Fractions of 3 ml were eluted from the column with the same buffer at increasing temperatures.

Fig. 5. Melting profiles of DNA fractions obtained from thermal fractionation shown in Fig. 4. The temperature at which a given fraction was obtained is indicated in the Figure.

temperatures have almost equal base compositions falling in the range of 58 - 60 mol% of A+T (Table 1). These fractions submitted to further renaturation to $C_0t = 10^{-3}$ showed differences in the regained hyperchromicity and in the amounts of helically paired regions or amounts of DNA involved in the helix (Fig. 5, Table 1). Only one fraction (eluted at 90°C amounting to about 20% of DNA reassociated to $C_0t = 10^{-3}$) showed almost complete reassociation after rapid cooling (see Fig. 5), with recovery of about 95% of hyperchromicity. This fraction may represent a sub-

fraction of the DNA with reassociation rate much higher (i.e. with $C_0t_{1/2}$ lower than 10^{-3}), as has been reported for the "fast" fraction.

Table 1

Composition of fractions obtained by thermal chromatography of DNA renatured to $C_0t=10^{-3}$

Temperature of fractionation (°C)	Eluted DNA (% of total load)	Hyperchromic effect (%)	A+T * (mol%)	Fraction of DNA involved in helix*
70	8	28.3	57.6	0.72
75	14	29.1	58.1	0.75
80	21	28.0	59.8	0.72
85	26	33.3	56.3	0.86
90	18	32.8	58.8	0.95
95	5	19.7	60.0	0.08

* Data calculated from ultraviolet spectral analysis according to Felsenfeld (1968).

These data show that heterogeneity of the DNA reassociated to $C_0t=10^{-3}$ is due more to the extent of sequence mismatching than to base composition.

The biphasic melting curve of DNA reassociated to $C_0t=10^{-3}$ may thus reflect rather differences in thermal stability of the DNA molecules with different molecular weight and with different length of reassociated regions than the differences in base composition.

Centrifugation of DNA in CsCl density gradient. Sheared, native DNA fractions obtained in the neutral CsCl density gradient were reassociated to $C_0t=10^{-3}$ and to $C_0t=2$ corresponding to "fast" fraction and total reiterated nucleotide sequences, respectively (Fig. 6). DNA reassociating at $C_0t=10^{-3}$ followed rather

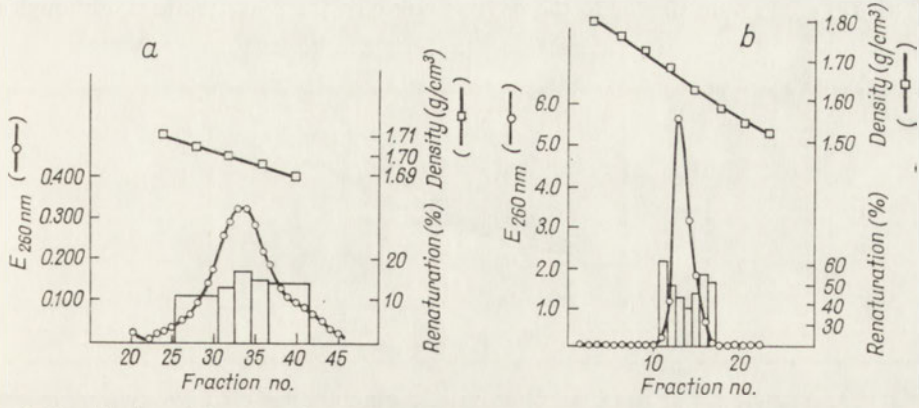

Fig. 6. Localization of the two reiterated DNA fractions in the band of native, sheared DNA on buoyant density gradient centrifugation. *a*, Fraction reassociated to $C_0t=10^{-3}$; *b*, fraction reassociated to $C_0t=2$. Percentage of renaturation of each fraction (outlined columns) is calculated from hydroxyapatite chromatography values.

closely the main band (Fig. 6a). On the other hand, DNA which reassociated to $C_0t=2$ was localized on the heavy and light side of the main peak (Fig. 6b). DNA reassociated at $C_0t=10^{-3}$ and sheared, unfractionated native DNA banded in neutral CsCl at the same buoyant density (1.695 ± 0.005 g/cm^3, compare Fig. 6a and 7a).

We attempted to use alkaline CsCl density gradient centrifugation for strand separation of fragmented DNA, reassociated to $C_0t=10^{-3}$. Only one broad band was found at a density of 1.758 ± 0.002 g/cm^3 (Fig. 7b). Unfractionated, native DNA showed in alkaline CsCl gradient also one main band with density $1.760\pm$ ±0.005 g/cm^3.

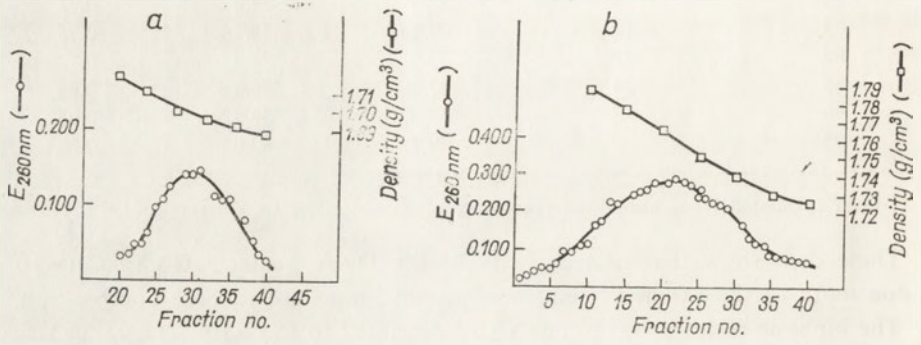

Fig. 7. Buoyant density gradient centrifugation in CsCl of the DNA reassociated to $C_0t=10^{-3}$. *a*, Neutral CsCl sedimentation pattern; 50 µg of DNA reassociated to $C_0t=10^{-3}$ was centrifuged at 33 000 rev./min for 60 h at 25°C in Spinco L fixed-angle rotor no. 40. The initial density of the solution, calculated from the refractive index, was 1.720 g/cm^3 at 25°C, pH 7.5. *b*, Alkaline CsCl sedimentation pattern. About 100 µg of DNA was centrifuged at 43 000 rev./min for 24 h at 13°C in Spinco L fixed-angle rotor no. 50. The initial density of the solution was 1.760 g/cm^3, pH 12.4.

The melting profiles of fractions taken both from leading ("heavy") and trailing ("light") edges were similar to the melting curve of the bulk fractions, although the

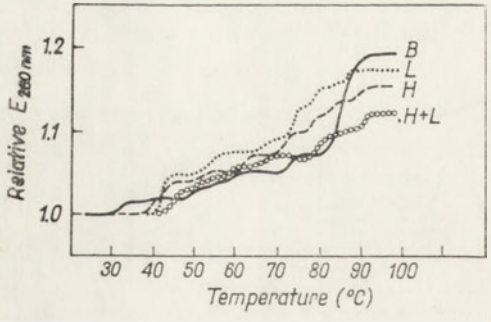

Fig. 8. Melting profiles of DNA fractions obtained from alkaline CsCl density gradient centrifugation in 0.125 M-Na-phosphate buffer, pH 6.8. Fraction *H* (heavy) includes DNA fractions 5 - 15 from Fig. 7b, fraction *L* (light) — 28 - 35, and bulk (*B*), fractions 16 - 27. *H*+*L*, equimolar mixture of fractions *H* and *L*. Fractions *H* and *L* were recentrifuged as in Fig. 7b prior to determination of melting curves.

transition of the last is somewhat sharper (Fig. 8). The mixture of the fractions from heavy and light density regions gave a very broad transition and low hyperchromic effect.

Chromatography on kieselguhr coated with poly-I. DNA reassociated to $C_0t = 10^{-3}$ submitted to chromatography on poly I-coated kieselghur showed only one peak (Fig. 9). The melting curve of this fraction showed relatively well paired polynucleotide strands indicating a highly ordered secondary structure (Fig. 10).

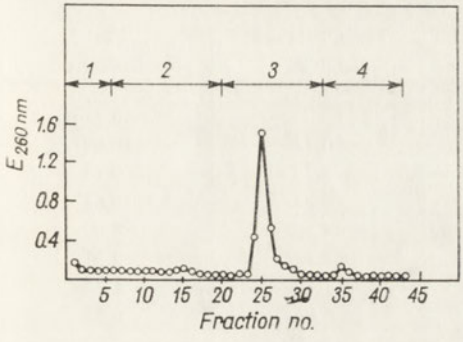

Fig. 9

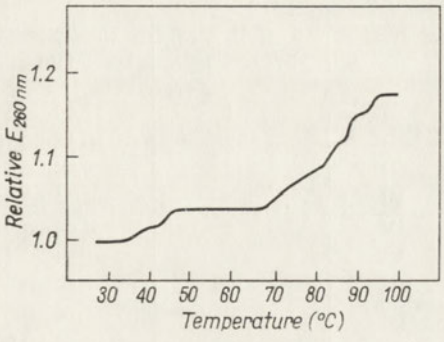

Fig. 10

Fig. 9. Fractionation of heat-denatured DNA reassociated to $C_0t = 10^{-3}$ on kieselguhr column coated with poly-I. After adsorption of the DNA, the column was washed with the following solvents: *1*, 50% dioxane - 1 mM-Na-phosphate buffer, pH 7.5; *2*, in gradient of 50% dioxane - 1 mM--Na-phosphate buffer to 1 mM-Na-phosphate buffer; *3*, 1 mM-Na-phosphate buffer; *4*, 0.1 M-NaCl solution.

Fig. 10. Melting profile of the DNA obtained from chromatography on kieselguhr coated with poly-I. The fractions no. 24, 25 and 26 from Fig. 9 were pooled, dialysed and dissolved in 0.125 M-Na-phosphate buffer, pH 6.8.

Pyrimidine isoplith distribution of DNA reassociated to $C_0t = 10^{-3}$. Separation according to chain length on DEAE-cellulose of the pyrimidine tracts of unfractionated DNA is shown in Fig. 11. The frequency and distribution of the nucleotides was calculated from the absorbance at pH 5.0 and at 270 nm (the isosbestic wavelength of the nucleotides). Results of similar analyses for DNA reassociated to $C_0t = 10^{-3}$ ("fast" component), native DNA and non-reiterated DNA are shown in Table 2. The total, unfractionated DNA and non-reiterated DNA have almost the same pattern of distribution of pyrimidine isostichs. Frequency of the occurrence of pyrimidine isostichs containing 1 - 4 monomers tends to be a little higher in the "fast" fraction than in the unfractionated DNA, whereas isostichs of 5 to 8 monomers have a reverse tendency.

Further fractionation of each pooled isostich fraction from Fig. 11 on DEAE--cellulose at pH 3.0 gave the profiles shown in Fig. 12. In fractions 1 to 4 all theoretically possible components were isolated. In fractions 5 to 7 the oligonucleotides containing a high proportion of cytidylic acid were less frequent than thymidylic oligonucleotides. In isostichs no. 5 and 6 only a negligible amount of C_5p_6 and

C_6p_7 are present. In fraction 7 no C_7p_8 was observed and a negligible amount of C_6Tp_8.

Table 2

Distribution of pyrimidine isostichs in rat liver DNA

The values are means of 5 (native DNA) or 2 (non-reiterated DNA and "fast" fraction) determinations, ±S.D.

Isostich	Number of pyrimidines	Pyrimidine (mol/100 gatoms P-DNA)		
		Unfractionated DNA	Non-reiterated DNA	"Fast" fraction
I	1	11.99±0.17	12.23	13.32
II	2	10.34±0.10	10.32	10.68
III	3	8.26±0.01	8.55	9.13
IV	4	6.14±0.10	6.01	6.63
V	5	4.48±0.01	4.55	4.09
VI	6	2.89±0.11	2.82	2.31
VII	7	1.88±0.07	1.72	1.20
VIII	8	1.14±0.07	1.04	0.33
	>8	2.86±0.09	2.76	2.22
Total		49.98	50.00	50.02

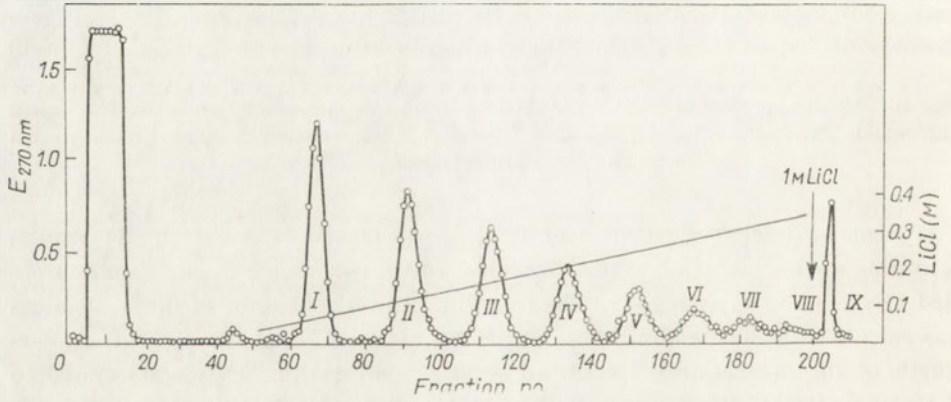

Fig. 11. Pyrimidine isopliths separation on DEAE-cellulose column. Unfractionated rat liver DNA, 5 mg, was degraded by diphenylamine-HCOOH as described in Material and Methods. The mixture of pyrimidine oligonucleotides was applied on DEAE-cellulose column (1×10 cm). The elution was carried out with a linear molarity gradient (0 to 0.35 M) of LiCl in 0.01 M-Li-acetate buffer, pH 5.0. Fractions of 35 drops were collected.

Distribution of the separated oligonucleotides (Table 3) shows a similar pattern for both unfractionated DNA, and DNA reassociated to $C_0t=10^{-3}$. All possible components in isostichs 1 to 5 were present and only negligible differences between these DNA's could be found in isostichs 1, 2 and 3. DNA reassociated at $C_0t=10^{-3}$ has higher amounts of Cp_2 and C_2p_3, and isostich 3 of the "fast" fraction

has a lower amount of T_3p_4 and higher value for CT_2p_4 than unfractionated DNA. All other isostichs of these DNA's reveal no significant differences. Therefore it

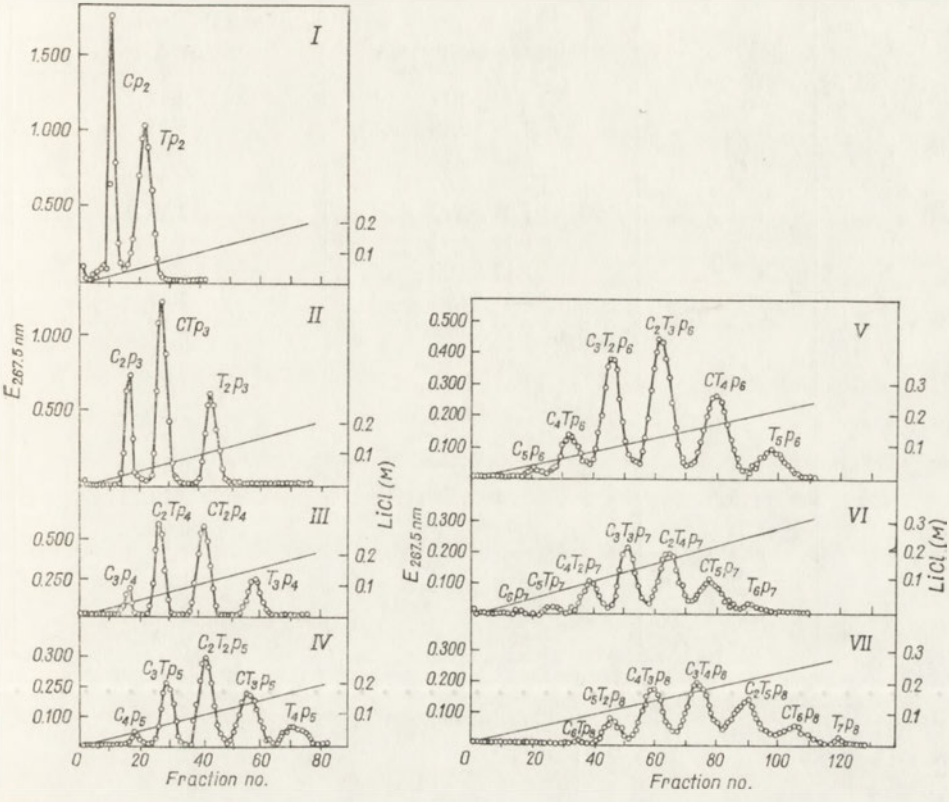

Fig. 12. Chromatography of the separated pyrimidine isopliths. The pooled and diluted fractions of pyrimidine oligonucleotides (*I-VIII*) obtained as in Fig. 11, were applied to a DEAE-cellulose column (1×5 cm). Elution was carried out with a linear gradient from 0 to 0.25 M or 0.35 M of LiCl in 0.01 M-Li-acetate buffer, pH 3.0. Fractions of 35 drops were collected.

might be supposed that the DNA reassociated to $C_0t=10^{-3}$ represents a more heterogeneous population of repeated sequences than would be expected from their kinetics of reassociation.

Kinetics of reassociation of the DNA fraction reassociated to $C_0t=10^{-3}$. The kinetics of reassociation of DNA fraction reassociated to $C_0t=10^{-3}$ ("fast" fraction), (Fig. 13), shows that about 40% of the "fast" fraction (i.e. 2 - 4% of total DNA) is bound to hydroxyapatite at very low value of C_0t ($C_0t<10^{-5}$). Approximately 20% (about 1 - 2% of total DNA) of the "fast" fraction renatures with $C_0t_{1/2}=5\times10^{-4}$, and the remaining 40% of DNA (2 - 4% of total DNA) was half reassociated at $C_0t=5$.

Table 3

Distribution of pyrimidine isostich components in rat liver DNA

The values are means of 4 (unfractionated DNA) or 2 ("fast" fraction and isostich V) determinations, ±S.D.

Isostich	Component	Pyrimidine (mol/100 gatoms P-DNA)	
		Unfractionated DNA	"Fast" fraction
I	Tp_2	7.37±0.14	7.57
	Cp_2	4.51±0.18	5.75
II	T_2p_3	3.13±0.11	2.66
	CTp_3	4.93±0.07	5.22
	C_2p_3	1.97±0.10	2.79
III	T_3p_4	1.74±0.03	1.38
	CT_2p_4	3.17±0.02	3.66
	C_2Tp_4	2.61±0.04	2.98
	C_3p_4	0.79±0.02	1.10
IV	T_4p_5	0.77±0.05	0.93
	CT_3p_5	1.62±0.07	1.58
	$C_2T_2p_5$	2.28±1.14	2.27
	C_3Tp_5	1.20±0.06	1.35
	C_4p_5	0.25±0.02	0.46
V	T_5p_6	0.34	0.29
	CT_4p_6	1.04	0.61
	$C_2T_3p_6$	1.47	1.23
	$C_3T_2p_6$	1.18	1.13
	$C_4T_1p_6$	0.40	0.61
	C_5p_6	0.06	0.03

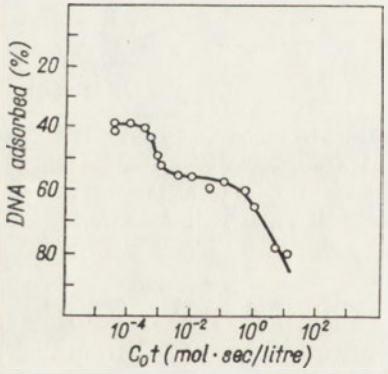

Fig. 13. Reassociation profile of the "fast" fraction of rat liver DNA (i.e. the DNA reassociated to $C_0t=10^{-3}$). Reassociation was done in 0.125 M-Na-phosphate buffer, pH 6.8, at 60°C. Reassociated DNA was measured on hydroxyapatite columns.

DISCUSSION

The presented results show that DNA of the rat genome reveals an extremely high degree of heterogeneity with respect to repeated nucleotide sequences. This finding is consistent with data of Melli & Bishop (1970) and McConaughy & McCarthy (1970) and also fits well into the general pattern of reiterated sequences found in a wide range of rodent species (Hennig & Walker, 1970). Three distinct

fractions, separated on an arbitrary basis, were found; they were named in respect to their reassociation rate as "slow" (or unique), "intermediate" and "fast" (Fig. 1). The most abundant is the "slow" fraction which amounts up to 75% in the rat genome, whereas "intermediate" and "fast" fractions represent 15 - 20% and 5 -10% of the total DNA, respectively.

The "fast" fraction (Fig. 1) consists of highly reiterated nucleotide sequences present in millions of copies according to the interpretation of Britten & Kohne (1968). The occurrence of higher proportion of circular forms found in this fraction (Szala, Chorąży & Kilarski, 1971), also indicates the presence of tandemly repeated sequences. The biological role of this fraction is obscure. It could be analogous to satellite DNA found in several species of mammals (Coudray, Quetier & Guille, 1970; Arrighi, Mandel, Bergendahl & Hsu, 1970) and known to contain more than 10^6 copies of repetitive sequences (Britten & Kohne, 1968; Walker, 1971). Although satellite DNA in the rat was not observed (Walker, 1968) and the buoyant density of the reassociated fraction is identical with that of unfractionated rat DNA (see Fig. 6a and 7a), all other structural features of the "fast" fraction in this species are similar to those of satellite DNA: it reanneals at low C_0t values after denaturation, constitutes a small fraction of the genome, and is poorly transcribed by RNA polymerase *in vitro* (data to be published).

After reannealing, the melting profile of the "fast" fraction shows narrow transition range, high hyperchromic effect, and T_m value close to that of unfractionated DNA and of the slow component of DNA (Fig. 2). However, this "fast" fraction contains at least two components differing in the degree of precision of the double stranded form (Fig. 5, Table 1). They show no difference in their average A+T content which amounts to 58 - 60 mol% (Table 1). The uniform distribution of the "fast" fraction over CsCl density fractions of sheared, total DNA (Fig. 6a) implies that this fraction is evenly dispersed throughout the rat genome and also that their length is shorter than the physical length of DNA fragments used (Southern, 1970).

We are not able to segregate complementary strands of the "fast" fraction by alkaline CsCl density gradient centrifugation and it seems therefore that the "fast" fraction of the rat has equal proportions of G+C in its complementary strands like, for example, the satellite DNA of *Apodemus sylvaticus* (Hennig & Walker, 1970). Chromatography on kieselguhr columns coated with poly-I was also unsuccessful in separating complementary strands, thus it seems probable that distribution of G+C between complementary chains is equal. These failures may be alternatively explained by the fact that the "fast" fraction is still so complex that it averages out any differences in the base distribution between the complementary strands, or that its molecular weight is too low to allow sharp banding of the strands in alkaline CsCl. The latter can be ruled out, since the estimated molecular weight of 200 000 to 400 000 daltons is high enough to enable successful strand separation.

The failure to separate the "fast" fraction into complementary strands limits to a great extent the meaning of our pyrimidine isostichs analysis, and though from such an analysis Southern (1970) was able to present the basic repeating sequence in the α-satellite of guinea-pig DNA, we may only present a general pattern of the

frequency of distribution of pyrimidine isostichs. The absence of longer tracts of cytosine oligonucleotides and the occurrence of thymidine oligonucleotides at considerably greater amounts (Table 3) is in good agreement with the observation of Chargaff (1968). Our results confirm also the suggestion of this author (for review see: Chargaff, 1968), about rather "random" distribution pattern of oligonucleotides in DNA. No large differences in pyrimidine oligonucleotide pattern are observed in the "fast" fraction as compared to the total, unfractionated DNA.

The reassociation kinetics of the "fast" fraction shows that it is still a heterogeneous population of DNA sequences (Fig. 13), so that the precise calculation of the number of repeated sequences by hydroxyapatite chromatography should be taken with caution. In the course of the second heating and renaturation DNA is probably broken down further, so that three components again could be disclosed. One of the two most aboundant components amounting to 40% of the "fast" fraction shows instantaneous reassociation on hydroxyapatite chromatography. This subfraction reassociationg at $C_0t < 10^{-5}$ could represent DNA fragments either with natural cross-links, or with an ability to self-anneal in a collision-independent manner. The second component reassociates with $C_0t_{1/2}$ of about 5, i.e. with the rate shown by "intermediate" fraction. Its presence in the "fast" fraction in rather large amounts (40%), as disclosed by the second course of reannealing, is enigmatic. It could partly correspond to the less stable fraction obtained by thermal chromatography at a temperature range of 70 - 80°C (Fig. 3, Table 1).

The most plausible explanation of this behaviour is that the $C_0t\ 10^{-3}$ fraction is composed of at least two types of sequences, tandemly arranged; on isolation these sequences separate because DNA is broken down. The two or more types of sequence could be distinguished by their reassociation rates and it is an open question, at the present time, as to whether these different rates are due to different frequency of the sequences or to differences in the extent of mismatching in the douplex formed after reassociation. In either case, the fact that they are tandemly arranged raises intriguing questions as to how such sequences are introduced into the genome.

We are grateful to Dr E. M. Southern for reading this manuscript and comments. The excellent technical assistance of Miss Elżbieta Gara is highly appreciated. This work was supported by the Polish Academy of Sciences within the project 09.3.1.

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NIEKTÓRE CECHY POWTARZAJĄCEGO SIĘ DNA W GENOMIE SZCZURA

Streszczenie

1. Zdegradowany do 200 000 - 400 000 daltonów DNA wątroby szczura poddany reasocjacji i frakcjonowaniu na hydroksyapatycie wykazuje trzy frakcje: ,,szybką", ,,pośrednią" i ,,wolną".

2. Frakcja ,,szybka" stanowiła 5 - 10% całkowitego DNA, wykazywała wąskie przejście fazowe, wysoki efekt hiperchromowy i wartość T_m zbliżoną do niefrakcjonowanego DNA. Zawartość G+C frakcji ,,szybkiej" wynosiła 40 - 42 mol%. Nie udało się rozdzielić frakcji szybkiej na pojedyncze pasma przy pomocy wirowania w alkalicznym gradiencie CsCl ani przy pomocy chromatografii na kolumnie poli-I. Analiza oligonukleotydów pirymidynowych frakcji ,,szybkiej" nie wykazała większych różnic w porównaniu z niefrakcjonowanym DNA.

3. Frakcja ,,szybka" poddana chromatografii termicznej wykazała dwufazowe przejście spowodowane obecnością cząsteczek o różnym stopniu dokładności odtworzonej struktury podwójnej spirali.

4. Frakcja ,,szybka" poddana ponownej denaturacji i reasocjacji wykazała obecność trzech komponentów. Pierwszy komponent stanowiący 40% frakcji ,,szybkiej" reasocjował przy $C_0t < 10^{-5}$,

drugi stanowiący 20% reasocjował przy $C_0t_{1/2}=5\times10^{-4}$, a pozostały, trzeci komponent reasocjował przy $C_0t_{1/2}=5$, to jest z szybkością odpowiadającą frakcji „pośredniej".

5. Dokładne obliczanie stopnia powtarzalności na zasadzie kinetyki reasocjacji musi być przyjmowane ostrożnie, ponieważ frakcje izolowane metodą chromatografii na hydroksyapatycie są nadal heterogenne.

Received 4 March, 1972.

ACTA BIOCHIMICA POLONICA
Vol. 19 1972 No. 3

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MUTANTS OF *SALMONELLA TYPHIMURIUM* RESISTANT TO TRIAZOLE

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1. Triazole-resistant mutants of *S. typhimurium* have been isolated. One group of the isolated mutants, designated *trzA*, showed 50% contransducibility with *cysA* gene. 2. It has been found that in *trzA* mutants triazole has no effect on the induction of cysteine biosynthetic enzymes by *O*-acetyl-L-serine. 3. The level of sulphate activating enzymes and sulphite reductase is 2 - 3 times higher in *trzA* mutants than in the parental strains.

As we have reported previously, triazole prevented the induction of sulphate permease and sulphate-activating enzymes by *O*-acetyl-L-serine (OAS)[1] in *Salmonella typhimurium* (Hulanicka, Kłopotowski & Smith, 1972). This indicates that interference with regulation of cysteine biosynthesis could be responsible for the inhibitory effect of triazole on growth of *S. typhimurium*. Isolation of triazole-resistant mutants and information on their properties may therefore shed some light on the regulation of cysteine biosynthesis.

We have isolated several triazole-resistant mutants of *S. typhimurium*, which have been classified into two distinct groups, designated *trzA* and *trzB* on the basis of genetic analysis. Locus *trzA* is cotransducible with *cysA*. It has been found that triazole does not prevent, in these mutants, the induction of cysteine enzymes by OAS. *trzB*, plasmid-like mutants, will be the subject of a separate report.

METHODS

Strains. All bacterial strains used were derivatives of *Salmonella typhimurium* LT-2 and are listed in Table 1. The transducing phage was the L4 mutant of phage P22 (Smith, 1968).

Culture media. Sulphur-free minimal medium (BS) was the medium C of Vogel & Bonner (1956) in which magnesium sulphate was replaced by magnesium chloride.

[1] Abbreviations: triazole, 1,2,4-triazole; CT, 3-chloro-1,2,4-triazole; ATT, 5-amino-1,2,3,4-tetrazole; OAS, *O*-acetyl-L-serine; NG, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine.

Ammonium sulphate was added to final concentrations of 0.1 mM or 1.0 mM where indicated; 0.1 M solution of L-cystine or L-djenkolic acid in 1.0 N-HCl were added to a final concentration of 1.0 mM (Kredich, 1971).

Table 1

Strains of Salmonella typhimurium *used*

Strain designation	Genotype	Source
LT-2	wild type	B. N. Ames
	purF145	P. E. Hartman
	argD5	K. E. Sanderson
	cysA20	D. A. Smith
	purC7	J. S. Gots
	argE116	K. E. Sanderson
	purD312	J. S. Gots
	purB12	J. S. Gots
TK168	*trzA168 purF145*	J. Bogusławski
TK292	*hisD3512 purF145 HfrK5*	W. Walczak
TK293	*his3501 purF145 HfrK5*	W. Walczak
TK302	*hisD3512 HfrK5*	by transduction of TK292
TK303	*trzA54 purF145*	isolated in *purF145*
TK306	*trzA45 hisD3512 HfrK5*	isolated in TK302
TK307	*trzB46 hisD3512 HfrK5*	isolated in TK302
TK310	*trzA48 hisD3512 HfrK5*	isolated in TK302
TK311	*trzA48 cysL1360 hisD3512 HfrK5*	isolated in TK310
TK312	*his3517 HfrK2*	by transduction of TK627
TK313	*his3517 trzA21 HfrK2*	isolated in TK312
TK318	*trzA55 his3501 purF145 HfrK5*	isolated in TK293
TK320	*purE8 trzA69 HfrK4*	isolated in SA540
TK627	*his3517 serA13 HfrK2*	W. Walczak
DW25	*cysB1352*	N. M. Kredich
SA540	*purE8 HfrK4*	K. E. Sanderson

Final concentration of glucose was 0.2%. For growth of auxotrophs, adenine (0.2 mM) with thiamine (0.01 mM) or amino acids (0.1 mM) were added. Medium C containing 2% glucose was solidified by addition of 2% agar.

Culture conditions. Liquid cultures were grown at 37°C with aeration and their growth followed turbidimetrically at 420 nm with a Unicam SP 500 spectrophotometer. Optical densities were converted to μg dry weight using a calibration curve.

Mating was performed by mixing male and female strains in 1:10 ratio and plating on selective plates.

Phage lysates. Log phase bacteria in broth medium were infected with phage L4-P22 at multiplicity of 0.1 and incubation continued for 8 - 18 h, followed by centrifugation at 6600 *g* for 20 min. Phage was then spun down at 32 000 *g* for 50 min, the pellet was soaked for 2 h in medium C, phage resuspended by vortexing and kept over chloroform at 4°C.

Transduction. Aliquots (0.1 ml) of overnight bacterial cultures in broth infected with phage at a multiplicity of at least 5 were spread on selective solid medium and incubated for 36 - 48 h at 37°C. Screening for triazole and selenate resistance was done by replica-plating on selective plates containing 10 mM-triazole or 2 mM-selenate.

Assay of sulphate-activating enzymes. The assay was based on the method of Pasternak (1962). Log phase bacteria were harvested, resuspended in 100 mM--tris-HCl buffer (pH 8.0) containing 5 mM-EDTA, and disrupted by sonification. After centrifugation at 10 000 *g* for 30 min the extract was passed through a Sephadex G-50 column equilibrated with tris buffer and centrifuged at 105 000 *g* for 3 h.

The incubation mixture contained in a volume of 0.5 ml: sodium ATP (3 μmol), magnesium chloride (3.7 μmol), potassium sulphate (0.3 μmol), carrier-free sodium [^{35}S]sulphate (10 μCi), tris-HCl buffer (pH 8.0, 25 μmol) and 0.12 - 1.25 mg protein. After 30 min incubation at 21°C the reaction was stopped by boiling for 2 min. For controls either ATP or the bacterial extract was omitted. Following centrifugation at 6600 *g* the supernatant was mixed with 90 mg of activated charcoal in 2 ml of 100 mM-sodium acetate buffer (pH 5.8) containing 30 mM-sodium sulphate (under these conditions 10 μmol of ATP as a model adenosine compound is adsorbed). After 10 min the suspension was passed through a membrane filter (Biomed Coli 5, average pore size 0.5 μ), the deposited charcoal washed extensively with acetate buffer, dried under infrared lamp and the radioactivity on the filter was counted with a thin mica window Geiger-Müller tube. A linear relationship between radioactivity and the quantity of the samples was obtained over the range of 0.12 - - 1.2 mg protein.

In an alternative method the enzymes were assayed in bacteria which had been washed with tris buffer and shaken with 10 μl of toluene per ml for 15 min at 37°C.

Assay of sulphite reductase. The method of de Vito & Dreyfuss (1964) was used.

Protein determination. Protein content in cell-free extracts was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Chemicals. O-Acetyl-L-serine (OAS) was synthesized by the method of Wiebers & Gardner (1967). 3-Chloro-1,2,4-triazole and 5-amino-1,2,3,4-tetrazole were kindly provided by Dr. J. L. Hilton. All other chemicals used were commercial products: disodium ATP (Sigma, St. Louis, Mo., U.S.A.), L-amino acids and 1,2,4- -triazole (Calbiochem, Los Angeles, Calif., U.S.A.), carrier-free sodium [^{35}S]sulphate (Instytut Badań Jądrowych, Świerk, Poland) and chloramphenicol (Polfa, Warszawa, Poland).

RESULTS

Isolation, mapping and properties of trz *mutants of* S. typhimurium. It was established previously that 2 mM-triazole in liquid medium inhibited transiently growth of *S. typhimurium* (Bogusławski, Walczak & Kłopotowski, 1967). However, growth on solid medium containing 10 mM-triazole was permanently inhibited. Therefore mutants resistant to triazole (*trz*$^-$) could be isolated on minimal agar plates con-

taining 10 mM-triazole and inoculated with about 2×10^8 bacteria. After three days of incubation 10 - 60 colonies appeared per plate. The number of *trz* mutants could be increased by putting a few crystals of NG, diethylsulphate or ICR 191 in centers of the plates seeded with bacterial cells.

Mutagen-induced mutants were isolated from dense rings of resistant colonies growing aroung inhibition zones produced by the mutagens.

For rough location of *trz* mutations, male *trz* mutants were crossed with female auxotrophic recipients and the prototrophic recombinants were scored for *trz* character by replication on plates containing 10 mM-triazole. The mapping data of typical *trz* mutants are shown in Table 2. The results obtained indicated the presence of two distinct groups of *trz* mutants. Mutants designated *trzA* are phenotypically stable and gave normal linkage pattern with several chromosomal markers, the highest linkage values being obtained with *purC* and *cysA* recipients. These genes are localised at 80 min of *Salmonella* map (Sanderson, 1970). The linkage with *purC* was about 80%, and that with *cysA* was always over 90%.

Table 2

Mapping of trz *mutants by conjugation*

The number of tested recombinant colonies from crosses with *trzA45* (TK306) and *trzB46* (TK307) were 100 and 200, respectively.

Recipient	Map position (min)	Donors			
		trzA45 (TK306)		*trzB46* (TK307)	
		trz colonies	linkage (%)	*trz* colonies	linkage (%)
argD6	11	20	20	0	<0.5
purB12	45	18	18	0	<0.5
cysA20	79	98	98	26	13.0
purC7	79	82	82	0	<0.5
argE116	102	46	46	0	<0.5
purD312	126	38	38	0	<0.5

Mutants of the second group designated *trzB* were phenotypically unstable and gave linkage only with *cysA*; the linkage values differed significantly from those obtained with *trzA* mutants as the linkage was never higher than 20%.

About 70% of spontaneously arising *trz* mutants were found to be *trzA*; however, 96% of ICR 191-induced *trz* mutants were found, on the basis of their phenotypic instability, to be *trzB*.

Fine mapping of these mutations was performed by transduction and the results are shown in Table 3. All of the *trzA* mutants tested were cotransducible with *cysA*. *CysA* gene is also cotransducible with a mutation, designated as *cysL*, which rendered bacteria resistant to selenate (Hulanicka, 1970).

As the three mutations: *cysA*, *cysL* and *trzA* are contransducible, the gene order in this region could be established by transduction. In order to perform three-point

Table 3

Mapping of trzA *mutants by transduction*

Strain *cysA20* was recipient for all the crosses; the number of tested colonies in each cross was 200.

Donors	*trz* colonies	linkage (%)
trzA54 (TK303)	96	48
trzA55 (TK318)	82	41
trzA48 (TK310)	102	51
trzA45 (TK306)	88	44

crosses, double mutants *cysL trzA* were isolated by direct selection on the plates containing selenate (2 mM). Strain *trzA48* (TK310) was used for isolation of selenate-resistant mutant. Phage grown on *trzA48 cysL1360* (TK311) was used as donor with *cysA20* as a recipient. The results are shown in Table 4. The rarest recombinant class was $cysA^+$ $cysL^+$ trz^-. This result points to gene order *cysA cysL trzA*. Linkage with *cysA*, calculated from the data of Table 4, is: *cysL* — 93%; *trzA* — 50%. These values are consistent with the gene order deduced from the frequency of recombinant classes.

Table 4

Three-point transduction cross

Donor: *trzA48 cysL1360* (TK311) Recipient: *cysA20* Selected marker: *cys*					Possible gene orders: a — *cysA cysL trzA* b — *cysL cysA trzA* c — *cysA trzA cysL*	
Unselected recombinant type		Number of crossovers required for the indicated gene order			Unselected type found	
cysL	*trzA*	a	b	c	number	%
+	+	2	2	2	36	6.7
+	48	4	2	2	5	0.9
1360	+	2	2	4	232	43.2
1360	48	2	2	2	264	49.2
Total					537	100.0

Similar results were obtained in crosses in which point mutants in cistrons *a*, *b* or *c*, of gene *cysA* were used as recipients.

Growth behaviour of typical *trzA* and *trzB* mutants and their parental strain is shown in Fig. 1. It can be seen that 2 mM-triazole affects the growth of trz^+ pa-

rental strain. However, even 20-fold higher concentration of triazole (40 mM) did not noticeably affect growth of either *trzA* or *trzB* mutants, and no difference was found between *trzA* and *trzB* mutants in the degree of resistance to triazole.

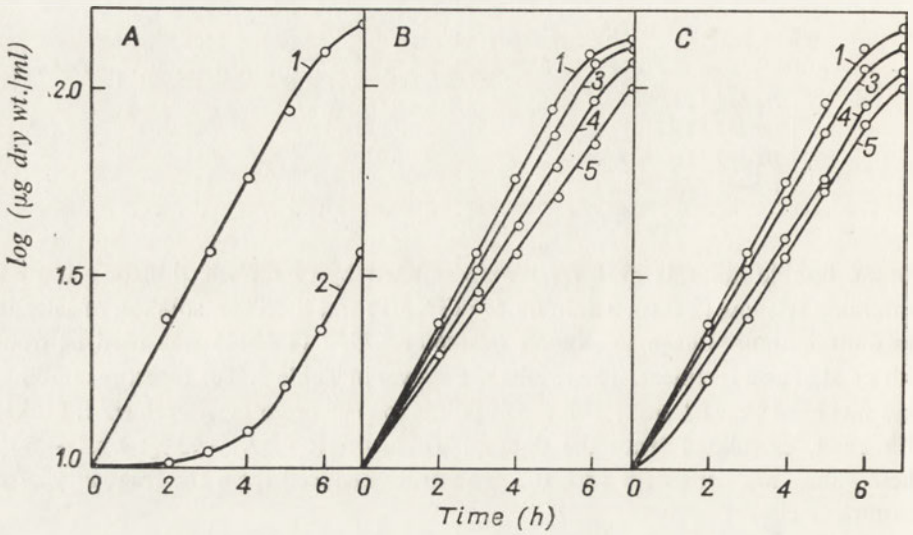

Fig. 1. Resistance of *trz* mutants to triazole. BS medium + sulphate (0.1 mM) was inoculated with washed bacterial suspension to a final density of 10 µg dry wt/ml. *A*, TK302 (*trz*$^+$); *B*, TK306 (*trzA*); *C*, TK307 (*trzB*); *1*, control without triazole; triazole added: *2*, 2 mM; *3*, 10 mM; *4*, 20 mM; *5*, 40 mM.

Response of trzA *mutants to triazole compounds.* Several triazole compounds are known to inhibit growth of *S. typhimurium* (Hilton & Kaufman, 1968). The effect of some of these derivatives on parental strain and *trzA* mutants are shown in Table 5. As may be seen from these data: (1) The effect of ATT was similar to that of triazole while that of CT was stronger. (2) *TrzA* mutants were also resistant to ATT and slightly less resistant to CT. (3) The inhibitory effect of triazole and ATT on *trz*$^+$ strain was fully reversed by cysteine, methionine or serine. The inhibitory effect of CT on *trz*$^+$ strain was reversed exclusively by cysteine whereas the inhibition of *trzA* mutants was abolished also by methionine or serine. (4) Glycine potentiated the effect of triazole and ATT but not of CT in wild type. (5) Glycine did not influence the effect of triazole on *trzA* mutants, however, it enhanced the effect of ATT and CT on these mutants.

Resistance of cysteine constitutive mutants to triazole. Some *cysB* mutants have high constitutive level of cysteine biosynthetic enzymes even in the presence of cysteine (Spencer, Collins & Monty, 1967; Kredich, 1971). It seemed of interest to check the resistance of such mutants to inhibitors of the cysteine pathway, selenate and triazole. It appeared that constitutive cysteine mutant *cysB1352* (DW25) was resistant to both inhibitors, whereas *trzA* mutants were resistant only to triazole. The resistance of DW25 to triazole was of the same order

of magnitude as that of *trz* mutants. Among several hundred newly isolated *trz* mutants, none has shown linkage with *cysB* region and none was resistant to selenate.

Table 5

Reversion and potentiation of growth-inhibitory effect of triazole and its analogues

Bacteria, 2×10^8, and L-cysteine (20 µmol), L-methionine (20 µmol), L-serine (200 µmol), or glycine (200 µmol) were added to 2 ml of melted 0.6% agar in isotonic saline kept at 45°C, mixed and poured over minimal agar plate containing 0.1 mM-L-histidine. After solidification three thick sterile filter paper discs were put on each plate. To the discs 10 µmol of triazole, 8 µmol of ATT or 8 µmol of CT were pipetted. After overnight incubation at 37°C, inhibition zones were measured as net radius in mm (i.e. distance between the edge of the disc and the outer limit of the zone).

Compound tested	Inhibition zone (net radius mm)	
	TK302 (*trz*$^+$)	TK306 (*trzA*)
Triazole	3	0
Triazole+cysteine	0	0
Triazole+methionine	0	0
Triazole+serine	0	0
Triazole+glycine	9	0
Aminotetrazole (ATT)	3	0
Aminotetrazole+cysteine	0	0
Aminotetrazole+methionine	0	0
Aminotetrazole+serine	0	0
Aminotetrazole+glycine	8	6
Chlorotriazole (CT)	17	13
Chlorotriazole+cysteine	0	0
Chlorotriazole+methionine	15	0
Chlorotriazole+serine	3	0
Chlorotriazole+glycine	17	17

Effect of triazole on the induction of cysteine enzymes in trzA *mutants.* Hulanicka *et al.* (1972) reported that triazole prevented OAS-mediated induction of sulphate permease and sulphate-activating enzymes in *S. typhimurium.* Similar experiments were performed with *trzA* mutants.

Logarithmic phase cultures of parental strain and *trzA* mutant were supplemented with OAS, triazole and OAS, or triazole only. After 2 h incubation, bacteria were harvested and assayed for sulphate-activating enzymes. The data (Table 6) show that OAS induced sulphate-activating enzymes in both *trz*$^+$ and *trzA* strains. In the parental *trz*$^+$ and in *trzA* strains the induction was 5-7-fold. However, in the *trz*$^+$ strain practically no induction by OAS in the presence of triazole was observed, whereas in *trzA* mutants the addition of triazole did not affect significantly the induction of the enzymes activating sulphate.

These findings suggest that triazole resistance of *trzA* mutants could result from the lack of triazole effect on the induction of sulphate-activating enzymes.

Table 6

Effect of triazole on the induction of sulphate-activating enzymes in trzA *mutant and wild type strain*

Cells were grown on BS medium, OAS (1 mM) or triazole (2 mM) were added where indicated. The activity of sulphate-activating enzymes in the control was taken as 1.00.

Supplements	Sulphate-activating enzymes	
	TK293 (*trz*$^+$)	TK318 (*trzA55*)
None (control)	1.00	1.0
OAS	6.7	5.5
Triazole	1.17	0.95
OAS+triazole	1.38	5.0

The activity of cysteine enzymes in trzA *mutants.* Bacteria resistant to inhibitory compounds have often been shown to have regulatory anomalies of the pathway affected by the inhibitors. Therefore activity of cysteine enzymes were assayed in *trzA* mutants grown on different sulphur sources in order to check the possibility that triazole resistance could result from the derepression of cysteine enzymes. It can be seen (Table 7) that the level of the assayed enzymes: sulphate-activating enzymes and sulphite reductase are 2 - 3 times higher in *trzA* than in parental strains. The degree of derepression was slightly lower than that of the constitutive *cysB1352* strain (DW25).

Table 7

The activity of sulphate-activating enzymes and sulphite reductase in trzA *mutants and parental strains growing on different sulphur sources*

The activity of either enzyme in parental strains grown on sulphate was taken as 1.00. The growth medium contained as sulphur source: 0.1 mM-sulphate, 0.1 mM-L-cystine or 0.1 mM-L-djenkolate.

Strain	Sulphate-activating enzymes			Sulphite reductase		
	Sulphate	Cystine	Djen-kolate	Sulphate	Cystine	Djen-kolate
Experiment 1						
SA540 (*trz*$^+$)	1.00	—	4.90	1.00	—	5.60
TK320 (*trzA69*)	2.63	—	4.15	3.10	—	5.56
Experiment 2						
TK312 (*trz*$^+$)	1.00	0.02	4.20	1.00	0.06	2.80
TK313 (*trzA21*)	2.18	0.32	3.70	1.60	0.10	2.32
Experiment 3						
purF145 (*trz*$^+$)	1.00	0.30	4.44	1.00	0.04	1.70
TK168 (*trzA168*)	3.30	0.13	5.20	1.54	0.12	2.70
DW25 (*cysB1352*)	3.80	2.70	5.60	3.70	3.70	4.55

It is known that the activity of the enzymes involved in cysteine biosynthesis depends to a large extent on sulphur source in the growth medium. High activity

of cysteine biosynthetic enzymes was found in the cells grown on poor sulphur sources such as L-djenkolate, on the other hand, cells grown in the presence of good sulphur source as sulphide or L-cysteine, had low activity (Dreyfuss & Monty, 1963; Kredich, 1971).

It is apparent from the data presented in Table 7 that in *trzA* mutants the assayed enzymes were repressed on L-cysteine-containing medium. Cysteine enzymes of DW25 were not repressed by L-cystine. In this respect *trzA* mutants differed also from the constitutive cysteine mutant.

DISCUSSION

We have previously reported that triazole inhibits cysteine biosynthesis in *S. typhimurium* by interfering with induction of enzymes of this pathway mediated by OAS (Hulanicka *et al.*, 1972). This suggested that in mutants resistant to triazole induction should proceed normally in the presence of triazole.

In this study we have isolated mutants of *S. typhimurium* resistant to triazole. They were classified into two groups on the basis of their phenotypic stability and linkage with chromosomal markers. Stable *trzA* mutants were found to map close to *cysA* gene coding for sulphate permease (Sanderson, 1970). As expected, in these mutants triazole did not inhibit the induction of sulphate-activating enzymes by OAS. This result suggests that *trzA* mutations affect a factor mediating triazole effect on the induction.

The lack of triazole interference with induction in *trzA* mutants could result from the fact that triazole does not penetrate into *trzA* cells. However, this alternative cannot be reconciled with the higher level of cysteine biosynthetic enzymes observed in *trzA* mutants (Table 7).

In addition, *trzA* mutants differed from triazole-sensitive strains by their higher activity of cysteine biosynthetic enzymes. Sulphite reductase activity in the individual *trzA* mutants grown on sulphate as a sole sulphur source was 1.5 - 3.1 times higher than that in parental strains.

Properties of *trzA* mutants were therefore compared with those of a mutant showing high activity of all the enzymes of cysteine biosynthesis and mapping in *cysB* locus. In *cysB* cells grown on sulphate medium sulphite reductase activity was twice as high as in the wild-type *S. typhimurium* strain LT-2 (Kredich, 1971).

As we have found, the resistance of mutant *cysB1352* was of the same order of magnitude as that of *trzA* mutants. However, their behaviour towards selenate, an inhibitor of sulphate permease and sulphate-activating enzymes (Hulanicka *et al.*, 1972) was different: *cysB1352* was resistant, but all the *trzA* mutants tested were sensitive.

Moreover, repressibility of cysteine biosynthetic enzymes was different in these mutants: growth in media containing cystine depressed the activities of sulphite reductase and sulphate-activating enzymes in *trzA* mutants, whereas these enzymes in *cysB1352* were not repressed. On the other hand, these mutants did not differ

in their response to poor sulphur sources such as djenkolate, or to OAS in sulphate medium (Kredich, 1971).

Other mutants of *cysB* gene showed low activity of all the enzymes of cysteine biosynthesis (Kredich, 1971). Therefore, *cysB* product seems to be involved in both repression and induction of cysteine biosynthesis. On the other hand, since *trzA* mutants are repressible by cysteine and another phenotype of *trzA* mutants is not known, it seems that *trzA* product is involved only in induction of cysteine enzymes.

The technical assistance of Mrs. W. Chabros is gratefully acknowledged.

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MUTANTY *SALMONELLA TYPHIMURIUM* OPORNE NA TRIAZOL

Streszczenie

1. Wyizolowano mutanty *S. typhimurium* oporne na triazol; mutanty jednej grupy, nazwane *trzA*, są w 50% kontransdukowalne z genem *cysA*.

2. Stwierdzono, że triazol u mutantów *trzA* nie ma wpływu na indukcję enzymów cysteinowych wywołaną dodaniem *O*-acetylo-L-seryny.

3. Poziom enzymów aktywujących siarczan oraz reduktazy siarczynowej u mutantów *trzA* jest 2 - 3 razy wyższy niż u macierzystych szczepów.

Received 8 March, 1972.

ACTA BIOCHIMICA POLONICA

Vol. 19 1972 No. 3

CELINA JANION

HYDROXYLAMINE MUTAGENESIS: ULTRAVIOLET ABSORPTION SPECTRA AND TAUTOMERIC FORMS OF N^4-HYDROXY, N^4-METHOXY AND N^4-METHYL,N^4-HYDROXY DERIVATIVES OF CYTOSINE, AND 5- AND 6-METHYLCYTOSINES

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1. Derivatives of methoxyamine and *N*-methylhydroxylamine cytosines have been obtained and their ultraviolet spectra in acid, basic and neutral forms examined. 2. The comparison of p*K* values, related to dissociation of the N^4-hydroxyl group in the investigated compounds, in the fixed amino, imino, or undetermined forms, and the comparison of their ultraviolet absorption spectra, indicated that N^4-hydroxy and N^4-methoxy derivatives of cytosine exhibit the imino form. 3. The dependence of ultraviolet spectra on alkaline pH values, observed for N^4-methoxy derivatives of cytosines, may be due alternatively to imino-amino tautomerism, or a loss of the proton from the ring $N_{(3)}$. 4. The mechanism of mutagenical action of hydroxylamine, methoxyamine and *N*-methylhydroxylamine in the light of their chemical similarities and differences, has been reviewed and discussed.

It is generally assumed that point mutations, i.e. those for which the genome exhibits differences confined to only one base-pair, result from the modification of a single base with concomitant modification of the base pairing normally encountered in twin-stranded DNA. It is presumed that such modifications may be due to: 1. a change in the tautomeric form of a naturally occurring base in DNA (spontaneous mutations); 2. to the presence in the replicating DNA strands, or in the precursor pool of base analogues with an enhanced ability either for tautomerization or for ionization at physiological pH (e.g. 5-bromouracils); 3. to a change in tautomeric form due to modification of a base by mutagen (e.g. chemical mutagen, radiation).

The use of various mutagenic agents is now widespread; however, the selection of a given agent should be dependent on the ability to determine the nature of the chemical modification it induces, since this is the only, although as yet rarely applied, method of evaluating the relationship between modification of the genome and the mechanism leading to this. Ideally, what we require is a mutagen which will react

with only one constituent of the genome to give a single defined product, being at the same time metabolically inert *in vivo*.

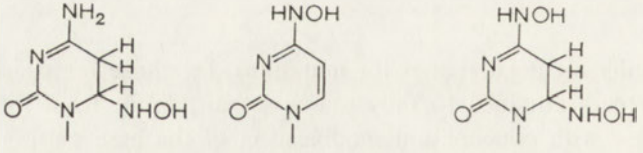
One of the more specific chemical mutagens is hydroxylamine, HA[1], as well as its *O*- and *N*-methyl analogues, NMeHA and OMeHA.

The present communication describes some of the products of reaction of cytosine with *O*- and *N*-methylated derivatives of HA: N^4-methyl, N^4-hydroxycytosine and N^4-methoxycytosine which, in addition to N^4-hydroxycytosine, are believed to be mutagenically significant. Some of these compounds have already been reported by Brown, Hewlins & Schell (1968), but their properties were not fully characterized. Furthermore, some emphasis is placed in this study on the properties of 5-substituted cytosines, which are of importance in relation to mutagenesis in the T-even bacteriophages and which have been little studied.

Reactions of HA, NMeHA and OMeHA with nucleic acid bases

Both biological and chemical investigations indicate that the mutagenic action of HA at concentration exceeding 1 M is the result of reaction with residues of cytosine or 5-substituted cytosines.

The reaction of HA with cytosine (or its glycosides) leads to formation of an extremely labile hypothetical compound I and of the two products: N^4-hydroxycytosine (II) and N^4-hydroxy-5,6-dihydro-6-hydroxylaminocytosine (III, Scheme 1). At slightly acid pH the latter readily eliminates HA from the 5,6 bond to give II; the ratio of II to III being dependent on HA concentration, pH of the reaction medium, temperature, and the form of cytosine residue (Janion & Shugar, 1968; Budowsky, Sverdlov, Shibaeva, Monastyrskaya & Kochetkov, 1971).



I II III

Scheme 1

With 5-substituted cytosines (e.g. 5-methylcytosine or 5-hydroxymethylcytosine) only one reaction product is formed with HA in aqueous medium, viz. that corresponding to II (Janion & Shugar, 1965a) from which it follows that the known mutagenic action of HA on the T-even bacteriophages, which contain only 5-hydroxymethylcytosine or glucosylated 5-hydroxymethylcytosine residues in place of cytosine, must be due to formation of analogues of the type II. This, in turn, has led

[1] The following abbreviations are employed: HA, hydroxylamine, NH_2OH; NMeHA, *N*-methylhydroxylamine, CH_3NHOH; OMeHA, methoxyamine, NH_2OCH_3.

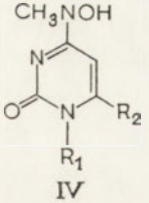
to the belief that products such as II are responsible for mutagenic effects of HA on other biological systems.

HA may also react with uracil (but not thymine), and not only at alkaline pH, as formerly presumed but even at neutral pH (Domkin, Pogorelov, Shibaev, Budowsky & Kochetkov, 1970) with formation of 5,6-dihydro-6-hydroxylaminouracil. It has been shown by studies on phage f2 that this does not lead to mutations (Budowsky & Pashneva, 1971). More recently it has been found that HA will likewise react with adenine, at a rate about 200-fold less than that with cytosine (Budowsky, Sverdlov & Monastyrskaya, 1971; Brown & Osborne, 1971). Various lines of evidence (Tessman, Poddar & Kumar, 1964; Brenner, Stretton & Kaplan, 1965; Vanderbilt & Tessman, 1970), including the fact that HA induces GC → AT transitions, argue against any mutagenic significance of this reaction.

OMeHA reacts with cytosine residues much the same as HA, the products of reaction differing from those formed by the latter only in that -OH is replaced by $-OCH_3$. By contrast it does not react at all with uracil (or thymine) (Kochetkov, Budowsky & Shibaeva, 1963) and to an even lesser extent with adenine than HA (Budowsky, Sverdlov & Monastyrskaya, 1971).

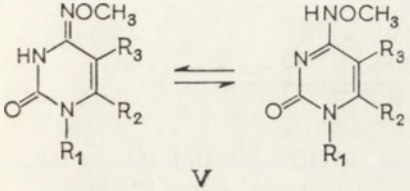
NMeHA, on the other hand, reacts uniquely with cytosine, the principal product being N^4-methyl,N^4-hydroxycytosine, i.e. an analogue of II. The presence of a product similar to III has been demonstrated, but the ease with which this is transformed to the analogue of II has made its isolation difficult if not impossible. Formation of an analogue of type III is further reduced in the reaction of NMeHA with cytosine glycosides. Furthermore, as a result of steric hindrance, NMeHA does not react with 5-substituted cytosines, in agreement with its reported lack of mutagenic activity against the T-even phages (Janion & Shugar, 1971).

If we ignore the side reactions involving HA and NMeHA at concentrations below 1 M, the mutagenic effects of which are less clear (Bautz-Freese, Gerson, Taber, Rhaese & Freese, 1967), the one feature common to HA, NMeHA and OMeHA as mutagens is their ability to react with cytosine residues in the genome



IV

a — H H — N^4-methyl,N^4-hydroxycytosine
b — CH_3 H — 1,N^4-dimethyl,N^4-hydroxycytosine
c — CH_3 CH_3 — 1,6,N^4-trimethyl,N^4-hydroxycytosine



V

a — H H H — N^4-methoxycytosine
b — CH_3 H H — 1-methyl-N^4-methoxycytosine
c — CH_3 H CH_3 — 1,5-dimethyl,N^4-methoxycytosine
d — CH_3 CH_3 H — 1,6-dimethyl,N^4-methoxycytosine

Scheme 2

to form products of type II. There are nonetheless certain differences between the individual products, which we shall now examine in the light of their UV spectra.

UV spectra of products of reaction of cytosine with HA and its methyl analogues

The compounds embraced in this study may be divided into two classes: (a) derivatives of N^4-methyl,N^4-hydroxycytosine, which can exist only in the amino form; (b) derivatives of N^4-methoxycytosine, where amino-imino tautomerism is possible (see Scheme 2). All of these exhibit pH-dependent modifications of their absorption spectra related to the presence of two functional groups.

Spectra of acid forms. Figures 1*a*, *b*, and *c* (see also Table 1) exhibit the spectra in neutral and acid media of N^4-methyl,N^4-hydroxycytosine and its analogues and Figs. 2*a* - 2*d* the spectra for the corresponding N^4-methoxycytosine derivatives.

From a comparison of the limiting spectral curves at neutral pH, it can be seen that the spectra of the N^4-methyl-N^4-hydroxycytosine, although they exhibit a ten-

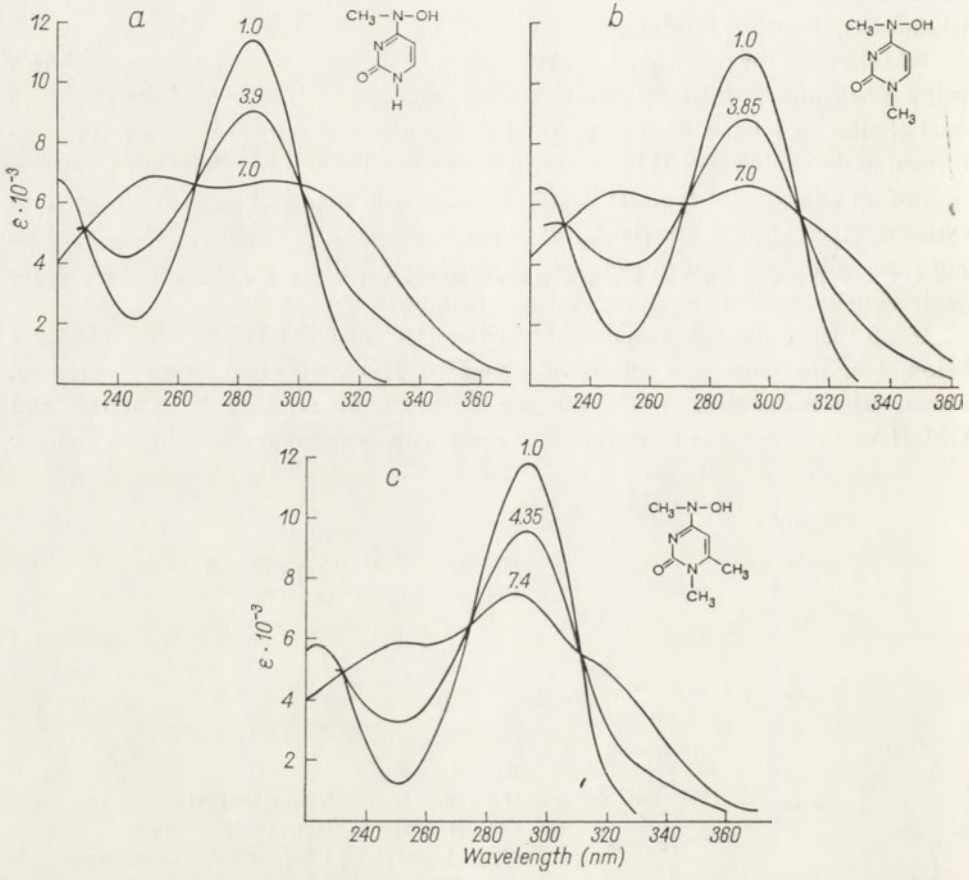


Fig. 1. Ultraviolet absorption spectra in aqueous medium, at pH values indicated, showing the neutral and acid forms of: (*a*), N^4-methyl,N^4-hydroxycytosine; (*b*), 1-methyl,N^4-methyl,N^4-hydroxycytosine, and (*c*), 1,6-dimethyl-N^4-methyl,N^4-hydroxycytosine.

dency towards the formation of two peaks, which is characteristic for the N^4-methoxy derivatives, nonetheless differ markedly from the latter. This could conceivably be due to some real difference in structure or, more likely, the presence on the exocyclic nitrogen of both methyl and hydroxy groups.

An illustration of the modification induced by an N^4-substituent is provided by the spectrum of 1-methyl,N^4-methyl,N^4-methoxycytosine (Fig. 3), which is in the fixed amino form, like IV, but with the N^4 substituent $-OCH_3$ in place of -OH. The resultant change in the spectrum is striking, it resembles rather 1,N^4,N^4-trimethyl cytosine (Szer & Shugar, 1966) rather than V.

Furthermore the spectra of the neutral forms of the N^4-methoxy derivatives in

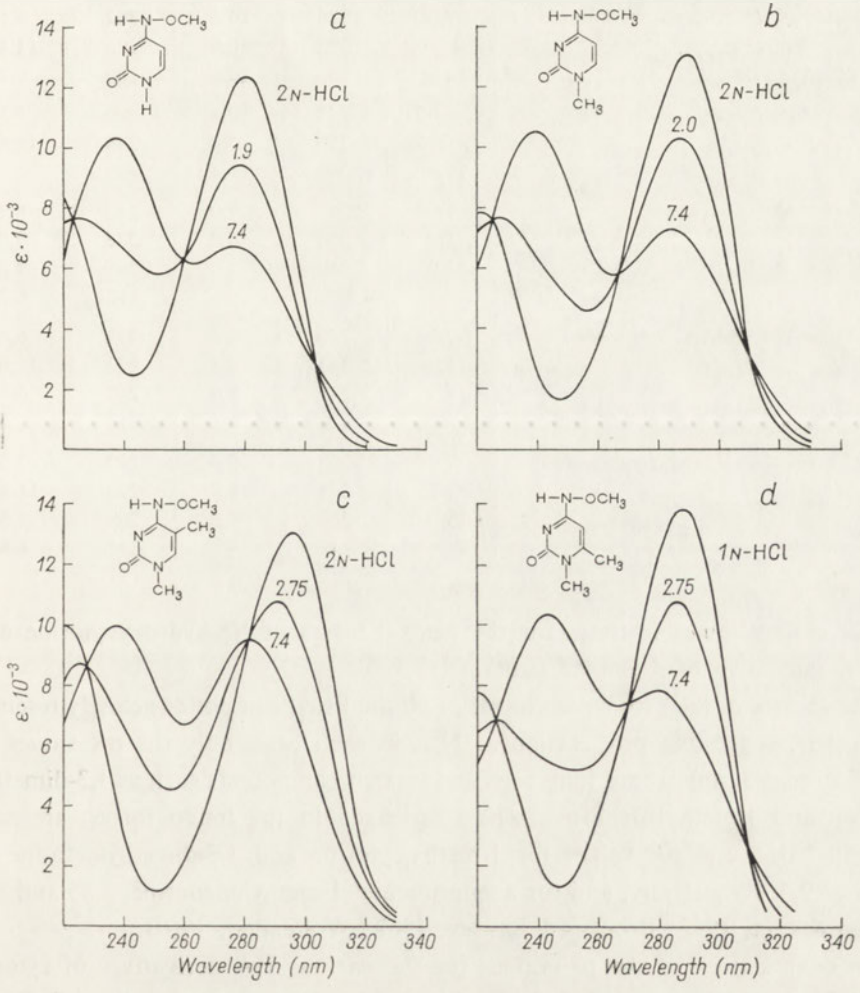


Fig. 2. Absorption spectra in aqueous medium, at pH values indicated, showing the neutral and acid forms of: (*a*), N^4-methoxycytosine; (*b*), 1-methyl-N^4-methoxycytosine; (*c*), 1,5-dimethyl,N^4--methoxycytosine, and (*d*), 1,6-dimethyl,N^4-methoxycytosine.

Table 1

Molar extinction coefficients of analogues of N^4*-methoxycytosine and* N^4*-methyl,*N^4*--hydroxycytosine*

Compounds	pH	λ_{max} (nm)	ε_{max} $\times 10^{-3}$	λ_{max} (nm)	ε_{max} $\times 10^{-3}$
N^4-Methoxycytosine	7.00	238	10.3	275	6.7
	2N-HCl			280	12.3
	14.00	251	10.4	292	6.1
1-Methyl,N^4-methoxycytosine	7.00	240	10.7	285	7.3
	2N-HCl	220	7.8	290	13.0
	14.00	258	11.6		
1,5-Dimethyl,N^4-methoxycytosine	7.00	238	10.0	282	9.5
	2N-HCl	222	10.0	295	13.1
	14.00	258	10.6	280	9.2
1,6-Dimethyl,N^4-methoxycytosine	7.00	245	10.4	280	7.8
	1N-HCl	220	7.8	288	13.8
	14.00	260	11.4		
N^4-Methyl,N^4-hydroxycytosine	7.00	253	7.0	292	6.7
	1.0	220	6.8	285	9.0
	13.0	240	9.0	265	7.2
				312	6.2
1,N^4-Dimethyl,N^4-hydroxycytosine	7.00	250	6.4	292	6.6
	1.0	223	6.5	292	11.0
	13.0	242	8.9	267	6.4
				317	5.5
1,6,N^4-Trimethyl,N^4-hydroxycytosine	7.00	253	5.9	290	7.5
	1.0	223	5.8	292	11.8
	13.0	242	8.2	268	6.5
				312	6.6

general closely resemble those for the neutral forms of N^4-hydroxycytosine derivatives, indicative of a similar structure.

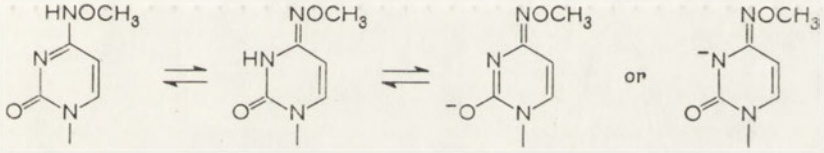
The spectra of the protonated forms of all the listed compounds closely resemble each other, suggesting protonation at $N_{(3)}$ in each case, only the pK values for protonation differing. It has long been known that compounds such as 1,3-dimethylcytosine and 1-methyladenosine, which are fixed in the imino forms, are more strongly basic, e.g. pK values for 1-methylcytosine and 1,3-dimethylcytosine are 4.55 and 9.4, respectively, and for adenosine and 1-methyladenosine, 3.65 and 8.25 (Shugar & Fox, 1952; Brookes & Lawley, 1962; Wolfenden, 1969).

An examination of the pK values for the various HA derivatives of cytosine (Table 2) shows that, with reference to N^4-methyl,N^4-hydroxycytosine, all possess pK values shifted to lower values characteristic for the amino forms. On the other hand, the fixed imino derivative 1,3-dimethyl,N^4-hydroxycytosine has a pK of

2.7, which led Brown *et al.* (1968) to conclude that N^4-hydroxycytosine derivatives are in the imino form. Therefore the presence of oxygen at the exocyclic nitrogen greatly influenced the basicity of $N_{(3)}$.

From the pK_a values listed in Table 2, it will further be noted that replacement of $H_{(6)}$ by a methyl group markedly influences the basicity of the ring, as previously observed for N^4-hydroxycytosine derivatives (Janion & Shugar, 1965b).

Spectra of basic forms. Table 2 includes the pK_b values for the various derivatives in alkaline medium. Figs 4*a*, *b* and *c* exhibit the spectra of the neutral and alkaline forms of N^4-methyl,N^4-hydroxycytosines from which these pK values were calculated; and Figs, 5*a*, *b*, *c* and *d* the corresponding spectra of N^4-methoxycytosines. A comparison of the spectra of the free bases and their $N_{(1)}$-methyl analogues shows that, notwithstanding the possibility of the unsubstituted bases to undergo enolization, due to the presence of a hydrogen on $N_{(1)}$ ($N_{(1)}H—C_{(2)}=O \leftrightarrow N_{(1)}= =C_{(2)}OH$), such enolization apparently does not occur, at least over the pH range herein examined. The forms of the spectra, as well as the isosbestic points, testify to the presence of only one functional group, the origin of which appears to be different for each group of compounds: (a) in the case of the N^4-methyl,N^4-hydroxy derivatives, the changes in absorption in alkaline medium can originate only from the hydroxyl substituent on N^4; (b) by contrast two possibilities may be envisaged for the N^4-methoxy derivatives, either amino-imino tautomerism or ionization of the hydrogen on the ring $N_{(3)}$, these being shown schematically in Scheme 3.



VI VII VIII IX

Scheme 3

UV absorption spectra alone do not provide sufficient information to resolve the origin of the foregoing modifications, at least with the derivatives presently available.

The idea that the alkali-induced spectral modifications are related to amino--imino tautomerism is particularly attractive. Notwithstanding the numerous studies and discussions on the possibility of the existence of amino purines and pyrimidines at least partially in the imino form, it is not known (at least there is no experimental data) whether the transition from the amino to imino form (or conversely) is accompanied by such marked spectral changes as in the case of keto-enol tautomerism.

Table 2

pK values for various derivatives spectrally determined at 20°C

Compounds	pK_a	pK_b
N^4-Methoxycytosine	1.9	11.1
1-Methyl,N^4-methoxycytosine	2.0	11.65
1,5-Dimethyl,N^4-methoxycytosine	2.05	12.10
1,6-Dimethyl,N^4-methoxycytosine	2.75	11.75
N^4-Methyl,N^4-hydroxycytosine	3.9	8.8
1,N^4-Dimethyl,N^4-hydroxycytosine	3.85	9.1
1,6,N^4-Trimethyl,N^4-hydroxycytosine	4.35	9.1
1,3-Dimethyl,N^4-hydroxycytosine	2.7*	12.3
1,N^4-Dimethyl,N^4-methoxycytosine	3.65	—
1-Methyl,N^4-hydroxycytosine	2.9**	10.4**
1,5-Dimethyl,N^4-hydroxycytosine	2.9**	11.1**

* Data from Brown, Hewlins & Schell (1968).
** Data from Janion & Shugar (1965b).

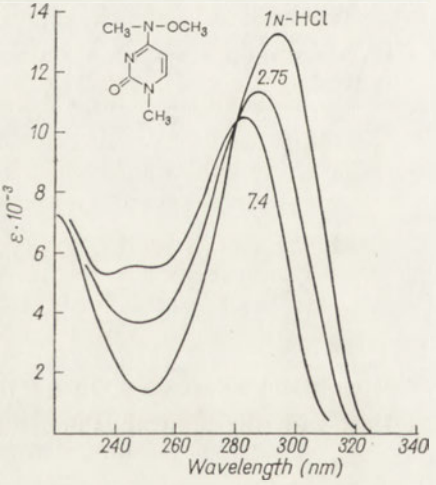


Fig. 3. Absorption spectra at pH values indicated of 1-methyl,N^4-methyl,N^4-methoxycytosine, showing neutral and acid forms.

The problem is further complicated in the case of the modifications undergone by II in alkaline medium, since the structure of these analogues is such as to permit of amino-imino tautomerism, dissociation of the ring $N_{(3)}$ hydrogen, and dissociation of the N^4-OH hydrogen. Nonetheless, the spectral changes provoked by passage from neutral to alkaline pH are consistent with the existence of only one pK value, suggesting that only one of these possibilities exists.

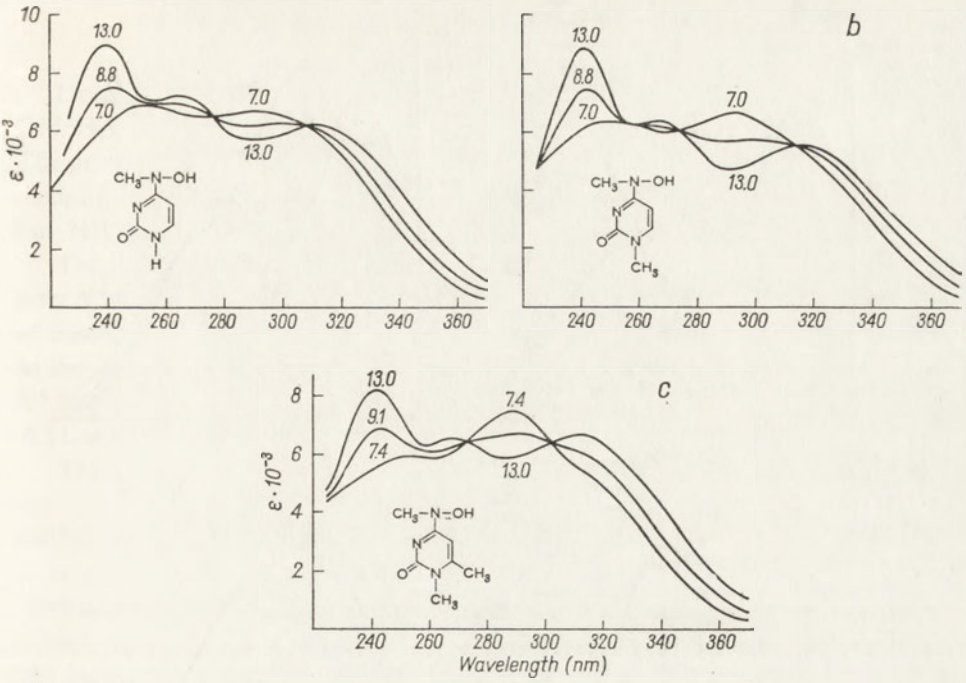


Fig. 4. Absorption spectra, at pH values indicated, showing the neutral and basic forms of: (*a*), N^4-methyl,N^4-hydroxycytosine; (*b*), the 1-methyl analogue, and (*c*), the 1,6-dimethyl analogue.

Comparing once again the pK_b values for the various analogues (Table 2), it will be seen that dissociation of the N^4-OH hydrogen, in N^4-methyl,N^4-hydroxycytosines, occurs at pH values considerably lower than those at which the alkaline forms of N^4-methoxycytosines (V) appear. This is not a characteristic feature of hydroxyl group function, but is due rather to the amino form of the compound. Dissociation of the N^4-hydroxyl in 1,3-dimethyl,N^4-hydroxycytosine, which is in the fixed imino form, occurs in more basic medium (Fig 6, pK 12.3 as compared to the pK of IVb, in the fixed amino form, pK of which is 9.1).

Summing up, we may draw the following conclusions: (1) Bearing in mind that the N^4-OH hydrogen dissociates more readily when the compound is in the imino form (as compared to the amino), it follows that 1-methyl,N^4hydroxycytosine (1-methyl analogue of II, pK 10.4) is in the imino form in neutral medium; (2) The resemblance of the neutral absorption spectra of N^4-methoxy and N^4-hydroxycytosines indicates that the N^4-methoxy analogues (V) are likewise in the imino form; (3) For the N^4-methoxycytosines (V) in alkaline medium, IX (Scheme 3) is the preferred form; (4) The form of type II compounds in alkaline medium remains unresolved, and there may even exist differences between them, e.g. the alkaline forms of 5-substituted derivatives of N^4-hydroxycytosines may differ from those of the non-substituted.

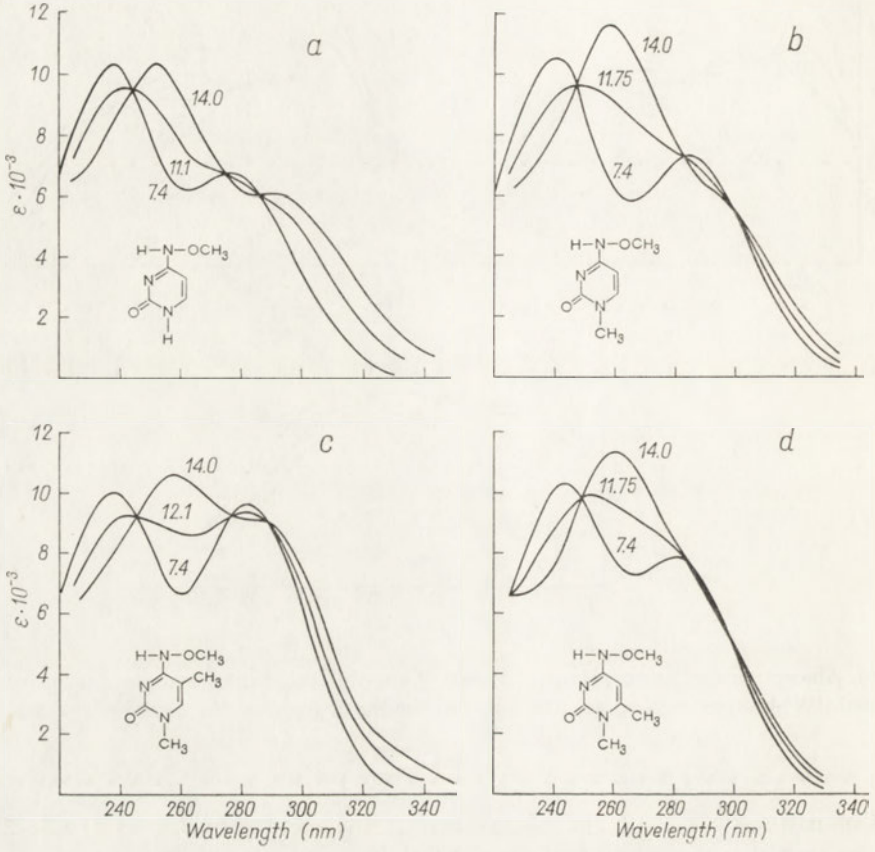


Fig. 5. Absorption spectra, at pH values indicated, showing the neutral and basic forms of: (*a*), N^4-methoxycytosine; (*b*), 1-methyl,N^4-methoxycytosine; (*c*), 1,5-dimethyl,N^4-methoxycytosine and (*d*), 1,6-dimethyl,N^4-methoxycytosine.

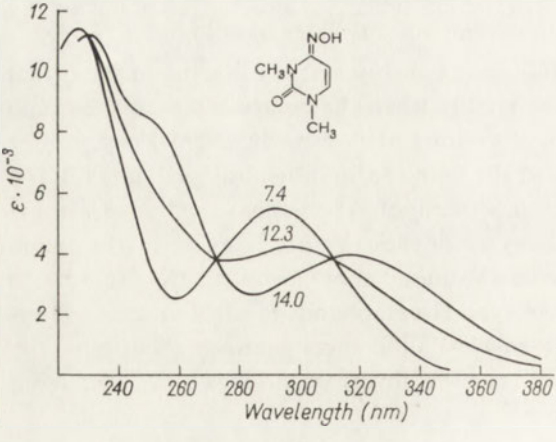


Fig. 6. Absorption spectra, at pH values indicated, of 1,3-dimethyl,N^4-hydroxycytosine, showing neutral and basic forms.

DISCUSSION

Despite the apparent similarity in reactions between cytosine residues and HA, OMeHA or NMeHA, the properties of the compounds formed may be different. The present investigations confirm the notion of Brown *et al.* (1968) that, in aqueous medium, HA and OMeHA derivatives of cytosine (II and V) accept the imino form, but NMeHA derivatives of cytosine (IV) exist only in the amino form.

The imino structure of II and V explains the lack of complex formation between poly N^4-hydroxycytidylic acid and poly I (Janion & Shugar, 1968) and the lack of association ability of 1-methyl,N^4-methoxycytosine with 9-ethylguanine, as well as the very weak association between 1-methyl,N^4-methoxycytidine or 1-cyclohexyl, N^4-hydroxycytosine and adenosine derivatives in carbon tetrachloride (Brown & Hewlins, 1969).

This association ability is so poor, that we were unable to show in salt solution any base pairing properties between poly A and N^4-hydroxycytosines residues included in the homopolymers, or in the copolymers (Janion & Shugar, 1968, 1969). It is also possible that complex formation between adenosine derivatives and 1--methyl,N^4-methoxycytosine, observed in tetracarbon chloride, is not due to Watson--Crick base pairing, but reversed Watson-Crick base pairing, and this may explain the lack of base pairing ability between residues of N^4-hydroxy, or N^4-methoxy cytosine and adenosine included in the polymer.

Furthermore, the hydroxy (or methoxy) group at N^4 in N^4-hydroxy (methoxy)--5-methyl derivatives of cytosines, the compounds responsible for HA (or OMeHA) mutagenesis in T-even phages, probably is located *trans* to the 5-methyl group and this can be a serious hindrance to the pairing of modified cytosine residues.

The amino structure of IV ruled out the explanation of mutagenic activity of this compound by change in base pairing properties *a priori*. Theoretically IV still can form complexes with guanine (if steric hindrance allows for), but only with two hydrogen bonds (without N^4), or lose its complexing ability at all.

That IV can act mutagenically is suggested by the following: 1) IV is the main (if not only) reaction product of NMeHA with glycoside derivatives of cytosine; 2) The lack of mutagenic activity of NMeHA on T-even phages is in accord with the lack of reaction of NMeHA on 5-substituted derivatives of cytosine (Janion & Shugar, 1971). If there is a side reaction with mutagenic significance, apart from the reaction with cytosine residues, these should be revealed after treatment of T-even phages with NMeHA.

The other compound, the mutagenic activity of which cannot be interpreted by simple change in complexing ability, is 3-methylcytosine (Singer & Fraenkel--Conrat, 1969). Investigations with DNA-dependent RNA polymerase have revealed that the presence of 3-methylcytosine residues in a polynucleotide template leads to the transition C(3MeC) → U, or to the transversion C(3-MeC) → A (Singer & Fraenkel-Conrat, 1970; Ludlum, 1970, 1971).

Mutagenic properties of NMeHA remain conjectural. It is highly probable that NMeHA leads to entirely different types of mutation than HA or OMeHA.

Such a conclusion was reached by Malling (1967) after an investigation of the mutants of *Neurospora crassa* obtained by treatment with one of these three mutagens.

In view of the possibility of existence of differences between alkaline forms II and V, it is not exluded that even OMeHA may lead to a different type of mutation than HA. Perhaps the infrared spectra which are under study now may provide a more precise insight into the structure of HA, OMeHA and NMeHA derivatives of cytosine. Significance of the mutagenic action of OMeHA should be estimated in straight-forward biological experiments.

EXPERIMENTAL

1,3-Dimethyl,N^4-hydroxycytosine and 1,N^4-dimethyl,N^4-methoxycytosine were kindly made available by Dr. D. M. Brown.

N-Methylhydroxylamine·HCl and methoxyamine were products of Aldrich and Eastman Kodak (U.S.A.).

In the syntheses of investigated compounds, 4-ethoxy or 4-methoxy 2-ketopyrimidine derivatives were used as starting products, since it was previously demonstrated that a 4-alkoxy substituent is a good leaving group (Janion & Shugar, 1965b). All reactions were carried out in methanolic medium, using freshly prepared solutions. The course of the reactions was followed chromatographically, or spectrally by following the UV absorption at neutral pH of the samples withdrawn at various time intervals. For most of the compounds, a good solvent system for separation of products from starting substances was water-saturated butanol (Table 3). Good separations of 1,5-dimethyl-4-ethoxy and 1,6-dimethyl-4-methoxy pyrimidine derivatives from their products of reaction with OMeHA were obtained by TLC on silica gel with benzene - ether, 8:2 (v/v).

On following the course of a reaction, it was necessary to take account of the fact that both OMeHA and NMeHA exhibit UV absorption at short wavelengths and, furthermore, that the absorption of the latter in aqueous medium varies with time (Janion & Shugar, 1971).

For preparation of methanolic solutions of NMeHA and OMeHA the required amount of either of these compounds was dissolved in the minimal volume of methanol, and to this was added a methanolic solution of NaOH to obtain the appropriate pH. The precipitated salt was filtered off and the molarity of the solution calculated from the volume of the filtrate.

N^4-*Methyl*,N^4-*hydroxycytosine (IVa):* 20.4 mg 4-ethoxyuracil was dissolved in 0.4 ml 2.6 M-NMeHA, pH 6, and the solution left at 37°C for 72 hours, then concentrated on a water bath to about 10% of the initial volume, leading to a drop in pH. A slight precipitate, devoid of UV absorption, was centrifuged off and two volumes of acetone was added to the supernatant to precipitate the HCl salt of III. Occasionally it was found necessary to add 10 - 20 µl methanol to initiate crystallization. The precipitate was washed several times with acetone, which also served to remove unreacted NMeHA and its products of decomposition, and recrystallized from methanol to yield 12.5 mg of IVa hydrochloride, m.p. 181 - 182°C.

Table 3

R_F *values of 4-alkoxy-2-ketopyrimidines and their reaction products with OMeHA or NMeHA*

Ascending chromatography with Whatman no. 1 paper and water-saturated *n*-butanol was used. Values in brackets refer to the crude reaction products.

Compounds	R_F
N^4-Methoxycytosine	0.67 (0.37)
4-Ethoxyuracil	0.71
1-Methyl,N^4-methoxycytosine	0.78 (0.37)
1-Methyl-4-ethoxyuracil	0.84
1,5-Dimethyl,N^4-methoxycytosine	0.85 (0.84)
5-Dimethyl-4-ethoxyuracil	0.88
1,6-Dimethyl,N^4-methoxycytosine	0.75 (0.3)
1,6-Dimethyl-4-methoxyuracil	0.77
N^4-Methyl,N^4-hydroxycytosine	0.31 (0.18)
1,N^4-Dimethyl,N^4-hydroxycytosine	0.49 (0.18)
1,6,N^4-Trimethyl,N^4-hydroxycytosine	0.55 (0.18)

1,N^4-*Dimethyl*,N^4-*hydroxycytosine (IVb):* 30 mg of 1-methyl-4-ethoxyuracil was dissolved in 0.5 ml of a methanolic solution of 4 M-NMeHA, pH 6, the solution stored at 37°C for 48 hours, and proceeded as above to give 32 mg of IVb hydrochloride, m.p. 223°C.

1,6,N^4-*Trimethyl*,N^4-*hydroxycytosine (IVc):* 25 mg of 1,6-dimethyl-4-methoxyuracil was dissolved in 0.6 ml of a 4 M solution of NMeHA in methanol, pH 6. Following 48 hours at 37°C, methanol was removed under reduced pressure and the residue dissolved in hot redistilled water. Cooling was accompanied by precipitation of crystals of IVc hydrochloride which, after washing with cold water, yielded 10 mg, m.p. 223 - 224°C.

N^4-*Methoxycytosine (Va):* 30 mg of 4-ethoxyuracil was dissolved in 350 µ of a methanolic solution of 2.6 M-OMeHA, pH 6.5 - 7 and stored at 37°C for 24 hours. The solution was brought to dryness on a water bath, dissolved in a small volume of water and deposited on an 80×1.5 cm column of Sephadex G-10, followed by elution with water. The successive fractions, following the void volume (93 ml), contained salts and N^4-methoxy-5,6-dihydro-6-methoxyaminocytosine (42 ml), OMeHA (52 ml) and, finally, N^4-methoxycytosine (58 ml). The evaporated last fraction yielded 25 mg of Va, m.p. 166 - 167°C. Removal of water under reduced pressure of the fraction containing N^4-methoxy-5,6-dihydro-6-methoxyaminocytosine led to discoloration and partial decomposition, as well as formation of Va. Both products were soluble in acetone.

*1-Methyl,*N^4*-methoxycytosine (Vb):* 30 mg of 1-methyl-4-ethoxyuracil was dissolved in 0.5 ml of 2.6 M-OMeHA at pH about 6.5, and kept at 37°C for 24 hours. The methanol was removed, the residue dissolved in water and deposited on a Sephadex G-10 column as above. Elution with water gave the same profile as above. Concentration to dryness of the fraction containing Vb yielded 15 mg 1-methyl,N^4--methoxycytosine, m.p. 122 - 123°C. An appreciable percentage of the products, isolated in one of the fractions, proved to be 1-methyl,N^4-methoxy-5,6-dihydro--6-methoxyaminocytosine which, during removal of solvent, was transformed to Vb.

1,5-*Dimethyl,*N^4*-methoxycytosine (Vc):* 50 mg of 1,5-dimethyl-4-ethoxyuracil was dissolved in 0.75 ml of 2 M-OMeHA at pH about 5.2, and kept at 37°C for 4 days; methanol was then removed and the residue dissolved in 0.8 ml water on a water bath. Subsequent cooling led to precipitation of crystalline 1,5-dimethyl,N^4--methoxycytosine hydrochloride which, following washing with cold water, yielded 34.6 mg, m.p. 117°C. It should be pointed out that control of pH is, in this case, particularly important; whereas 4-ethoxyuracil and 1-methyl-4-ethoxyuracil readily react with OMeHA even at pH 7, 1,5-dimethyl-4-ethoxyuracil fails to react unless the pH is reduced to 5.2.

The author is indebted to Mrs. Krystyna Myszkowska for excellent technical assistance. This investigation was supported by the Polish Academy of Sciences (Project 09.3.1) and profited also from the support of the Wellcome Trust, the World Health Organization and the Agricultural Research Service, U.S. Dept. of Agriculture.

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MUTAGENICZNE DZIAŁANIE HYDROKSYLAMINY. WIDMA W ULTRAFIOLECIE I TAUTOMERIA N^4-HYDROKSY, N^4-METOKSY I N^4-METYLO,N^4-HYDROKSY POCHODNYCH CYTOZYNY ORAZ 5 I 6-METYLOCYTOZYNY

Streszczenie

1. Otrzymano metoksyaminowe i *N*-metylohydroksylaminowe pochodne cytozyny i przebadano widma w ultrafiolecie ich form kwaśnych, zasadowych i obojętnych.

2. Porównanie wartości p*K* związanych z dysocjacją grupy hydroksylowej przy N^4, jak i porównanie widm w ultrafiolecie badanych związków o utrwalonej strukturze aminowej, iminowej lub nieokreślonej wskazuje, że N^4-hydroksy i N^4-metoksy pochodne cytozyny występują w formie iminowej.

3. Zależność widma w ultrafiolecie N^4-metoksy pochodnych cytozyny od pH w środowisku alkalicznym może być wynikiem tautomerii imino-aminowej, lub utraty protonu z $N_{(3)}$.

4. Omówiono mechanizm mutagennego działania hydroksylaminy, metoksyaminy i *N*-metylohydroksylaminy, zwracając uwagę na podobieństwa i różnice ich chemicznego działania.

Received 24 March, 1972.