POLSKA AKADEMIA NAUK
KOMITET BIOCHEMICZNY I BIOFIZYCZNY

POLISH ACADEMY OF SCIENCES
COMMITTEE OF BIOCHEMISTRY AND BIOPHYSICS

ACTA BIOCHIMICA POLONICA

QUARTERLY

Vol. 16
No. 3

WARSZAWA 1969
PAŃSTWOWE WYDAWNICTWO NAUKOWE

ADDRESS

Krakowskie Przedmieście 26/28, Warszawa 64.
Poland

Państwowe Wydawnictwo Naukowe — Warszawa, Miodowa 10

Nakład 1816+134 egz. Ark. wyd. 6,0 ark. druk. 5,125+0,25 wkł.
Papier druk. sat. kl. III, 80 g. B1
Oddano do składania 21.IV.1969. Podpisano do druku 28.VIII.1969.
Druk ukończono we wrześniu 1969.

Zam. 376/69 P-79 Cena zł 25.—

Warszawska Drukarnia Naukowa — Warszawa Śniadeckich 8

ACTA BIOCHIMICA POLONICA
Vol. 16 1969 No. 3

JADWIGA PURZYCKA-PREIS and M. ŻYDOWO

PURIFICATION AND SOME PROPERTIES OF MUSCLE AMP-AMINOHYDROLASE FROM CARP (*CYPRINUS CARPIO*)

Department of Biochemistry, Medical School, Al. Zwycięstwa 42, Gdańsk 6

1. A procedure for purification of AMP-aminohydrolase from the muscle of *Cyprinus carpio* has been described. 2. By applying ammonium sulphate fractionation, calcium phosphate gel adsorption and cellulose phosphate chromatography, a 400-fold purification of the enzyme was achieved. 3. The high specificity of the enzyme, its pH optimum, Michaelis constant, inhibition by fluoride and *p*-chloromercuriphenyl sulphonate, and activation by univalent cations resemble very much the properties of analogous enzymes obtained from muscles of mammals.

AMP-aminohydrolase (EC 3.5.4.6), an enzyme which catalyses the deamination of adenylic acid, was first described by Schmidt (1928, 1932, 1933) who demonstrated also its specificity for 5′-AMP. The enzyme is widely distributed in animal tissues (Conway & Cooke, 1939; Purzycka, 1962), the greatest amounts being found in muscle. The enzyme was purified by Kalckar (1947) and Nikiforuk & Colowick (1956), and Lee (1957) obtained a crystalline preparation from rabbit muscle. Recently a rapid method for preparation of crystalline enzyme from rabbit skeletal muscle was presented by Smiley, Berry & Suelter (1967). Using cellulose phosphate chromatography, they obtained directly from the crude extract a preparation, of which the yield and degree of purification were higher than reported previously. Since neither of these methods could be applied to fish muscle, a new procedure has been elaborated.

MATERIALS AND METHODS

Reagents. AMP and other nucleotides were purchased from Koch-Light, Colnbrook, England, and from Sigma, St.Louis, Mo., U.S.A. Tris(hydroxymethyl)aminomethane was from Koch-Light and *p*-chloro-

mercuriphenyl sulphonic acid from Sigma. Whatman cellulose phosphate (Balston Ltd., Maidstone, Kent, England) was successively washed with 0.5 M-KOH, water, 0.5 N-HCl, water, and finally with 5 mM-EDTA. All other reagents were reagent grade, purchased from Centrala Odczynników Chemicznych (Gliwice, Poland). Bidistilled water from an all-glass apparatus was used throughout.

Enzyme assays. A. When low substrate concentration was used, the deamination of 5′-AMP was followed according to Kalckar (1947). The reaction mixture contained in a final volume of 3 ml.: 0.1 M-potassium succinate buffer, pH 6.4, 0.5 M-KCl and 0.054 mM-5′-AMP. In the blank sample the substrate was omitted. The mixture was placed in a 1 cm. silica cell in the constant temperature cell housing of a Unicam SP-500 spectrophotometer, and the temperature was equilibrated to 30°. Then the reaction was started by adding 20 µl. of enzyme solution and the decrease in extinction was followed at 265 mµ. The amount of enzyme which produced a decrease of extinction at 265 mµ from 0.55 to 0.40 during 1 min. was taken as 10 units of activity.

B. For higher substrate concentrations the reaction was followed by reading the extinction at 285 mµ according to Smiley *et al.* (1967). The reaction mixture contained in a final volume of 3 ml.: 0.1 M-potassium succinate buffer, pH 6.4, 0.1 M-KCl and 0.2 mM-5′-AMP. The reaction was started by the addition of 20 µl. of enzyme and the increase in extinction was measured at 285 mµ.

C. The K_m value and enzyme specificity were determined by measuring the amount of ammonia liberated. The incubation was carried out at 30° in test tubes calibrated for 10 ml. The incubation mixture contained in a final volume of 1 ml.: 0.1 M-potassium succinate buffer, pH 6.4, 0.1 M-KCl and 5′-AMP or another substrate. The reaction was started by the addition of 20 µl. of enzyme solution, and stopped by the addition of 1 ml. of 0.215 M-sodium phenolate. The ammonia formed was determined by the phenol-hypochlorite method of Brown, Duda, Korkes & Handler (1957). It was found possible to omit the diffusion procedure and perform the determination of ammonia directly in the incubation mixture. After the reaction had been stopped by the addition of sodium phenolate, the following reagents were added: 1 ml. of 0.172 mM-sodium nitroferricyanide, 1 ml. of 0.05 M-sodium carbonate, and 1 ml. of 0.06 M-sodium hypochlorite. After 30 min. the volume was made up to 10 ml. with ammonia-free water, and the colour intensity was measured at 625 mµ in a Leitz spectrophotometer.

Protein concentration was determined by the biuret method of Gornall, Bardawill & David (1949), except in the eluate from cellulose phosphate column, for which the method of Warburg & Christian (Layne, 1957) was used.

RESULTS

Purification procedure

Extraction of muscle. The muscles of carp (*Cyprinus carpio*) were excised immediately after decapitation, washed with tap water and chilled on ice. The muscle was passed through a chilled meat chopper and then homogenized for 2 min. in a Waring blendor at 3000 rev./min. with 3 vol. of a solution containing 0.08 M-K_2HPO_4, 0.05 M-KCl and 0.75 mM-$MgCl_2$, pH 7.0. The homogenate was extracted for 3 hr. with efficient stirring, centrifuged 35 min. at 3000 *g*, and the supernatant was passed through four layers of muslin to remove debris and a lipid layer.

Ammonium sulphate fractionation. Most of the AMP-aminohydrolase activity was precipitated at 2.26 M-ammonium sulphate concentration. To the muscle extract, appropriate amount of solid ammonium sulphate was added very slowly during 4 hr. The mixture was allowed to stand overnight, then the precipitate was collected by centrifugation at 20 000 *g* for 15 min. and dissolved in 0.05 M-KCl - 0.05 M-tris buffer, pH 7.2 (1 ml. per 2 - 3 g. of original ground muscle). The solution was dialysed against two changes of 0.5 M-KCl (each of 20 volumes) with stirring for 10 hr.

Calcium phosphate gel fractionation. Calcium phosphate gel was prepared according to Keilin & Hartree (1938). To the dialysed ammonium sulphate fraction, calcium phosphate gel, 1 mg. per 1 mg. protein, was gradually added. The suspension was stirred for 2 - 3 hr., and the sediment collected by gentle centrifugation (5 min., 600 *g*). If not all the enzyme was adsorbed, successive small amounts of calcium phosphate gel were added, and, after 3 hr. with stirring, centrifuged in the same manner. The combined calcium phosphate gel sediments were eluted for 12 hr. with 0.1 M-K_2HPO_4, pH 8.5 (less than half the volume of the gel suspension added).

Separation on cellulose phosphate. The eluate from calcium phosphate gel was dialysed against two changes (50 volumes each) of a KCl--phosphate buffer containing 0.18 M-KCl, 0.054 M-KH_2PO_4 and 0.035 M--K_2HPO_4, pH 6.5, with stirring for 3 hr. Then 10 g. of washed cellulose phosphate equilibrated with the same buffer was added and the suspension stirred gently for 12 hr. After this time the cellulose phosphate was washed repeatedly under suction on a sintered glass filter; a total volume of 1 litre of the KCl-phosphate buffer was used in 100 - 200 ml. portions. During this process the cellulose phosphate pad was not permitted to become dry. Then the cellulose phosphate was packed to a column 2.5 cm. in diameter and washed with approximately 100 ml. of a solution of 0.45 M-KCl - 1 mM-mercaptoethanol, adjusted with 1 M-K_2HPO_4 to pH 7.0. When no more protein was eluted with the wash, the adsorbed

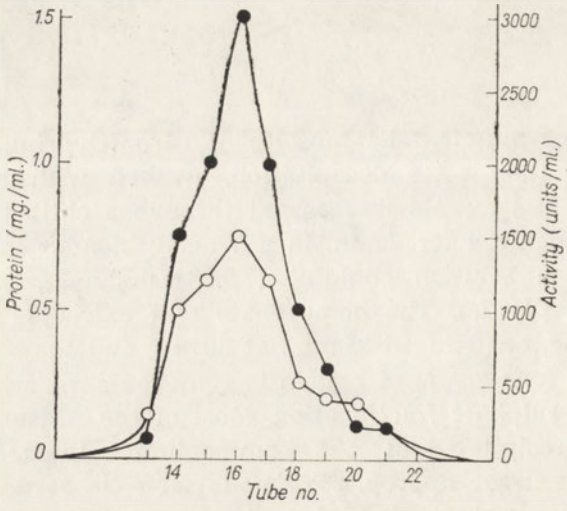

Fig. 1. Elution of AMP-aminohydrolase from cellulose phosphate column. The calcium phosphate gel eluate was applied to the column and the enzyme eluted with a KCl concentration gradient from 0.45 to 1.0 M, pH 7.0. Fractions of 3 ml. were collected at 7.5 min. intervals. (○), Protein; (●), activity.

enzyme was eluted with 1 M-KCl - 1 mM-mercaptoethanol (adjusted to pH 7.0 with 1 M-K_2HPO_4) or with a linear KCl concentration gradient from 0.45 to 1 M. The gradient elution gave one symmetrical protein peak coincident with the enzyme activity peak (Fig. 1).

The purification procedure summarized in Table 1 gave a 300 - 500--fold purification. All steps were carried out at 0 - 5°.

Table 1

Procedure of purification of AMP-aminohydrolase from carp muscle

Fresh carp muscle, 546 g., was used as starting material. For details see text.

Fraction	Total protein (mg.)	Activity		Purification factor	Yield (%)
		total (units)	specific (units/mg. protein)		
Homogenate	62 400	624 000	10	1	100
Extract	14 420	520 000	36	3.6	83
2.26 M-Ammonium sulphate ppt.	4 480	340 000	75	7.5	65
Calcium phosphate gel eluate	598	149 000	246	24.6	24
Cellulose phosphate eluate	68.5	28 700	4190	419	4.6

Some properties of the purified enzyme

AMP-aminohydrolase lost only 2 - 5% of its activity when stored at 4° for 2 months. The enzyme preparation in 1 M-KCl at pH 7.0 showed the absorption maximum at 278 mμ and the minimum at 250 mμ. The E_{280}/E_{260} ratio was 1.2 to 1.8 in different preparations.

The substrate specificity was tested by incubating for 1 hr. appropriate compounds in 0.2 mM concentration with 0.1 N-potassium succinate buffer, pH 6.4, 0.1 M-KCl and 20 μl. of enzyme solution in a final volume of 1 ml., and determining the liberated ammonia. No ammonia was formed from adenine, adenosine, deoxyadenosine, ADP, 3′-AMP, cytidine, CMP, NAD, NADP or CoA; only 5′-deoxyadenylic acid was deaminated but very slowly.

The enzyme showed a rather broad pH optimum around pH 6.4 when measured in 0.1 M-tris-succinate - 0.1 M-KCl buffer (Fig. 2). This value is similar to those reported by Lee (1957) for the crystalline enzyme

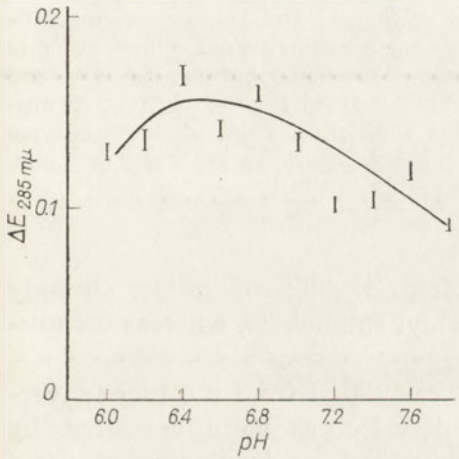

Fig. 2

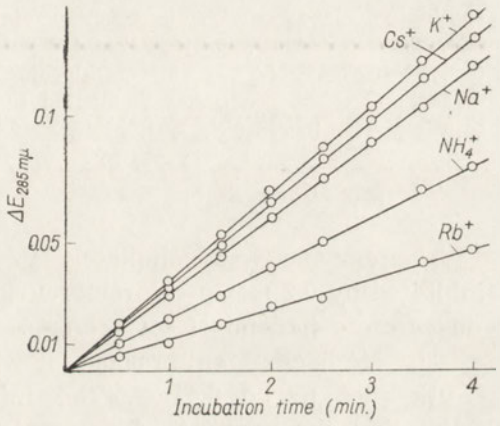

Fig. 3

Fig. 2. Effect of pH on AMP-aminohydrolase activity. The reaction mixture contained in a final volume of 3 ml.: 0.2 mM-5′-AMP, 0.1 M-KCl - 0.1 M-tris-succinate buffer of appropriate pH value. The reaction was started by the addition of 20 μl. of enzyme solution. The readings of extinction at 285 mμ were taken at 30 sec. intervals for 5 min. at 30° and during this time the increase in extinction at all pH values was linear with time. The activity is expressed as ΔE_{285} after 5 min. Perpendicular dashes indicate the range of values obtained in 4 experiments.

Fig. 3. The effect of univalent cations on AMP-aminohydrolase activity. The reaction mixture contained 0.2 mM-5′-AMP, 0.1 M-tris-succinate buffer, pH 6.4, and chlorides of the indicated cations in concentration of 0.15 M. The reaction was started by adding 20 μl. of the purified enzyme which had been diluted with tris-succinate buffer, pH 6.4. Final volume 3 ml. The increase in extinction at 285 mμ was measured at 1 min. intervals for 5 min. at 30°. No activity was observed when the univalent cations were omitted.

from rabbit muscle and by Makarewicz (1969) for the purified enzyme from elasmobranch fish.

The effect of univalent cations on enzyme activity is presented in Fig. 3. The highest activity was observed in the presence of K^+ ion. No activity was observed in the absence of univalent metal ions.

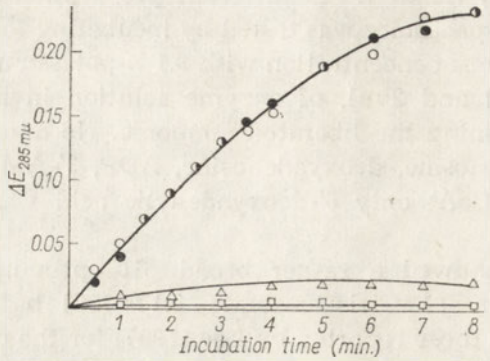

Fig. 4. Effect of NaF, *p*-chloromercuriphenyl sulphonate and iodoacetate on AMP--aminohydrolase activity. The reaction mixture contained in a final volume of 3 ml.: (●), 0.2 mM-5′-AMP, 0.1 M-KCl - 0.1 M-potassium succinate buffer, pH 6.4, and (□), 20 mM-NaF, (△), 0.2 mM-*p*-chloromercuriphenyl sulphonate, or (○), 20 mM--iodoacetate. The reaction was started by the addition of 20 µl. of dilute enzyme solution. The increase in extinction at 285 mµ was measured at 30 sec. or 1 min. intervals for 8 min.

The enzyme was completely inhibited by 20 mM-fluoride, strongly inhibited by 0.2 mM-*p*-chloromercuriphenyl sulphonate, whereas 0.2 mM--iodoacetate had no effect (Fig. 4).

The Michaelis-Menten constant was calculated from the Lineweaver--Burk plot to be 0.4 mM. The initial velocities were measured by estimating the amount of ammonia liberated on incubation of 0.05--0.45 mM-AMP with 1.5 µg. enzyme in 0.1 M-KCl - 0.1 M-potassium succinate buffer, pH 6.4, for 5 and 10 min.

DISCUSSION

Of the few procedures described hitherto for purification of AMP--aminohydrolase from mammalian skeletal muscle, that of Smiley, Berry & Suelter (1967) is the simplest one, consisting in one-step purification. However, this procedure is not suitable for fish muscle. The proteins of carp muscle are much easier to extract than those of mammalian muscle, and almost the whole protein material can be solubilized by exhaustive extraction with a salt solution of high ionic strength. Therefore selective adsorption of AMP-aminohydrolase directly from the

crude extract was unsuccessful, and it appeared necessary to purify the extract prior to cellulose phosphate chromatography. Ammonium sulphate fractionation and calcium phosphate gel adsorption were found to be the most efficient steps of purification. By the procedure described in this paper, a preparation was obtained from fish muscle with a specific activity comparable with that of pure preparations obtained from muscles of mammals.

AMP-aminohydrolase is known to be present in muscles of almost all classes of animals. Umiastowski (1964) studied muscles of 12 vertebrates and 6 invertebrates, and only in the freshwater clam, *Unio pictorum*, he did not detect the enzyme. The wide occurrence of AMP--aminohydrolase points to the physiological importance of the enzyme, although its biological role is still unclear. Setlow, Burger & Lowenstein (1966) found that the enzyme was activated by ATP and inhibited by GTP, and suggested that it might be involved in the regulation of interconversion of purine nucleotides. This suggestion is compatible with the finding of Smiley & Suelter (1967) that the enzyme is an allosteric protein, alkali metal cations and ATP being the allosteric effectors. Potassium, as well as some other univalent cations, have been found to activate also the carp muscle enzyme.

The properties of AMP-aminohydrolase from carp muscle are very similar to those of the analogous enzymes obtained from mammalian muscles. The results reported by several workers are summarized in Table 2 and it seems possible to suppose that these enzymes are not only analogous but homologous as well.

Table 2

Comparison of some properties of 5'-AMP aminohydrolase from rabbit and fish muscle

	Lee (1957)	Smiley, Berry & Suelter (1967)	Makarewicz (1969)	present paper
	Rabbit	Rabbit	*Raja clavata*	*Cyprinus carpio*
Specificity	very high*	not tested	very high*	very high*
Optimum pH	6.4	6.5** - 6.7	6.6	6.4 - 6.8
K_m (mM)	1.43	0.5 - 0.7	1.52 ± 0.66	0.4
Activation by monovalent cations	not tested	$K^+ > Na^+ > Li^+ > Rb^+ > NH_4^+ > Cs^+$	$K^+ > Rb^+ > Na^+ > Li^+ > NH_4^+$	$K^+ > Cs^+ > Na^+ > NH_4^+ > Rb^+$
Effect of iodoacetate	none	not tested	none	none
Effect of PCMBS	inhibition	not tested	inhibition	inhibition
Effect of NaF	inhibition	not tested	inhibition	inhibition

* Also 5'-dAMP was deaminated but at a rate 1/100 that for 5'-AMP.
** pH of optimal stability.

In previous work from this laboratory, different temperature susceptibility of the enzyme activity in poikilothermic and homeothermic animals has been observed (Żydowo, Makarewicz, Umiastowski & Purzycka, 1965). The obtained purified enzyme preparation from carp muscle may facilitate the elucidation of the molecular mechanism of this phenomenon.

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

REFERENCES

Brown R. H., Duda G. D., Korkes S. & Handler P. (1957). *Arch. Biochem. Biophys.* **66**, 301.
Conway E. J. & Cooke R. (1939). *Biochem. J.* **33**, 479.
Gornall A. G., Bardawill C. J. & David M. M. (1949). *J. Biol. Chem.* **177**, 751.
Kalckar H. M. (1947). *J. Biol. Chem.* **167**, 461.
Keilin D. & Hartree E. F. (1938). *Proc. Roy. Soc.* **124**, 397.
Layne E. (1957). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) Vol. III, p. 451. Academic Press, New York.
Lee Y. P. (1957). *J. Biol. Chem.* **227**, 987.
Makarewicz W. (1969). *Comp. Biochem. Physiol* **29**, 1.
Nikiforuk G. & Colowick S. P. (1956). *J. Biol. Chem.* **219**, 119.
Purzycka J. (1962). *Acta Biochim. Polon.* **9**, 83.
Schmidt G. (1928). *Hoppe-Seyler's Z. Physiol. Chem.* **179**, 243.
Schmidt G. (1932). *Hoppe-Seyler's Z. Physiol. Chem.* **208**, 185.
Schmidt G. (1933). *Hoppe-Seyler's Z. Physiol. Chem.* **219**, 191.
Setlow B., Burger R. & Lowenstein J. M. (1966). *J. Biol. Chem.* **241**, 1244.
Smiley K. L. Jr., Berry A. J. & Suelter C. H. (1967). *J. Biol. Chem.* **242**, 2502.
Smiley K. L. Jr. & Suelter C. H. (1967). *J. Biol. Chem.* **242**, 1980.
Umiastowski J. (1964). *Acta Biochim. Polon.* **11**, 459.
Żydowo M., Makarewicz W., Umiastowski J. & Purzycka J. (1965). *Acta Biochim. Polon.* **12**, 319.

OCZYSZCZANIE I NIEKTÓRE WŁASNOŚCI AMP-AMINOHYDROLAZY Z MIĘŚNI KARPIA (*CYPRINUS CARPIO*)

Streszczenie

1. Opisano metodę oczyszczania AMP-aminohydrolazy z mięśni karpia (*Cyprinus carpio*).

2. Stosując wytrącanie siarczanem amonu, adsorpcję na żelu Ca-fosforanowym i chromatografię na fosforanie celulozy osiągnięto 400-krotne zagęszczenie enzymu.

3. Wysoka specyficzność enzymu, jego optimum pH (6.4), stała Michaelisa (0.4 mM), hamowanie fluorkiem i *p*-chlororteciofenylosulfonianem, a aktywowanie jednowartościowymi kationami, bardzo przypomina własności analogicznych enzymów otrzymanych z mięśni ssaków.

Received 2 January, 1969.

ACTA BIOCHIMICA POLONICA
Vol. 16 1969 No. 3

H. SIEMIENIEWSKI and T. BARANOWSKI

THE REACTION OF AZLACTONE WITH CHYMOTRYPSIN AND TRYPSIN

Department of Physiological Chemistry, Medical School, ul. Chałubińskiego 10, Wrocław

1. In the reaction of azlactone of *p*-nitrobenzoylvaline with trypsin and chymotrypsin, the number of bound *p*-nitrobenzoylvaline (NBVal) residues was found to depend on the pH value. 2. The enzymic activity of chymotrypsin decreased when the number of introduced NBVal residues increased up to 5, whereas further blocking of amino groups had little influence. 3. No relationship was found between the decrease of activity of modified trypsin and the number of bound NBVal residues.

The chemical modification of various reactive groups in proteins has been used extensively in studies on the correlation between their structure and biological activity. Of the agents supposed to react with amino groups azlactones were little used for the modification of proteins. Saturated azlactones (2,4-substituted oxazolones-5) were obtained for the first time by Mohr & Stroschein (1909) by action of acetic anhydride on α-acylamino acids. The formation of azlactones as intermediates in reactions of chemical synthesis of peptides has been observed by Siemion & Nowak (1960). Detailed studies on the reaction of azlactone with proteins were performed by Baranowski, Kochman, Nowak & Siemion (1963). The reaction of azlactone of *p*-nitrobenzoylvaline with rabbit muscle aldolase was studied by Kochman & Baranowski (1967).

The aim of the present work was to examine the course of the reaction of azlactone of *p*-nitrobenzoylvaline with chymotrypsin and trypsin under possibly mild conditions, and to elucidate the changes in enzymic activity as well as the nature of the bonds formed.

MATERIALS

α-Chymotrypsin was prepared according to Kunitz & Northrop (1935) by slow activation by trypsin of a six times crystallized chymotrypsinogen A. The enzyme was crystallized four times, then dialysed and

freeze-dried. Trypsin was a preparation of Sigma Chem. Co. (St. Louis, Mo., U.S.A.), lot no. 46B-0480. Azlactone of *p*-nitrobenzoylvaline was prepared from acylamino acid and cyclohexylcarbodiimide as described by Siemion & Nowak (1960). p-Nitrobenzoylvaline[1] was obtained according to Karrer & Christoffel (1944). Universal buffer solution of Britton & Robinson (1931) was used throughout.

METHODS

The activity of chymotrypsin and trypsin was measured with casein as substrate either spectrophotometrically (Kunitz, 1947; Wu & Laskowski, 1955) or using the Folin-Ciocalteu reagent (McDonald & Chen, 1965).

Proteins prior to modification were determined spectrophotometrically using for chymotrypsin $E^{1\%}_{280} = 20.0$ (Dixon & Neurath, 1957) and for trypsin $E^{1\%}_{280} = 14.4$ (Davie & Neurath, 1955). Modified proteins were determined according to Lowry *et al.* (Layne, 1955), using as standard chymotrypsin and trypsin, respectively. Molecular weight of chymotrypsin was taken as 24 800 (Schonbaum, Zerner & Bender, 1961) and of trypsin as 24 000 (Mares-Guia & Shaw, 1965). Free amino groups in proteins were assayed by the manometric method of Van Slyke (1929). The preparation of chymotrypsin contained 17 amino groups, and that of trypsin 15 groups per mole.

The number of NBVal residues bound to the protein was estimated by measuring the difference in absorption at 270 mμ between modified and non-modified protein. For calculation, the $E^{1\,cm.}_{270} = 11.3$ for 1 μmole of NBVal in 1 ml. was taken (Kochman & Baranowski, 1967).

The reaction of protein with alzactone was carried out at 0°. To the protein dissolved in universal buffer, the solution of azlactone of NBVal in ethyl alcohol was added. The final concentrations were: alcohol 10%, chymotrypsin 0.24 μmole/ml., trypsin 0.25 μmole/ml. After 3 hr. the reaction was stopped by adding hydroxylamine solution and the mixture was centrifuged. The supernatant was placed on a Sephadex G-25 column and in the effluent the amount of azlactone bound to protein was determined spectrophotometrically. Control samples were submitted to the same procedure except that NBVal instead of the azlactone was used.

Simultaneously additional tests were carried out on samples which after the reaction with azlactone were dialysed and freeze-dried. In all cases the obtained results were identical.

---

[1] Abbreviation used: NBVal, *p*-nitrobenzoylvaline.

RESULTS

Modification of chymotrypsin. The effect of time on the reaction between azlactone of NBVal and chymotrypsin is presented in Fig. 1. The experiments were carried out at four pH values, the concentration of azlactone corresponding to a threefold excess with respect to all free

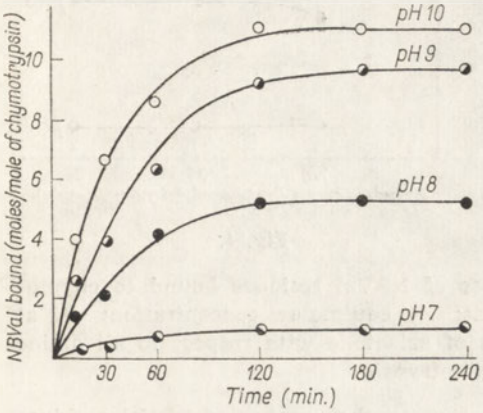

Fig. 1.

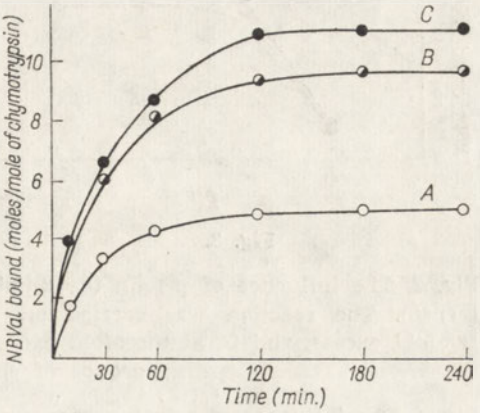

Fig. 2.

Fig. 1. The effect of time and pH on the number of NBVal residues introduced into chymotrypsin. The reaction was carried out at 0° in universal buffer, with a threefold excess of azlactone in relation to protein amino groups. The reaction was stopped by adding hydroxylamine solution and the sample was centrifuged. The supernatant was placed on a Sephadex G-25 column and in the effluent the number of NBVal residues bound to protein was calculated on the basis of spectrophotometric measurements.

Fig. 2. The effect of azlactone concentration on the reaction with chymotrypsin at pH 10. *A*, Equimolar concentration; *B*, twofold and *C*, threefold excess of azlactone with respect to all amino groups of chymotrypsin.

amino groups in chymotrypsin. Figure 2 present the effect of time on the reaction at pH 10 at different azlactone concentrations. The results given in Figs. 1 and 2 indicate that after 2 hr. the reaction was almost completed. In further experiments a time of 3 hr. was applied to eliminate the influence of the time of penetration of the reaction mixture into the column.

The influence of pH and the concentration of azlactone is shown in Figs. 3 and 4. At pH 6 no reaction occurred and beginning with pH 7 the number of introduced NBVal residues increased with increasing pH value. This effect was probably due to a stepwise loss of the charge of the ε-amino groups of lysine residues, and at pH 10, where these groups are non-ionized, the highest incorporation was obtained. The concentration of azlactone had no influence at pH 7 or 8 (Fig. 4). At these pH values only a few amino groups are reactive, and 17 moles of azlactone per mole of chymotrypsin made the concentration of

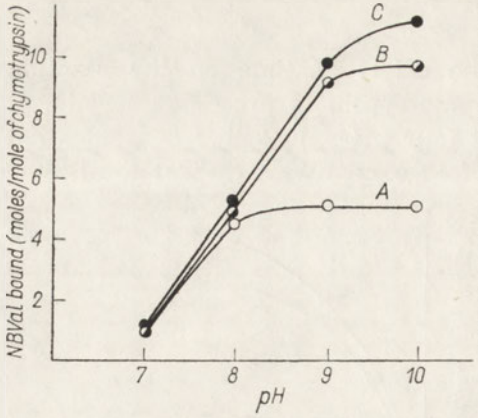

Fig. 3.

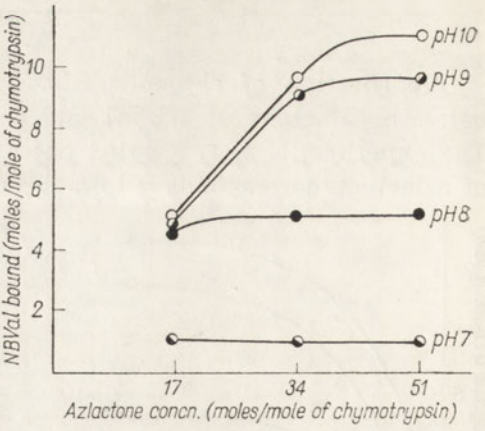

Fig. 4.

Fig. 3. The influence of pH on the number of NBVal residues bound to chymotrypsin. The reaction was carried out at: *A*, equimolar concentration; *B*, at twofold excess, and *C*, at threefold excess of azlactone with respect to all amino groups of chymotrypsin.

Fig. 4. The influence of azlactone concentration on the number of NBVal residues bound to chymotrypsin at different pH values.

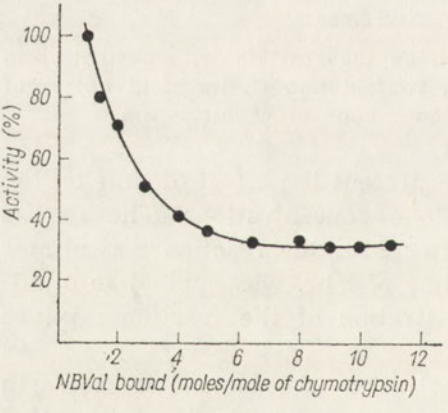

Fig. 5.

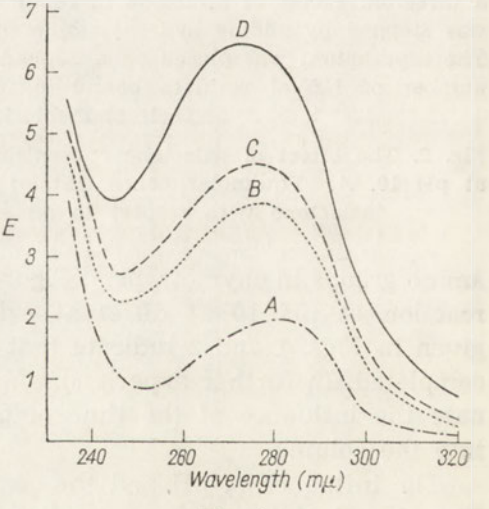

Fig. 6.

Fig. 5. Proteolytic activity of modified chymotrypsin as a function of the number of introduced NBVal residues. The results are expressed in percentage on control sample basis. The reactions with azlactone were carried out at pH values from 7 to 10, to obtain different degrees of modification.

Fig. 6. Ultraviolet absorption spectra of chymotrypsin: *A*, non-modified, and containing: *B*, 4 moles, *C*, 6 moles, and *D*, 10 moles of NBVal per mole protein. The extinctions were recalculated for the concentration of 1 mg. of protein per 1 ml.

azlactone sufficient to complete the reaction. On the other hand, at pH 9 and 10 the concentration of azlactone markedly influenced the reaction.

Proteolytic activity of chymotrypsin as a function of the number of NBVal residues introduced, is presented in Fig. 5. When up to 5 NBVal residues were introduced, the activity decreased almost linearly, whereas no further decrease in activity was observed when more amino groups were substituted.

Table 1

Acylation of chymotrypsin by azlactone of NBVal

Acylation was performed at 0° during 3 hr. at different pH values. The concentration of azlactone corresponded to a threefold excess with respect to all amino groups in protein. The reaction was stopped by adding hydroxylamine solution, the mixture was centrifuged and the supernatant was dialysed for 72 hr. and then freeze-dried. The number of NBVal residues bound was determined spectrophotometrically, and the free amino groups according to Van Slyke (1929). Control sample contained NBVal instead of azlactone. The results are expressed in moles per mole of chymotrypsin.

pH of reaction	NBVal bound	Amino groups	
		found	difference
Control	0	17.6*	
pH 8	5.1	12.3	5.3
pH 9	9.6	8.4	9.2
pH 10	11.0	6.9	10.7

* High Van Slyke value is attributed to unspecific reaction of guanidino groups with nitrous acid (Oppenheimer, Labouesse & Hess, 1966).

The amounts of azlactone bound to protein determined spectrophotometrically, were compared with the number of free amino groups. The results summarized in Table 1 show that the decrease of free amino groups in the modified chymotrypsin preparation was in agreement with the number of introduced residues of NBVal. This permits to conclude that the azlactone reacts only with amino groups of chymotrypsin.

Absorption spectra of modified chymotrypsin compared with that of non-modified chymotrypsin are presented in Fig. 6.

Modification of trypsin. The influence of time on the reaction of azlactone with trypsin at pH 8 is presented in Fig. 7; the concentration of azlactone was 30 moles per mole of trypsin which corresponded to a twofold excess with respect to the total number of amino groups. The time course of the reaction was analogous to that observed in the case of chymotrypsin. The data shown in Fig. 8 point to the decisive influence of pH on the number of bound azlactone. In contrast to chymotrypsin, the reaction began at pH 6. At an azlactone concentration equimolar with respect to the amino groups, the decrease in NBVal binding at pH values

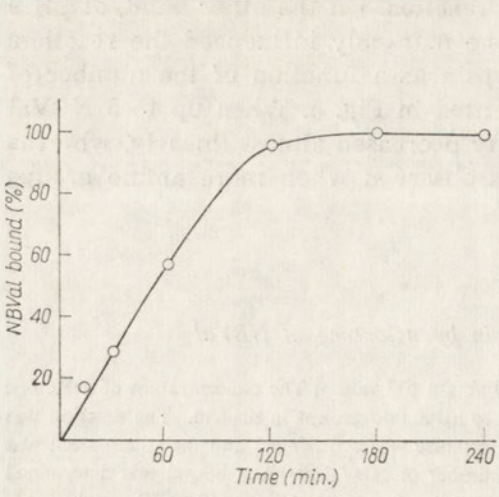

Fig. 7.

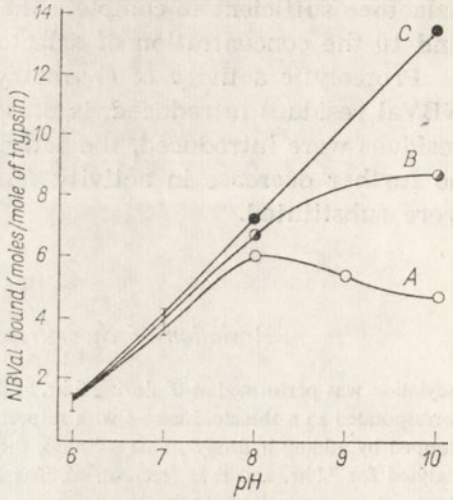

Fig. 8.

Fig. 7. The influence of time on the reaction of azlactone with trypsin. The reaction was carried out at pH 8 and the concentration of azlactone was 30 moles per mole of trypsin. The results are expressed in percentage on the 4-hr. result basis.

Fig. 8. The influence of pH on the number of NBVal residues bound to trypsin. The reaction was carried out at the following concentrations of azlactone: *A*, equimolar, *B*, twofold excess, and *C*, threefold excess with respect to all amino groups of trypsin.

above 8 (Fig. 8, curve *A*) could not be due to the hydrolysis of azlactone because such an effect of pH was not observed in the case of chymotrypsin (cf. Fig. 3, curve *A*). With higher azlactone concentration, no points are given for pH 9 (curves *B* and *C*) because the solubility of the reaction products was too low.

The results presented in Fig. 9 show that the concentration of azlactone did not affect the number of NBVal residues introduced at pH 8 but at pH 10 it played a dominant role.

During the modification of trypsin, a loss of solubility of the obtained products in the presence of 10% alcohol was observed. The amounts of the soluble product expressed in percentages of the control samples are given in Fig. 10 as a function of the pH values at which the reaction was carried out. The number of substituted amino groups per mole of trypsin was similar in all preparations (4.5 to 6.0) except that in the case of trypsin modified at pH 6 the number of introduced NBVal residues was lower (1.1). In spite of small differences in the extent of modification, there are significant differences in solubility, especially for the pH values 9 and 10.

A comparison between the number of introduced NBVal residues determined spectrophotometrically and the decrease of free amino groups

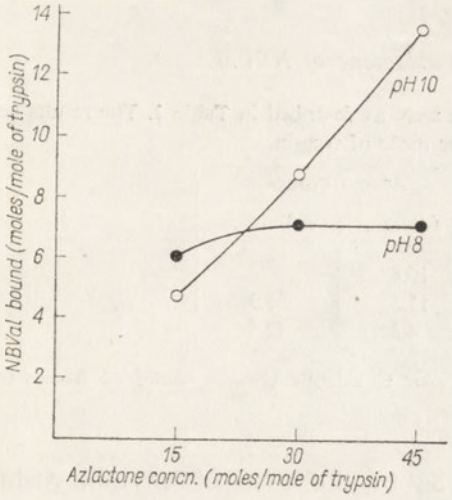

Fig. 9.

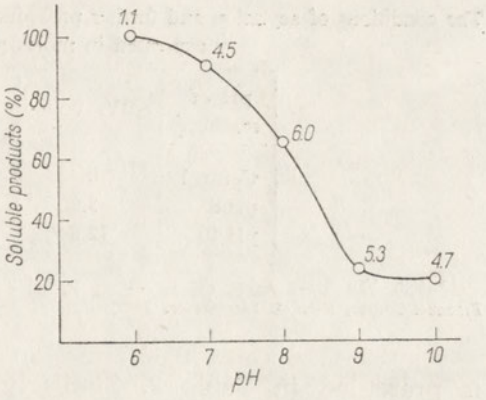

Fig. 10.

Fig. 9. The influence of azlactone concentration on the number of NBVal residues introduced into trypsin at two pH values.

Fig. 10. The effect of pH on the amount of soluble reaction products. The reaction was carried out at an equimolar concentration of azlactone with respect to all amino groups of trypsin. The results are expressed in percentage on control sample basis. The figures in the diagram denote the number of NBVal residues bound.

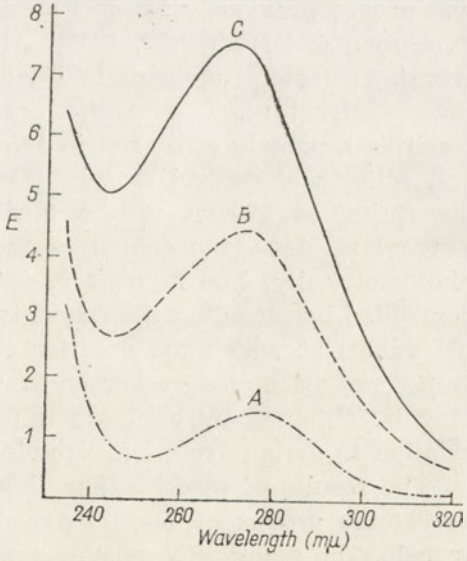

Fig. 11. Ultraviolet absorption spectra of trypsin: *A*, non-modified, and containing *B*, 6.5 moles and *C*, 13.5 moles of NBVal per mole protein. The extinctions were recalculated for the concentration of 1 mg. of protein per 1 ml.

Table 2

Acylation of trypsin by azlactone of NBVal

The conditions of acylation and further procedure were as described in Table 1. The results are expressed in moles per mole of trypsin.

pH of reaction	NBVal bound	Amino groups	
		found	difference
Control	0	16.8*	
pH 8	5.9	11.5	5.3
pH 10	12.8	4.3	12.5

*High Van Slyke value for trypsin is probably caused by autolytic processes during the reaction (cf Fraenkel-Conrat, Baen & Lineweaver, 1949).

is presented in Table 2. Similarly as in the case of chymotrypsin, azlactone reacted only with amino groups of protein. However, in contrast to chymotrypsin, no relation between the number of substituted amino groups and the enzymic activity was observed. The activity of modified trypsin was about 60% of that of the control sample regardless of the number of blocked groups. Only the preparation modified at pH 6 possessed about 80% of activity.

The ultraviolet absorption spectra of modified and non-modified trypsin preparations are presented in Fig. 11.

DISCUSSION

The applicability of azlactone of NBVal as a protein modifying agent was proved previously for gelatine (Baranowski *et al.*, 1963) and aldolase (Kochman & Baranowski, 1967). Also in the case of chymotrypsin and trypsin, azlactone appears to be a suitable agent for modification of protein structure. The reaction can be carried out under very mild conditions, namely at 0° and in 10% alcohol, which permit to retain the full activity of control samples. Owing to considerable changes in the absorption at 270 mμ of the modified protein caused by the nitrobenzoyl residues, the course of the reaction can be followed spectrophotometrically. The number of introduced residues of NBVal may be controlled by changing the concentration of the reactants as well as the pH values at which the reaction is carried out. The required concentrations of azlactone are low and the maximum amount of NBVal bound may be obtained by applying a threefold only excess of azlactone in relation to amino groups in proteins.

The results of modification of both investigated enzymes show that the amino groups of trypsin are more reactive than those of chymotrypsin. The reaction of azlactone with trypsin begins at pH 6, and with

chymotrypsin at pH 7. The number of substituted groups in chymotrypsin under the same conditions is always somewhat lower than in trypsin but the enzymic activity of chymotrypsin is more affected by acylation.

The authors are indebted to Mrs. J. Gołębiowska for excellent technical assistance. The technical assistance of Mr. R. Wojtowicz is acknowledged.

REFERENCES

Baranowski T., Kochman M., Nowak K. & Siemion I. (1963). *Bull. Acad. Polon. Sci. Ser. Biol.* **11**, 107.
Britton H. T. S. & Robinson R. A. (1931). *J. Chem. Soc.* 1456.
Davie E. W. & Neurath H. (1955). *J. Biol. Chem.* **212**, 507.
Dixon G. H. & Neurath H. (1957). *J. Biol. Chem.* **225**, 1049.
Fraenkel-Conrat H., Baen R. S. & Lineweaver H. (1949). *J. Biol. Chem.* **177**, 385.
Karrer P. & Christoffel C. (1944). *Helv. Chim. Acta* **27**, 622.
Kochman M. & Baranowski T. (1967). *Acta Biochim. Polon.* **14**, 221.
Kunitz M. (1947). *J. Gen. Physiol.* **30**, 291.
Kunitz M. & Northrop J. H. (1935). *J. Gen. Physiol.* **18**, 433.
Layne E. (1955). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. III, p. 448. Academic Press, New York.
McDonald C. & Chen L. L. (1965). *Analyt. Biochem.* **10**, 175.
Mares-Guia M. & Shaw E. (1965). *J. Biol. Chem.* **240**, 1579.
Mohr E. & Stroschein F. (1909). *Ber. chem. Ges.* **42**, 2521.
Oppenheimer L. H., Labouesse B. & Hess G. P. (1966). *J. Biol. Chem.* **241**, 2720.
Schonbaum G. R., Zerner B. & Bender M. L. (1961). *J. Biol. Chem.* **236**, 2930.
Siemion I. Z. & Nowak K. (1960). *Roczniki Chem.* **34**, 1479.
Van Slyke D. D. (1929). *J. Biol. Chem.* **83**, 425.
Wu F. C. & Laskowski M. (1955). *J. Biol. Chem.* **213**, 609.

MODYFIKACJA CHYMOTRYPSYNY I TRYPSYNY AZLAKTONEM *p*-NITROBENZOILOWALINY

Streszczenie

1. W reakcji między azlaktonem *p*-nitrobenzoilowaliny a chymotrypsyną i trypsyną stwierdzono decydujący wpływ pH na ilość związanych reszt *p*-nitrobenzoilowaliny (NBVal).

2. Enzymatyczna aktywność chymotrypsyny zmniejszała się stopniowo aż do wprowadzenia 5 reszt NBVal, zaś dalsze blokowanie grup aminowych miało już tylko nieznaczny wpływ.

3. Nie stwierdzono zależności między spadkiem aktywności zmodyfikowanej trypsyny a ilością wprowadzonych reszt NBVal.

Received 15 January, 1969.

ACTA BIOCHIMICA POLONICA
Vol. 16 1969 No. 3

J. MENDECKI, ZOFIA WIĘCKOWSKA and M. CHORĄŻY

INHIBITION OF RNA SYNTHESIS BY 9-AMINOACRIDINE IN REGENERATING RAT LIVER AND CELL CULTURE

Department of Tumour Biology, Institute of Oncology, Wybrzeże Armii Czerwonej 15, Gliwice

A new acridine derivative, 1-nitro-9(3′-dimethylaminopropylamine)-acridine (C-283) inhibited both the incorporation of [^{32}P]phosphate into RNA in regenerating rat liver and of [^{14}C]uridine and [^{3}H]uridine into RNA of the Ehrlich-Landschütz diploid cells grown in culture. The inhibitory effect was roughly proportional to the administered dose of C-283. Sucrose gradient centrifugation and gel filtration on Sephadex G-200 showed that the biosynthesis of the high-molecular-weight fractions of RNA as measured by ^{32}P incorporation was more suppressed than that of low-molecular-weight ones.

Some of acridine derivatives are known as mitotic inhibitors which interfere with nucleic acid synthesis *in vivo* and *in vitro*. They induce a series of ultrastructural alterations in the nucleolus and cytoplasm of cells grown in culture (Simard, 1966). They are also strong mutagens that intercalate between adjacent base pairs of the DNA molecule, causing errors during replication. A series of new acridine derivatives have been synthesized at the Department of Drug Chemistry and Technology of the Polytechnical School in Gdańsk. One of them, 1-nitro-9-(3′-dimethylaminopropylamine)-acridine · 2HCl (compound C-283) [1] is of particular interest due to its strong cytostatic activity. This activity has been observed in several screening tests including transplantable mouse and rat tumours (Radzikowski *et al.*, 1967a) and established cell lines in tissue culture (Radzikowski *et al.*, 1967b).

The purpose of the present study was to examine the influence of C-283 on RNA biosynthesis using regenerating rat liver and cell culture as model systems. A preliminary report of the obtained results has been presented (Chorąży, Mendecki & Więckowska, 1967).

[1] The following abbreviations are used in this text: C-283, 1-nitro-9(3′-dimethylaminopropylamine)-acridine; ELD, Ehrlich-Landschütz diploid cells; rRNA, ribosomal RNA; sRNA, soluble RNA; PVS, polyvinyl sulphate; SDS, sodium dodecyl sulphate.

MATERIAL AND METHODS

Reagents. Carrier-free $H_3{}^{32}PO_4$ in 0.1 N-HCl was supplied by the Institute for Nuclear Research (Świerk, Poland). Before injection the sample was neutralized with 0.1 N-NaOH and diluted with sterile 0.85% NaCl. [2-^{14}C]Uridine (spec. act. 26.7 mc/mole) and [^{3}H]uridine (spec. act. 2.7c/m-mole) were products of Schwarz BioResearch Inc. (Orangeburg, N.Y., U.S.A.). [2-^{14}C]Uridine (spec. act. 28 mc/m-mole) was also supplied by N. V. Philips Duphar (Amsterdam, Netherlands).

Ribonuclease (RNase), cryst., was from Sigma Chem. Co. (St. Louis, Mo., U.S.A.); to inactivate DNase, the solution of RNase was heated for 10 min. at 95°. Other reagents were from the following sources: Sephadex G-200 (Pharmacia, Uppsala, Sweden); TC Medium 199 (Difco Lab., Detroit, Mich., U.S.A.); calf serum (Biomed, Serum and Vaccine Manufacturers, Lublin, Poland); sodium dodecyl sulphate (SDS), and methyl green (British Drug Houses, Poole, Dorset, England); Macaloid, the trade name for Bentonite (G. M. Langer Co., Ritterhude bei Bremen, G.F.R.); kanamycine (Pierree S.P.A., Milan, Italy); neomycine sulphate and penicillin, cryst. (Polfa, Tarchomin, Poland). Polyvinyl sulphate (PVS) was kindly supplied by the Department of Physiological Chemistry of the Medical School in Łódź (Poland). Sucrose, phenol, perchloric acid and pyrronine were purchased from P.O.CH. (Gliwice, Poland).

Evaluation of RNA biosynthesis in regenerating rat liver

Randombred Wistar rats of both sexes, 3 months old, weighing about 200 g. were used. Partial hepatectomy was performed by the method of Higgins & Anderson (1931). At 1 - 2.5 hr. after hepatectomy the rats were injected intraperitoneally with 5 mg. of C-283 per 1 kg. body weight, dissolved in 0.15 M-NaCl (1.0 mg/ml.). In preliminary experiments it was found that administration of C-283 at 8 or 16 hr. after hepatectomy had a smaller effect. Control animals received a corresponding volume of 0.15 M-NaCl. Eighteen hours after hepatectomy the rats were injected with 1 - 2 mc of [^{32}P]phosphate; 30 min. to 4 hr. later the animals were killed by decapitation under light ether anaesthesia, thoroughly bled and livers immediately removed, washed in chilled saline and stored on solid CO_2.

For RNA extraction, liver tissue was minced with scissors, then homogenized in a Potter-Elvehjem type homogenizer at 0° in 9 vol. of 0.14 M-NaCl containing Macaloid, 1 mg./ml., and PVS, 2 µg./ml. From the homogenate, RNA was extracted by the hot phenol method (Scherrer & Darnell, 1962) or by phenol extraction at stepwise increased tempe-

rature according to Lerman, Mantieva & Georgiev (1964). In the first procedure the homogenate was treated with an equal volume of water-saturated phenol warmed up to 60°and after 10 min. of shaking at this temperature chilled in the mixture of solid CO_2 and acetone to 0°. Following centrifugation at 3000 *g* for 10 min., the aqueous phase was removed and extracted with one half volume of phenol containing 0.5% SDS. This procedure was repeated, the aqueous layers combined and extracted 3 times with ether; then traces of ether were removed by bubbling nitrogen through the solution. RNA was precipitated with 2 vol. of ethanol, dissolved in 3 ml. of 0.05 M-NaCl, and dialysed for about 18 hr. against three changes of 0.05 M-NaCl.

In the second procedure the homogenate was mixed with an equal volume of water-saturated phenol at pH 6.0 and shaken for 10 min. at 0°. After centrifugation, the water phase was collected. The interphase was mixed with 5 vol. of 0.14 M-NaCl, an equal volume of phenol was added and, after shaking for 5 min. at 0°, centrifuged; the pooled water phases were deproteinized with phenol containing SDS, and RNA precipitated with ethanol. This preparation was called the 0° fraction. In a similar way further RNA fractions were isolated from the interphase at 10°, 40°, 55° and 65°. The obtained RNA preparations were dialysed against several changes of 0.15 M-NaCl.

Absorption spectra were determined in a Beckman spectrophotometer model DU and the concentration of RNA was estimated assuming that 0.022 extinction unit at 260 mμ corresponds to a concentration of RNA of 1 μg./ml. in a cell of 1 cm. light path.

To determine the radioactivity, three samples of 0.05 ml. of RNA solution were placed on planchettes, dried and counted in a thin-window gas-flow counter type VA-Z-530, Vakutronik (Dresden, G.D.R).

In some cases the preparations were separated into high-molecular-weight fractions corresponding to rRNA, and low-molecular-weight sRNA. For this fractionation, the salting-out technique (Milman, 1960) or gel filtration on Sephadex G-200 (Dirheimer, Weil & Ebel, 1962) were employed.

Sucrose density gradient centrifugation was performed on Spinco L 50 ultracentrifuge (rotor SW 39). The RNA solution containing 6.0 E units in 0.1 ml., was layered on 5 ml. of a linear sucrose gradient (2.5 - 20%) in 0.1 M-NaCl - 0.01 M-sodium acetate buffer, pH 5. The gradient was prepared in the vessel of Britten & Roberts (1960). The samples were centrifuged at 35 000 rev./min. for 5 hr. at 4°. Then the bottom of the tube was perforated and three-drop fractions were collected, diluted by adding 2.5 ml. of water, then the concentration of RNA was determined spectrophotometrically and the radioactivity was measured in the Tracerlab (Waltham, Mass., U.S.A.) window-less gas-flow counter type SC-50B.

Cell culture

Ehrlich-Landschütz diploid (ELD) cell line (kindly supplied by Dr. J. Kieler, Fibiger Laboratory, Copenhagen, Denmark) were grown as a monolayer culture either in Leighton tubes or in Carell flasks in TC medium 199 supplemented with 10% of calf serum, kanamycine (0.09 mg./ml.), neomycine (0.05 mg./ml.) and penicillin (20 units/ml.).

The cells were exposed for 12 hr. to different amount of C-283 dissolved in sterile 0.15 M-NaCl. Then [2-^{14}C]uridine (0.1 μc/ml.) was added to control and experimental cultures and after 5 hr. of growth the cells were detached from the glass by freezing and thawing, transferred to centrifuge tubes, and separated from the medium by repeated washing with cold saline and subsequent centrifugation. In each experimental and control group, six separate cultures were used.

Free nucleotides were extracted with 0.5 N-$HClO_4$, and the radioactivity of RNA was determined either in alkaline or RNase hydrolysates. In some experiments, the cells were suspended in 0.14 M-NaCl and disintegrated in a TUR sonicator, type US 2-2 (Transformatoren u. Röntgenwerk, Dresden, G.D.R.), and after extraction with perchloric acid total activity was determined in the sonicated cell homogenate. Details of these procedures are given in Table 2.

Autoradiography. ELD cells were grown on cover slips in Leighton tubes for 48 hr. Then C-283 at concentrations of 0.0001, 0.001 and 0.01 μg./ml. was added, and after 14 or 20 hr. of growth, fresh medium and [^{3}H]uridine (0.02 μc/ml.) were added. After a further 5 hr. the cultures were washed with glucosole solution (Eagle, 1955), the cells attached to cover slips fixed in Carnoy solution (1 vol. conc. acetic acid and 5 vol. absolute ethanol) for 10 min., washed in 80% and 50% ethanol, then in water, extracted with 0.5 N-$HClO_4$ for 8 min. at 2° and washed again in water. The cover slips were attached to microscopic slides and covered either with Kodak AR-10 stripping film or with liquid emulsion Illford G-4. The exposure time was six weeks. The autoradiograms were then developed, stained with methyl green-pyrronine according to Brachet (Pearse, 1961) and examined in light microscope under oil-immersion objective.

RESULTS

Effect of the amount of administered C-283 on RNA biosynthesis. In all experiments, C-283 inhibited the incorporation of ^{32}P into RNA of regenerating rat liver, and the inhibitory effect was roughly proportional to the administered dose (Table 1). The inhibition amounted to 86 - 88% of control values after administration of 5 mg. of C-283 per 1 kg. body weight, and did not increase with a dose of 10 mg./kg. In

Table 1

Inhibition by C-283 of incorporation of [^{32}P]phosphate into RNA in regenerating rat liver

C-283 was administered 2 hr. after partial hepatectomy. [^{32}P]Phosphate (1.0 mc/rat) was injected intraperitoneally 18 hr. after the operation, and 4 hr. later liver RNA was isolated. rRNA and sRNA were separated by the salting-out technique (Milman, 1960). Each figure is the mean obtained from 3 animals.

Dose of C-283 (mg./kg. body wt.)	RNA synthesis					
	total RNA		rRNA		sRNA	
	counts/min./mg. RNA	%	counts/min./mg. RNA	%	counts/min./mg. RNA	%
Experiment 1						
None (control)	122 000	100	74 000	100	87 000	100
1.25	73 200	60	56 000	75	70 400	81
2.50	37 820	31	44 820	60	30 450	35
5.00	17 080	14	11 900	16	18 270	21
Experiment 2						
None (control)	182 100	100	105 000	100	126 000	100
2.5	47 300	26	—	—	42 960	34
5.0	22 100	12	21 500	20	38 700	31
10.0	22 260	12	20 200	18	26 200	20

Table 2

The effect of C-283 on labelling of RNA of ELD cells with [^{14}C]uridine

The cells after 48 hr. of growth were incubated with C-283 for 12 hr., then with [2-^{14}C]uridine (0.2 μc/ml.) for 5 hr. After removal of free nucleotides with 0.5 N-$HClO_4$, *A*, RNA was hydrolysed with RNase (20 μg./ml., 2 hr., 37°, pH 5.2), and radioactivity determined (practically no activity was found in the $HClO_4$ precipitate). *B*, RNA was hydrolysed with 0.1 N-KOH (18 hr., 37°), treated with activated charcoal, eluted with 50% ethanol in 1% NH_4OH solution, and radioactivity was determined. *C*, The cells were submitted to sonication and free nucleotides extracted; the precipitate was suspended in water, 0.05 ml. of the suspension deposited on a planchette and radioactivity determined. The radioactivity was expressed as percentage of the control values. Each figure is an average obtained from 4 - 6 cultures and triplicate radioactivity determinations.

Concentration of C-283 (μg./ml.)	Procedure							
	A			*B*		*C*		
	Expt. 1	Expt. 2	Expt. 3	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 3
None (control)	100	100	100	100	100	100	100	100
0.0001	90	119	92	—	81	88	67	77
0.001	5	38	64	74	79	64	68	22
0.01	1	28	34	50	4	31	32	13

four other experiments with samples of C-283 taken from different batches, the dose of 5 mg./kg. inhibited the specific activity of total RNA by 70 - 86%. This dose was applied in all further experiments.

A similar, even more pronounced, dose-dependent inhibition of RNA synthesis as in regenerating rat liver has been observed with ELD cells grown in culture (Table 2).

Effect of labelling time. To evaluate whether RNA fractions labelled in short and prolonged time differ in their susceptibility to C-283, two groups of control animals and four groups treated with C-283 were injected with [^{32}P]phosphate, and after 30 and 120 min. RNA was isolated and its specific activity compared (Table 3). In three out of four experiments C-283 caused stronger inhibition of the RNA fractions labelled for 30 min. than of those labelled for 120 min.

Table 3

The effect of C-283 on RNA synthesis in regenerating rat liver in pulse and 2-hr. labelling experiments

Two hours after partial hepatectomy, the rats were injected intraperitoneally either with NaCl solution (control) or with 5 mg. of C-283/kg. body weight. After a further 18 hr. [^{32}P]phosphate (1.4 mc/rat) was administered, and 30 or 120 min. later liver RNA was isolated. The radioactivity was determined and expressed as percentage of the control values taken as 100. The figures are means of 3 determinations.

Labelling time (min.)	Remaining synthesis (%)			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
30	65	6	0	18
120	27	25	9	50

Sensitivity of various RNA fractions to C-283. As shown in Table 1, the biosynthesis of both the high-molecular-weight fraction corresponding to rRNA and the low-molecular-weight one of the sRNA type was inhibited by C-283. From Sephadex G-200 gel filtration it is evident that the incorporation of ^{32}P into high-molecular-weight RNA fraction eluted from the column in the first peak, was always more strongly inhibited (Fig. 1), and even in some experiments the inhibition of incorporation of ^{32}P into the low-molecular-weight fraction could not be demonstrated.

The higher susceptibility of rRNA to C-283 as compared to sRNA was also demonstrated in the RNA preparation fractionated by sucrose density gradient centrifugation (Fig. 2). The labelling of RNA fractions

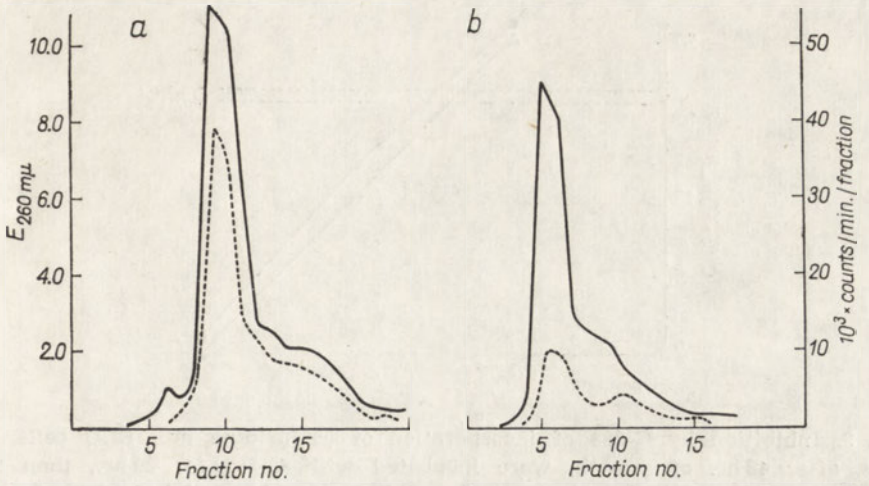

Fig. 1. Elution pattern from Sephadex G-200 column (1.5 × 25 cm.) of RNA isolated from rat liver 2 hr. after injection of [^{32}P]phosphate (1 mc/rat). The column was eluted with 0.05 M-NaCl, fractions of 3 ml. being collected. The first peak corresponds to rRNA, the second to sRNA. (a), RNA from control rat; (b), RNA from C-283 treated rat. (——), Extinction; (- - -), radioactivity

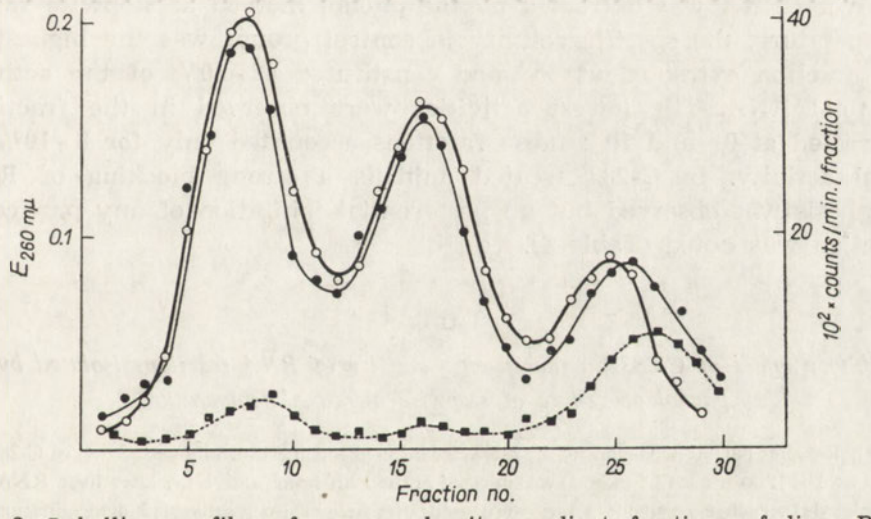

Fig. 2. Labelling profiles of sucrose density gradient fractions of liver RNA isolated from control and C-283 treated rats. At 1.5 hr. after partial hepatectomy the rats were injected with C-283, then after 19 hr. 1.4 mc of [^{32}P]phosphate was administered intraperitoneally and 4 hr. later liver RNA was isolated. (○), Extinction of RNA of control and C-283 treated animals; (●), radioactivity of RNA of control rats; (■), radioactivity of RNA of C-283 treated rats

in control animals was found to be dependent on time, and was uniform in all fractions 240 min. after ^{32}P administration. The inhibition by C-283 of labelling of both heavier fractions of RNA, presumably 28S and 18S rRNA, was much more pronounced than the inhibition of the

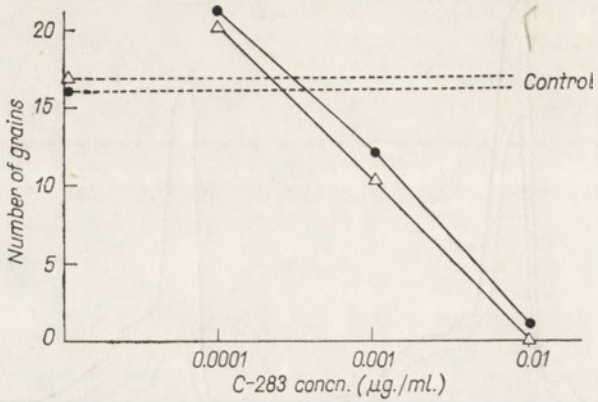

Fig. 3. Inhibition by C-283 of incorporation of [^{3}H]uridine into ELD cells. The cells, after 48 hr. of growth, were incubated with C-283 for 20 hr., then with [^{3}H]uridine, 0.02 μc/ml., for 4 hr. The (●) median and (△) modal number of grains was obtained from counting of 5000 cells

third fraction corresponding to sRNA. This was also true for the 30 min. labelling time.

When RNA was extracted by the phenol method at stepwise raised temperature, the specific activity in control groups was the highest in the fraction extracted at 65° and constituted 42 - 50% of the activity of total RNA. The lowest activities were observed in the fractions extracted at 0° and 10°; these fractions accounted only for 8 - 10% of total activity. In C-283 treated animals a strong blocking of RNA synthesis was observed but no preferential inhibition of any particular fraction was noted (Table 4).

Table 4

Inhibitory effect of C-283 on the specific activity of RNA fractions isolated by the phenol procedure at stepwise increased temperature

Two hours after partial hepatectomy the rats were injected intraperitoneally with 5 mg. of C-283/kg. body wt. [^{32}P]Phosphate (2.5 mc/rat) was injected at the 19th hour and 1 hr. later liver RNA was isolated. In each experiment 3 rats were used; the figures are means of 3 determinations.

Fraction of RNA obtained at °C	Experiment 1			Experiment 2		
	Counts/min./mg. RNA		Inhibition (%)	Counts/min./mg. RNA		Inhibition (%)
	Control	C-283		Control	C-283	
0	25 400	5 000	80	115 000	35 000	70
10	25 800	1 800	93	72 900	21 500	71
40	148 000	10 000	93	234 000	93 000	60
55	136 000	12 000	91	479 000	158 200	67
65	243 000	38 000	85	933 500	210 200	78

Autoradiography. After exposure of cell culture to [^{3}H]uridine, the average number of grains scored over the cells decreased with the increasing amount of the acridine derivative added to the medium. At C-283 concentration of 0.01 μg./ml. of medium almost no grains were found (Fig. 3). It should be noted that the number of grains at the lowest C-283 concentration, 0.0001 μg./ml., was found to be higher than in control cultures.

DISCUSSION

Our studies carried out on two model systems, i.e. regenerating rat liver and Ehrlich-Landschütz diploid cells in culture, have shown that a new compound, 1-nitro-9(3′-dimethylaminopropylamine)-acridine (C-283), similarly as other acridine derivatives (Scholtissek, 1965), is a strong inhibitor of RNA synthesis. The inhibition was dependent on the dose of C-283, the high-molecular-weight RNA fraction being more affected than the low-molecular one.

The inhibition by C-283 of RNA synthesis seems to explain its cytostatic effect on cell culture (Radzikowski *et al.*, 1967b) and inhibition of tumour growth (Radzikowski *et al.*, 1967a). Our results seem to indicate that the new acridine derivative, similarly as its other derivatives, becomes easily complexed with DNA. Lerman (1964) has stated that the derivatives of aminoacridine intercalate between two pairs of bases of DNA molecule. The acridine derivatives possess greater affinity for the spaces between successive AT and AT pairs than for those between the AT-GC and GC-GC pairs (Tubbs, Ditmars & Van Winkle, 1964). It has been calculated that in the acridine-DNA complex one molecule of acridine corresponds to 4-5 nucleotides (Peacocke & Skerrett, 1956). Increased ionic strength, lowered pH and compounds such as urea which weaken the intermolecular bonds, permit of rapid dissociation of the DNA-proflavin complex in the course of gel filtration (Liersch & Hartman, 1964). This indicates that electrostatic forces are also participating in formation of the complex of DNA with acridine. The intercalation of acridine into DNA stretches and unwinds the DNA molecule thus changing its secondary structure. This is reflected by greater viscosity, lowered sedimentation coefficient (Lerman, 1961) and elevated melting temperature (Lerman, 1964; Walker, 1965). Effective blocking of DNA template by C-283 seems to be responsible for subsequent inhibition of RNA biosynthesis.

This work was supported by the State Pharmaceutical Establishment "Polfa". We are indebted to Mrs. Z. Szweda, Mrs. H. Minc and Miss E. Polakiewicz for skilful technical assistance.

REFERENCES

Britten J. R. & Roberts R. B. (1960). *Science* **131**, 32.
Chorąży M. (1965). *Neoplasma* **12**, 333.
Chorąży M., Mendecki J. & Więckowska Z. (1967). *V Internationaler Kongress für Chematherapie, Wien, Österreich, 26.VI - 1.VII.1967.* p. 361. Verlag der Wiener Medizinischen Akademie, Wien.
Dirheimer G., Weil J. H. & Ebel J. P. (1962). *C. R. Acad. Sci.* **225**, 2312.
Eagle H. (1955). *Science* **122**, 501.
Higgins G. M. & Anderson R. M. (1931). *Arch. Pathol.* **12**, 186.
Lerman L. S. (1961). *J. Mol. Biol.* **3**, 18.
Lerman L. S. (1964). *J. Cell Comp. Physiol.* **64**, Suppl. 1, 1.
Lerman M. J., Mantieva V. L. & Georgiev G. P. (1964). *Biokhimija* **29**, 518.
Liersch M. & Hartman G. (1964). *Biochem. Z.* **340**, 390.
Milman Z. S. (1960). *Biokhimija* **25**, 796.
Peacocke A. R. & Skerrett J. N. H. (1956). *Trans. Faraday Soc.* **52**, 261.
Pearse A. G. E. (1961). *Histochemistry.* II ed., p. 825. J. & A. Churchill Ltd., London.
Radzikowski Cz., Ledóchowski Z., Ledóchowski A., Wrzołek St., Hrabowska M. & Konopa J. (1967a). *Arch. Immun. Ther. Exper.* **15**, 126.
Radzikowski Cz., Więckowska Z., Woźniakowska Z. & Chorąży M. (1967b). *Neoplasma* **14**. 121.
Scherrer K. & Darnell J. E. (1962). *Biochem. Biophys. Res. Commun.* **7**, 486.
Scholtissek Ch. (1965). *Biochim. Biophys. Acta* **103**, 146.
Simard R. (1966). *Canc. Res.* **26**, 2316.
Tubbs R. K., Ditmars W. E. & Van Winkle Q. (1964). *J. Mol. Biol.* **9**, 545.
Walker J. O. (1965). *Biochim. Biophys. Acta* **109**, 585.

HAMOWANIE 9-AMINOAKRYDYNĄ SYNTEZY RNA W REGENERUJĄCEJ WĄTROBIE SZCZURA I KULTURACH KOMÓRKOWYCH

Streszczenie

1. Nowa pochodna akrydyny, 1-nitro-9(3'-dimetyloaminopropyloamino)akrydyna (C-283), silnie hamuje zarówno wbudowywanie [^{32}P]fosforanu do RNA regenerującej wątroby, jak i [^{14}C]urydyny i [^{3}H]urydyny do diploidalnych komórek Ehrlicha-Landschütza rosnących w kulturach. Efekt hamujący jest proporcjonalny do dawki C-283.

2. Wirowanie w gradiencie sacharozy i filtracja na żelu Sephadex G-200 wykazały, że C-283 silniej hamuje biosyntezę wysokocząsteczkowych frakcji RNA mierzoną włączaniem ^{32}P niż biosyntezę frakcji niskocząsteczkowych.

Received 3 February, 1969.

ACTA BIOCHIMICA POLONICA
Vol. 16 1969 No. 3

M. ŚWIERKOWSKI and D. SHUGAR

A NEW THYMINE BASE ANALOGUE, 5-ETHYLURACIL: 5-ETHYLURIDINE-5′-PYROPHOSPHATE AND POLY-5-ETHYLURIDYLIC ACID

Department of Radiobiology, Institute of Oncology; and Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Rakowiecka 36, Warszawa 12

1. Syntheses are described for 5-ethyluridine, 5-ethyluridine-5′-pyrophosphate and the various intermediates involved in the preparation of the latter. The preparation of 5-hexyluridine is also described. The absorption spectra and dissociation constants are presented for 5-ethyluracil, 5-ethyluridine and 5-hexyluridine. 2. 5-Ethyluridine-5′-pyrophosphate proved to be a substrate for polynucleotide phosphorylase from *Escherichia coli*, but not for the enzyme from *Micrococcus lysodeikticus*, and was a very poor substrate (if at all) for the *Azotobacter vinelandii* enzyme. 3. The preparation and some of the properties of poly-5-ethyluridylic acid are described. It is readily hydrolysed by pancreatic ribonuclease. At room temperature, the polymer is in the form of a random coil; below 10°, in the presence of high salt concentration, or 0.01 M-Mg^{2+}, it forms a helical structure with a melting point of about -2°. The properties of poly-5-ethyluridylic acid are compared with those of poly-uridylic acid and poly-5-methyluridylic acid, and the structures of all three polymers are discussed.

The preparation of poly-5-ethyluridylic acid, poly-EtU [1], was originally undertaken with a view to making available an additional model polyribonucleotide, and a potential messenger RNA, which might conceivably assist in elucidating the origin of the enhanced stability of poly-rT as compared to poly-U (Szer, Świerkowski & Shugar, 1963;

[1] The following abbreviations are used in this text: DCC, *N,N′*-dicyclohexylcarbodiimide; 5MeC, 5-methylcytidine; EtUDP, 5-ethyluridine-5′-pyrophosphate; poly-U, poly-uridylic acid; poly-rT, poly-5-methyluridylic acid; poly-EtU, poly-5-ethyluridylic acid; poly-rA, poly-riboadenylic acid; poly-dA, poly-deoxyriboadenylic acid; poly-C, poly-cytidylic acid; poly-5MeC, poly-5-methylcytidylic acid; poly-I, poly-inosinic acid; poly-X, poly-xanthylic acid; poly-(A:U), twin-stranded helix of poly-A and poly-U, with similar connotations for other polymer complexes; tRNA, transfer RNA.

Świerkowski, Szer & Shugar, 1965). Under conditions where poly-U is a random coil, poly-rT exhibits a transition profile typical of that for a helix-coil transition (Shugar & Szer, 1962). Furthermore the helical complex of poly-rT with poly-A melts out with a T_m considerably higher than that for the corresponding complex of poly-U and poly-A (Szer *et al.*, 1963; Barszcz & Shugar, 1968). This pronounced enhancement of helix stability by a pyrimidine 5-methyl substituent is rather unexpected and unlikely to be due to modifications in strength of base-pair hydrogen bonds. A similar enhancement of helix stability is observed in poly-(X:rT) as compared to poly-(X:U) (Fikus & Shugar, 1969) and in poly-(I:5MeC) relative to poly-(I:C) (Szer & Shugar, 1966).

It is obviously desirable to establish the origin and nature of the effect of a pyrimidine 5-methyl substituent on helix structure and stability, the more so in that one of the two major differences in composition between RNA and DNA is the 5-methyl group in the thymine residues of DNA, while thymine is also one of the minor base components of tRNA.

Attempts to find some theoretical interpretation for the influence of a pyrimidine 5-methyl substituent on helix stability have hitherto proven unsuccessful. It was consequently desirable to obtain additional experimental data, and it therefore seemed appropriate to investigate the effect of a higher alkyl substituent. The most logical choice appeared to be the replacement of the 5-methyl by a 5-ethyl group.

The following communication describes the preparation of 5-ethyluridine-5′-pyrophosphate, which was found to be a suitable substrate for *Escherichia coli* polynucleotide phosphorylase, as well as the necessary intermediates, and the polymerization of 5-ethyluridine-5′-pyrophosphate to give poly-EtU. The properties of the latter have been described in part elsewhere (Świerkowski & Shugar, 1969a); some additional data is appended here.

It should be emphasized that 5-ethyluracil and its glycosides are of more than purely physico-chemical interest. Not only is 5-ethyluracil a thymine analogue, which may undergo incorporation into bacterial DNA, but 5-ethyldeoxyuridine readily replaces thymidine in bacteriophage DNA. Furthermore such incorporation occurs without any mutagenic effects, as might be expected (for brief summary, see Shugar, Świerkowski, Fikus & Barszcz, 1967). In addition 5-ethyldeoxyuridine is almost as effective an anti-viral agent as 5-iododeoxyuridine (Świerkowski & Shugar, 1969b).

5-Hexyluridine was also prepared during the course of this work. Although no attempt was made to phosphorylate this nucleoside, it has proven extremely useful in studies on the photochemical transformation of 5-alkyl pyrimidine nucleosides (Pietrzykowska & Shugar, 1968), the results of which will be reported elsewhere.

RESULTS AND DISCUSSION

Chemical syntheses

5-Ethyluridine-5′-pyrophosphate was obtained with only minor modifications of standard procedures by the following sequence of reactions:

The thymine analogue, 5-ethyluracil, was synthesized essentially as described by Burckhalter & Scarborough (1955), and the di(5-ethyluracil)mercury salt (I) according to the method of Fox, Yung, Davoll & Brown (1956) for the analogous mercury salt of thymine (Shapira, 1962).

1-*O*-Acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose was prepared by the method of Recondo & Reyderknecht (1959). This was converted to 2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl chloride (II) by standard procedures, but with added precautions for removal from the final product of traces of HCl (see Experimental).

5-Ethyluridine [1-(β-D-ribofuranosyl)-5-ethyluracil] (IV) was then prepared essentially according to the method of Fox *et al.* (1956). However, in the condensation reaction, the molar ratio of II to I employed was 4:3, with a view to increasing the yield of the benzoylated nucleoside (Griffin, Todd & Rich, 1958). In one such experiment the yield in the condensation reaction, estimated chromatographically, was of the order of 90%, but this dropped to 53% (with respect to 5-ethyluracil) following isolation and crystallization of the 1-(2′,3′,5′-tri-*O*-benzoyl-β-D-ribofuranosyl)-5-ethyluracil (III).

The benzoylated nucleoside (III) was debenzoylated in sodium methoxide to give 5-ethyluridine (IV) in 90% yield.

An additional route has been utilized to obtain 5-ethyluridine, based on the Hilbert & Johnson (1930) condensation of 2,4-diethoxy-5-ethylpyrimidine with 2,3,5-tri-*O*-benzoyl-D-ribofuranosyl chloride. The advantage of this procedure, to be described elsewhere (Kulikowski & Shugar, in preparation) is that it permits of the simultaneous preparation of 5-ethylcytidine and alkylamino analogues of the latter.

5-Ethyluridine (IV) was next converted to 2′,3′-*O*-isopropylidene-5--ethyluridine (V) according to the method of Levene & Tipson (1934) for uridine. This, in turn, was phosphorylated by the cyanoethylphosphate procedure of Tener (1961) to give 5-ethyluridine-5′-phosphate [1-(β-D--ribofuranosyl)-5-ethyluracil-5′-phosphate] (VI).

Compound VI was converted to the morpholidate (VII) as described by Moffatt & Khorana (1961) and phosphorylated to give the lithium salt of 5-ethyluridine-5′-pyrophosphate (VIII) in 40% yield.

Absorption spectra and dissociation constants

The absorption spectrum of 5-ethyluracil at several pH values is shown in Fig. 1. While the maximum at neutral pH is at 265 mμ as for thymine (Shugar & Fox, 1952), the spectrum for the monoanionic form (curve at pH 12) is more nearly like that for uracil. The shape of the

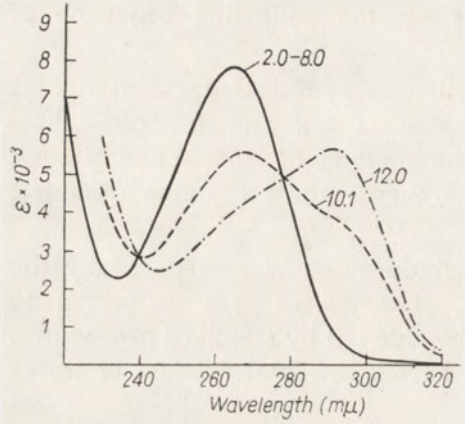

Fig. 1. Absorption spectrum of 5-ethyluracil at pH values indicated; pK_a, from spectral titration, 9.90

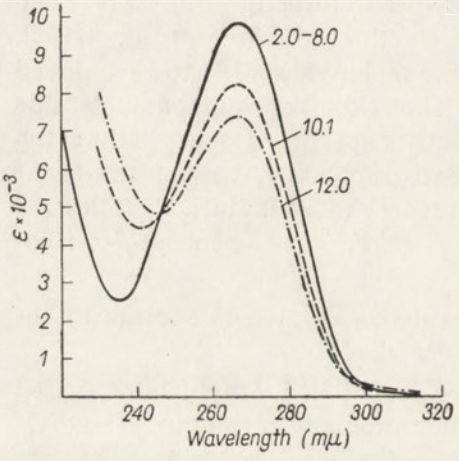

Fig. 2.

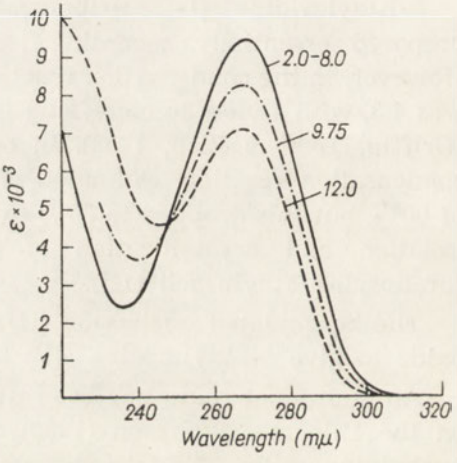

Fig. 3.

Fig. 2. Absorption spectrum of 5-ethyluridine at pH values indicated; pK_a, from spectral titration, 9.86

Fig. 3. Absorption spectrum of 5-hexyluridine at pH values indicated; pK_a, from spectral titration, 9.76

pH 12 curve demonstrates that, as for uracil and thymine (Wierzchowski, Litońska & Shugar, 1965), the monoanionic form of 5-ethyluracil consists of an almost equimolar equilibrium mixture of two anions, one involving ionization of the $N_{(1)}$ hydrogen, the other the $N_{(3)}$ hydrogen. Spectral titration gave a pK_a for 5-ethyluracil of 9.9, as compared to 9.9 for thymine and 9.5 for uracil.

The absorption spectrum of 5-ethyluridine (Fig. 2), as might be anticipated, closely resembles that for thymidine (Fox & Shugar, 1952). Spectral titration gave a pK_a, for dissociation of the $N_{(3)}$ hydrogen, of 9.86 as compared to 9.82 for thymidine, and 9.98 for 5-ethyldeoxyuridine (Świerkowski & Shugar, 1969b).

The solubility of 5-hexyluracil in aqueous medium was, as might be anticipated, too low to permit of accurate measurement of absorption spectra at various pH values; the pK_a value was therefore not measured, although this probably could be done at a more elevated temperature. The solubility of 5-hexyluridine was quite adequate for such measurements, and Fig. 3 exhibits the absorption spectrum at several pH values, from which the pK_a was calculated to be 9.76. Note that the molar extinction of this nucleoside, as well as its pK_a, are both lower than for 5-ethyluridine.

It seems reasonable to expect that 5-ethyluracil will base-pair like thymine or uracil, and this is supported by the fact that the pK_a for thymine and 5-ethyluracil are identical. However, the 5-ethyl substituent does influence the stability of the complex of poly-EtU with poly-rA (Świerkowski & Shugar, 1969a) and poly-dA (Barszcz & Shugar, 1968); and this is reflected in the stability of natural DNA containing incorporated 5-ethyluracil (Pietrzykowska & Shugar, 1967).

Polymerization of 5-ethyluridine-5′-pyrophosphate

Initial attempts to polymerize 5-ethyluridine-5′-pyrophosphate with polynucleotide phosphorylase from *A. vinelandii* and *M. lysodeikticus*, under standard conditions, proved unsuccessful. This was followed by trials involving a vide variation in reaction conditions, such as temperature (up to 50°), pH (up to pH 9.5 for the micrococcal enzyme), the use of various salts, urea, divalent cations, etc. In several experiments, low yields (5 - 10%) of polymer material were obtained with the azotobacter enzyme, but even these were not reproducible, and were finally abandoned.

Polymerization was then attempted with the *E. coli* enzyme (Kimhi & Littauer, 1968), employing conditions optimal for polymerization of ADP (Kimhi & Littauer, 1968), and described below (see Experimental). These same conditions were also found suitable for polymerization of ribosylthymine-5′-pyrophosphate, but gave no signs of polymerization of EtUDP. However, prolonged incubation of the latter substrate under the same conditions eventually led to formation of poly-EtU in approximately 50% yield. Additional prolongation of the incubation time resulted in a gradual decrease in polymer yield, probably because of the presence of traces of some nucleolytic enzyme in the polynucleotide phosphorylase (cf. Szer *et al.*, 1963) or as a result of bacterial growth.

Polymerizations were therefore carried out for about 16 hr. Pronase was then added and incubation continued for 1 hr. to destroy traces of nucleolytic enzymes, and the polymer isolated by the phenol method, followed by extensive dialysis against decreasing NaCl concentration and EDTA, then against distilled water, and finally freeze-dried or stored in solution in the deep-freeze.

It is somewhat difficult to conclude from the above results whether the *E. coli* enzyme exhibits a wider range of specificity than that from *A. vinelandii* or *M. lysodeikticus*. It must be borne in mind that the degree of purification of the three enzyme preparations is by no means the same, consequently no final conclusions can be drawn at this time. It is, however, pertinent to note that preliminary trials indicated that the *E. coli* enzyme can polymerize dihydrouridine-5′-pyrophosphate; it has previously been shown that this compound is not a substrate for the azotobacter enzyme (Ochoa & Heppel, 1957; Szer & Shugar, 1961).

Properties of poly-5-ethyluridylic acid

Individual preparations of poly-EtU varied somewhat in homogeneity and sedimentation constants. The preparation used in the following experiments was fairly homogeneous in the ultracentrifuge with an $S_{20,w}$ of 6.1.

At room temperature the residual hyperchromicity of the polymer was low and amounted on the average to 4-5% on enzymic or acid hydrolysis to monomers. Poly-EtU was completely hydrolysed to monomers by pancreatic ribonuclease.

The temperature profile for poly-EtU at neutral pH in the presence of 0.01 M-Mg^{2+} is shown in Fig. 4, which includes, for purposes of comparison, the profiles of poly-U (taken from Lipsett, 1960) and poly-rT (taken from Tramer, Wierzchowski & Shugar, 1969), under the same conditions. It is clear from Figs. 4 and 6a that although, like poly-U and poly-rT, poly-EtU possesses an ordered structure at low temperature, this structure is not fully developed at 0°; and that the melting point, T_m, must be in the neighbourhood of, or even below, 0°.

In order to place in evidence the complete helix-coil transition of poly-EtU, advantage was taken of the observation (Szer, 1966) that the transition profiles of poly-U and poly-rT are shifted to higher temperatures in the presence of 1 equivalent of various polyamines. The same was found to hold for poly-EtU, as can be seen from Fig. 5, which exhibits the temperature profiles, measured in each case at the λ_{max} of the absorption spectrum (Fig. 6b), for poly-EtU, poly-U and poly-rT, each in the presence of 1 equivalent of spermine or spermidine.

Note that the effect of either polyamine is to place in evidence the complete profile for poly-EtU, with a temperature hyperchromicity of

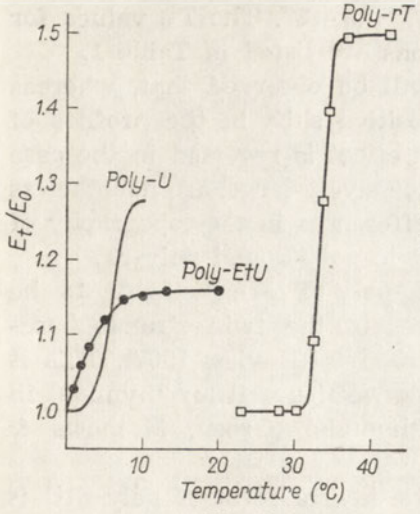

Fig. 4.

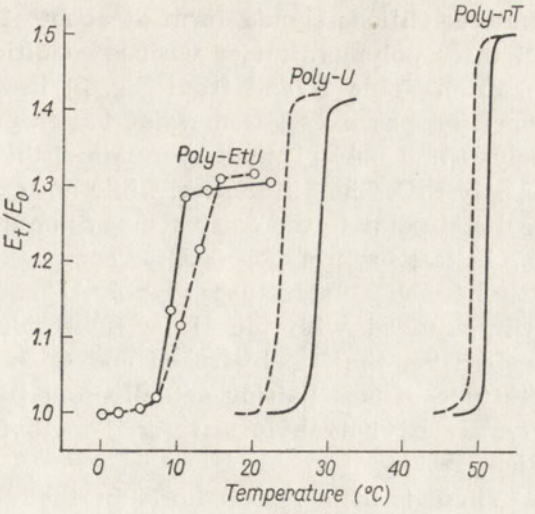

Fig. 5.

Fig. 4. Temperature transition profile, measured at 267 mμ, for poly-EtU in 0.01 M-Mg^{2+} at neutral pH, together with those for poly-U (Lipsett, 1960) and poly-rT (Tramer *et al.*, 1969) under the same conditions. Note that the profile for poly-EtU is incomplete, due to the fact that the T_m is below 0°

Fig. 5. Temperature transition profiles of poly-EtU, poly-U and poly-rT at neutral pH in the presence of 1 equivalent of spermine (——) and spermidine (— — —).

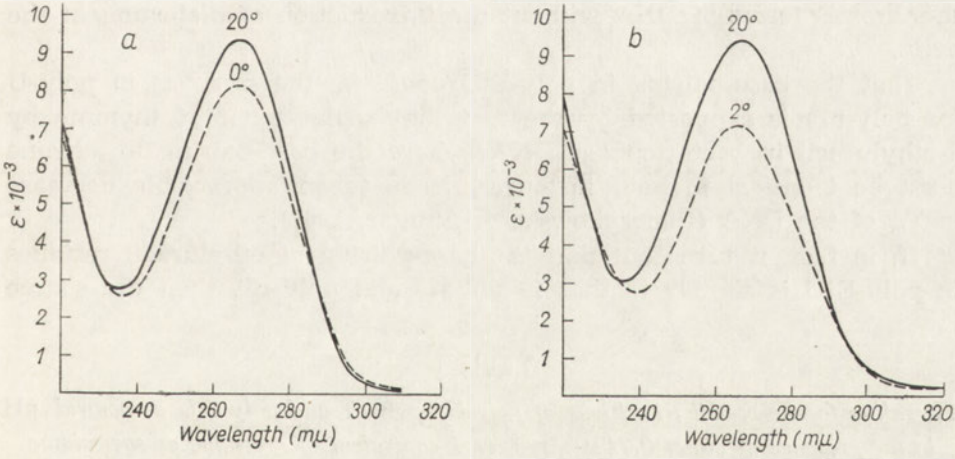

Fig. 6. Absorption spectra of poly-EtU at neutral pH: (a), in the presence of 0.01 M-Mg^{2+}, of the random coil form (at 20°) and partially helical form (at 0°); (b), in the presence of 1 equivalent of spermine, of the random coil form (at 20°) and the fully helical form (at 2°)

32%. This is to be compared with the hyperchromicity of 15% in the presence of 0.01 M-Mg^{2+} (Fig. 4), which indicates that in the presence of the divalent cation the complete profile for poly-EtU must have a T_m at about -2.0°, and that the fully ordered structure under

these conditions should form at about -10° to -12°. The T_m values for all three polymers under various conditions are listed in Table 1.

From Table 1 (and from Fig. 5) it will be observed that, whereas spermine provokes a somewhat larger positive shift in the profiles of poly-U and poly-rT than spermidine, this effect is reversed in the case of poly-EtU. The differences, while not excessively large, are nonetheless real and point to the existence of some differences in the topography of the helical form of poly-EtU as compared to poly-U and poly-rT.

The helical structure of poly-U and poly-rT are believed to be twin-stranded with the U (or T) residues of the twin strands base--paired *via* the $N_{(3)}$ hydrogen and O^4 carbonyls (Davies, 1967). This is the type of base-pairing actually found between uracil (or thymine) in crystals of 1-methyluracil (or 1-methylthymine) (Green, Mathews & Rich, 1962).

There is little reason to doubt that the helical form of poly-EtU is also twin-stranded. If, however, the type of base-pairing between 5-ethyluracil residues is similar to that in helical poly-U and poly-rT, it is by no means obvious as why the T_m of poly-EtU should be so much lower than for poly-rT, and even below that for poly-U (Table 1). It is most unlikely that the 5-ethyl substituent offers any steric hindrance to helix formation. Actual trials with molecular models have shown that 5-ethyluracil residues readily replace 5-methyluracil (thymine) in either the A or B forms of DNA without the introduction of distortion in the helix.

That the base-pairing in poly-EtU *could* be the same as in poly-U (or poly-rT) is supported by the fact that replacement of thymine by 5-ethyluracil in bacteriophage DNA (where the base-pairing to adenine must be identical in both instances) leads to an appreciable decrease in T_m of the DNA (Pietrzykowska & Shugar, 1967).

If, in fact, it turns out that the base-pairing of ethyluracil residues in poly-EtU is similar to that in poly-U and poly-rT, then the source

Table 1

T_m *values for helix-coil transitions of poly-U, poly-rT and poly-EtU at neutral* pH *in the presence of either 0.01* M-Mg^{2+} *or 1 equivalent of spermine or spermidine*

All values are taken as the mid-points of the profiles shown in Figs. 4 and 5.

	T_m in presence of		
	Mg^{2+}	Spermine	Spermidine
Poly-U	6.0° *	28° **	24° **
Poly-rT	33.5°	51° **	49° **
Poly-EtU	—2.0°	9.2°	11.5°

* From Lipsett (1960).
** From Szer (1966).

of the differences in stabilities of the helical forms of these three polymers are probably linked to differences in degree of stacking. As previously pointed out, it is most unlikely that the higher stability of poly-rT, as compared to poly-U, is due to more effective hydrogen bonding of the thymine, as compared to uracil, base pairs (Szer *et al.*, 1963). The more likely involvement of enhanced base stacking is supported by the observations of Ts'o and coworkers (see Ts'o, 1968) and Helmkamp & Kondo (1968) on the enhancement of association of purine and pyrimidine residues resulting from alkylation of the heterocyclic rings.

The foregoing considerations suggest a number of additional investigations which would undoubtedly contribute to a clarification of these and related problems. To begin with, it would be useful to examine by X-ray diffraction the structure of crystals of 1-methyl-5-ethyluracil, as compared to 1-methyluracil and 1-methylthymine (Davies, 1967). It would also be advantageous to compare the association, in aqueous medium, of 5-ethyluracil (or its glycosides) as compared to uracil and thymine (or their glycosides); the finding that thymidine in aqueous medium exhibits enhanced stacking as compared to deoxyuridine (Ts'o, 1968) is at least in qualitative agreement with the enhanced stability of poly-rT as compared to poly-U. It would, furthermore, also be of value to measure the association constant for self-association of 5-ethyluracil residues in non-polar media. Attempts in these directions are now under way.

Finally, attention should be drawn to the fact that poly-EtU forms a helical complex with poly-dA, most likely a triple-stranded helix (Barszcz & Shugar, 1968). The results of a more detailed study of the nature of the helical complexes of poly-EtU with poly-rA will be described in a separate paper (Świerkowski & Shugar, 1969a).

EXPERIMENTAL

General. The hydrochloride salts of the polyamines, spermine and spermidine, were products of Sigma Chemical Co. (St. Louis, Mo., U.S.A.). The phenol employed for deproteinization of polymer preparations was freshly distilled prior to use and saturated with the required buffer to neutrality.

All spectrophotometer measurements were carried out with a Unicam SP 500 instrument. Temperature profiles were run with the aid of a specially constructed thermostated cuvette carriage through which circulated a water-glycol mixture from a Hoeppler ultrathermostat. Temperature measurements were carried out with a thermistor inserted into a dummy cuvette. For temperatures below 10°, dry air was circulated through the cuvette compartment to prevent condensation on the cuvette surfaces.

Table 2

Paper chromatography of relevant compounds

Ascending technique on Whatman paper no. 1, with the following solvent systems: *A*, water-saturated butanol; *B*, ethanol - ammonium acetate, 5:2 (v/v); *C*, isopropanol - conc. NH_4OH - H_2O, 7:1:2 (by vol.); *D*, butanol saturated with sat. H_3BO_3; *E*, *n*-propanol - conc. NH_4OH - H_2O, 6:3:1 (by vol.).

Compound	R_F value in solvent				
	A	*B*	*C*	*D*	*E*
5-Ethyluracil	0.67	0.88	0.89	—	0.87
1-(2′,3′,5′-Tri-*O*-benzoyl-β-D-ribofuranosyl)-5-ethyluracil (III)	1	1	1	—	1
5-Ethyluridine (IV)	0.46	0.82	0.73	0.54	0.78
2′,3′-*O*-Isopropylidene-5-ethyluridine (V)	0.83	0.96	0.95	0.94	0.95
5-Ethyluridine-5′-phosphate (VI)	0.02	0.34	0.16	—	0.34
5-Ethyluridine-5′-phosphoromorpholidate (VII)	0.01	0.16	0.04	—	0.19
5-Ethyluridine-5′-pyrophosphate (VIII)	0.0	0.18	0.08	—	—

For the spectral titrations to determine the p*K* values of the pyrimidines and nucleosides, pH measurements were made with a Radiometer PHM22 instrument fitted with a glass microelectrode. Glass-distilled water was employed throughout.

Ultracentrifugations were performed with the aid of a Beckman Model E instrument fitted with ultraviolet optics.

Melting points (uncorrected) were carried out on a Boëtius microscope hot stage.

Details regarding paper chromatography are given in Table 2, a dark ultraviolet lamp being used to locate the spots on the paper.

Di(5-ethyluracil)mercury salt (I): This was prepared as described by Fox *et al.* (1956) for thymine. To 16 m-moles (2.24 g.) of 5-ethyluracil, prepared according to the method of Burckhalter & Scarborough (1955) and dissolved in 64 ml. hot water containing 640 mg. sodium hydroxide, was added dropwise 8 m-moles (2.16 g.) of mercuric chloride in ethanol. The mixture was heated for several minutes with constant stirring, and then cooled to room temperature. The resulting precipitate was washed with water until the washings were free of chloride ion, and then washed with absolute ethanol and ether and dried under vacuum over P_2O_5 to give 3.72 g. (theoret. 3.84 g., yield 97%) of product with m.p. 295 - 297°.

1-(2′,3′,5′-Tri-O-benzoyl-β-D-ribofuranosyl)-5-ethyluracil (III): 2 m-moles (1.01 g.) of 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose was converted to 2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl chloride (II) according to standard procedures (Recondo & Reyderknecht, 1959), except that special attention was devoted to the removal of HCl; in addition to a threefold evaporation of product from dry benzene, it was kept overnight under vacuum in

the presence of solid NaOH, and finally dissolved in 3 ml. anhydrous benzene. Then 1.5 m-moles (720 mg.) of dried I was suspended in 18 ml. anhydrous xylene, and about one-fourth of the solvent removed under vacuum, following which the 3 ml. benzene solution of II was added and the mixture heated under reflux for 2 hr. The molar ratio of II to I of 4:3 was analogous to that employed by Griffin *et al.* (1958) for ribosylthymine. Following cooling of the reaction mixture to room temperature, the precipitate was filtered off and washed with xylene. The latter was dissolved in chloroform and the solution filtered to about 15 ml. and petroleum ether added to give a white precipitate. The latter was dissolved in chloroform and the solution filtered to remove traces of insoluble mercuric chloride, then shaken with a 30% aqueous KI solution and finally washed with water and dried over anhydrous sodium sulphate. Following removal of the sodium sulphate, the filtrate was brought to dryness, the residue dissolved in ethyl acetate, petroleum ether added dropwise to slight turbidity, and the mixture then kept in the cold room for crystallization. The crystals were collected by filtration and washed with ether and dried to give 646 mg. of III (53% yield with respect to 5-ethyluracil), m.p. 149.5 - 151.5°.

5-Ethyluridine (IV): To 0.88 m-mole (516 mg.) of III suspended in 15 ml. anhydrous methanol was added 3 ml. of a methanolic solution of 65 mg. potassium. All the material dissolved within 1 hour, with continual shaking. Paper chromatography at this point showed that debenzoylation had proceeded quantitatively. The mixture was brought to dryness, the residue dissolved in a few milliliters of water, and the resulting solution extracted several times with ether. To the aqueous, strongly alkaline, phase was added 60% $HClO_4$, dropwise, to bring the pH to about 3.5 - 4.0. The resulting precipitate of $KClO_4$ was centrifuged off and washed with ethanol. The aqueous solution was brought to dryness and the residue dissolved in ethanol, which was filtered to remove traces of $KClO_4$, and then brought to small volume to induce crystallization, giving 210 mg. of IV (90% yield), m.p. 160.5 - 162°. Recrystallization brought this value up to 185° - 187°; Shapira (1962) reports 184° - 186°.

5-Hexyluridine: This nucleoside was obtained as described in the previous paragraph for 5-ethyluridine. From 0.45 m-mole (265 mg.) of di(5-hexyluracil)mercury salt and 0.62 m-mole of 2,3,5-tri-*O*-benzyol-*β*-D-ribofuranosyl chloride, the yield of 1-(2′,3′,5′-tri-*O*-benzoyl-*β*-D-ribofuranosyl)-5-hexyluracil was 245 mg. (43% with respect to 5-hexyluracil) with m.p. 144 - 146°. Debenzoylation gave 5-hexyluridine in 90% yield with m.p. 134 - 135°.

2′,3′-O-*Isopropylidene-5-ethyluridine (V):* Dried, finely powdered, 5-ethyluridine 1.8 m-moles (0.5 g.) and anhydrous $CuSO_4$ (1 g.) were suspended in 12.5 ml. anhydrous acetone, to which was added 12.5 µl. of

conc. H_2SO_4. The entire mixture, in a sealed ampoule, was shaken for 48 hr. at 37°, following which the ampoule was opened and paper chromatography employed to verify that the reaction had proceeded quantitatively. The copper sulphate was filtered off and washed with acetone. The washings and filtrate were combined and 0.5 g. anhydrous calcium hydroxide added. The resulting suspension was shaken for 1 hr., filtered, and the filtrate brought to dryness. The residue was crystallized from acetone to give 220 mg., m.p. 146 - 148°. No further crystals could be obtained from the mother liquor; it was therefore brought to a known volume and the remaining amount of IV estimated spectrophotometrically as 276 mg. Total yield 496 mg. (88%). The entire product was used directly for phosphorylation.

5-Ethyluridine-5′-phosphate (VI): 1.57 m-moles (495 mg.) of V and 3.14 m-moles (470 mg.) of β-cyanoethylphosphate in 15 ml. pyridine was concentrated under vacuum at 30° to an oil, to which was added 30 ml. anhydrous pyridine and the resulting solution evaporated to dryness. This latter operation was repeated once more, and the residue following evaporation taken up in 15 ml. anhydrous pyridine, to which was added 4.7 m-moles (970 mg.) DCC. The reaction mixture was kept under anhydrous conditions for 2 days at room temperature, although paper chromatography showed the complete disappearance of V after 36 hr. To the reaction mixture was added 4.5 ml. water, and the whole then set aside for 1/2 hr. Pyridine was removed by bringing the solution to dryness under vacuum, and the residue was then dissolved in 60 ml. of 10% acetic acid and heated for 90 min. at 100°. Acetic acid was removed by distillation and the residue taken up in 60 ml. of 9 N-ammonium hydroxide and heated at 60° for 90 min.

The reaction mixture was filtered to remove a small precipitate of cyclohexylurea and then concentrated to about 10 ml. (pH 6.9) and 1 M-barium acetate added dropwise to precipitate quantitatively barium phosphate. The latter was removed by centrifugation and washed with 2 - 3 ml. water. The filtrate and washings were combined and the amount of nucleotide estimated spectrally (490 mg., yield 88%). The solution of nucleotide, 14 ml., was transferred to a centrifuge tube to which was added 1.5 m-moles barium acetate in 1.5 ml. water. The barium salt of the nucleotide was then precipitated with 2 volumes ethanol. The product was washed with ethanol, acetone and ether. However, notwithstanding a number of trials, it did not prove possible to isolate the barium salt free of barium phosphate in this way without excessive loss of product. The nucleotide was therefore dissolved in water, brought to pH 8, and passed through a column of Dowex 50 (H^+), to give 495 mg. of free 5-ethyluridine-5′-phosphate.

5-Ethyluridine-5′-phosphoromorpholidate (VII) (salt of 4-morpholine *N,N′*-dicyclohexylcarboxamidine): A solution of DCC (0.6 m-mole,

123.6 mg.) in 2.2 ml. *tert.*-butanol was added dropwise to a solution of 5-ethyluridine-5′-phosphate (0.14 m-mole, 50 mg.) in 1.4 ml. water, 1.4 ml. *tert.*-butanol and 50 µl. morpholine, and heated under reflux. Following addition of the DCC solution (3 hr.), heating was continued for an additional 3 hr. Chromatography at this point showed 60% transformation to the morpholidate. An additional 25 µl. morpholine was added, following which 1.1 ml. of DCC solution was added dropwise and the entire mixture heated for a further 4 hr. At this point paper chromatography demonstrated complete conversion to the morpholidate.

The foregoing procedure was repeated on a somewhat larger scale (using 400 mg., 1.12 m-moles, of 5-ethyluridine-5′-phosphate), and the two reaction mixtures then combined. The crystalline precipitate was filtered off and washed with *tert.*-butanol. The filtrate and washings were combined, brought to dryness, the residue dissolved in water, and the resulting solution extracted with ether three times. The aqueous phase was brought to dryness, the residue taken up in methanol and transferred to a centrifuge tube, and carefully evaporated to a volume of about 0.5 ml. Ether was then added to give a gummy precipitate, which was centrifuged down and triturated with ether until it was transformed to a white powder (900 mg., yield 92%).

5-Ethyluridine-5′-pyrophosphate (VIII): 0.28 m-mole (100 mg.) of VII was dried by two evaporations from 5 ml. pyridine. At the same time 1.27 µmoles (87 µl.) of 85% phosphoric acid was dissolved in 5 ml. pyridine containing 1.27 m-moles of distilled tri-*n*-butylamine, and dried by three evaporations from 5 ml. pyridine. Each residue was taken up in 5 ml. pyridine, the two mixed, and the mixture again subjected to drying by evaporation twice with anhydrous pyridine. The final residue was taken up in anhydrous pyridine and shaken for 1 hr. to facilitate solution. The final clear solution was left for four days at room temperature, following which there were only traces of VII and VI, as revealed by paper chromatography. The solution was taken to dryness and traces of pyridine removed by evaporation from 5 ml. water. The residue was then dissolved in 4.5 ml. water containing 154 mg. lithium acetate, and the tri-*n*-butylamine extracted with ether. The pH of the aqueous phase was brought to 12 with lithium hydroxide and the mixture left at 0° for 30 min. The resulting precipitate of trilithium phosphate was centrifuged down and washed with 0.01 N-lithium hydroxide until the washings showed no UV absorption.

The supernatant and washings were combined and brought to pH 8 by addition of Dowex 50 (H^+). The resin was filtered off and washed with water. The filtrate and washings were applied to a 1×10 cm. column of Dowex 2 X 8 (Cl^-), through which water was passed for washing. Traces of 5-ethyluridine-5′-phosphate were then eluted with 0.04 N-LiCl in 0.003 N-HCl. Then 0.1 N-LiCl in 0.003 N-HCl was employed

to elute the pyrophosphate (70 mg, 56% yield), the eluate was brought to pH 4.5 with lithium hydroxide and brought to dryness under reduced pressure. The pyrophosphate was then isolated as the pure lithium salt in 40% yield.

Polymerization of EtUDP: The conditions used with *E. coli* polynucleotide phosphorylase were as suggested by Dr. U. Z. Littauer (Kimhi & Littauer, 1968). The stock enzyme preparation had an activity of 340 units/ml. (1 unit of phosphorolysis is the number of micromoles of ADP released from poly-A in 15 min. at 37°). The reaction mixture consisted of 0.10 ml. of 1 M-tris buffer, pH 8; 0.05 ml. of 0.1 M-$MgCl_2$; 0.05 ml. of 0.01 M-EDTA; 0.06 ml. of 0.175 M-substrate; 0.03 ml. of the stock enzyme solution; 0.71 ml. water to give a final volume of 1 ml. Incubation was at 37° and the progress of polymerization was followed by paper or thin-layer chromatography.

Under the above conditions polymerization of ribosylthymine-5′-pyrophosphate proceeded in good yield within 20 - 30 min. However, with EtUDP, no sings of polymerization were observed until after about 2 hr. incubation, following which the reaction proceeded for up to 18 hr. Further incubation led to a decrease in yield of polymer. Hence, after about 16 hr., 40 μg. pronase was added to the incubation mixture, and incubation continued for 1 hr. The incubation mixture was then deproteinized with freshly distilled phenol buffered at neutral pH, and then dialysed in the cold room against 0.05 M-NaCl and 0.005 M-EDTA, followed by 0.01 M-NaCl and 0.001 M-EDTA, and finally against water. The polymer samples were then freeze-dried.

In some instances incubation was terminated by heating for 1 min. at 100°, followed by deproteinization, dialysis and freezing-drying.

We are indebted to Dr. S. Ochoa, Dr. H. Matthaei, and Dr. U. Z. Littauer for samples of polynucleotide phosphorylase from *Azotobacter vinelandii, Micrococcus lysodeikticus* and *Escherichia coli,* respectively; and to Dr. W. Szer, who collaborated in the initial attempts at polymerization. This investigation profited from the support of the Wellcome Trust, the International Atomic Energy Agency, the World Health Organization, and the Agricultural Research Service, U.S. Dept. of Agriculture (UR-E21-(32)-30).

REFERENCES

Barszcz D. & Shugar D. (1968). *European J. Biochem.* **5**, 91.
Burckhalter J. H. & Scarborough H. C. (1955). *J. Amer. Pharm. Assoc.* **44**, 545.
Davies D. R. (1967). *Ann. Rev. Biochem.* **36**, 321.
Fikus M. & Shugar D. (1969). *Acta Biochim. Polon.* **16**, 55.
Fox J. J. & Shugar D. (1952). *Biochim. Biophys. Acta* **9**, 369.
Fox J. J., Yung N., Davoll J. & Brown G. B. (1956). *J. Amer. Chem. Soc.* **78**, 2117.

Green D. N., Mathews F. S. & Rich A. (1962). *J. Biol. Chem.* **237**, 3573.
Griffin B. E., Todd A. R. & Rich A. (1958). *Proc. Natl. Acad. Sci. (U.S.)* **44**, 1123.
Helmkamp G. K. & Kondo N. S. (1968). *Biochim. Biophys. Acta* **157**, 242.
Hilbert G. E. & Johnson T. B. (1930). *J. Amer. Chem. Soc.* **52**, 2001.
Kimhi Y. & Littauer U. Z. (1968). *J. Biol. Chem.* **243**, 231.
Levene A. & Tipson R. S. (1934). *J. Biol. Chem.* **106**, 113.
Lipsett M. N. (1960). *Proc. Natl. Acad. Sci. U.S.* **46**, 445.
Moffatt J. G. & Khorana H. G. (1961). *J. Amer. Chem. Soc.* **83**, 649.
Ochoa S. & Heppel L. A. (1957). In *The Chemical Basis of Heredity* (W. O. McElroy & B. Glass, eds.) p. 615. Johns Hopkins Press, Baltimore, Maryland.
Pietrzykowska I. & Shugar D. (1967). *Acta Biochim. Polon.* **14**, 169.
Pietrzykowska I. & Shugar D. (1968). *Science* **161**, 1248.
Recondo E. F. & Reyderknecht H. (1959). *Helv. Chim. Acta* **42**, 1171.
Shapira J. (1962). *J. Org. Chem.* **27**, 1918.
Shugar D. & Fox J. J. (1952). *Biochim. Biophys. Acta* **9**, 199.
Shugar D. & Szer W. (1962). *J. Mol. Biol.* **5**, 580.
Shugar D., Świerkowski M., Fikus M. & Barszcz D. (1967). *7th International Congress of Biochemistry, Tokyo*, Vol. I, Symp. 1, pp. 59 - 60.
Szer W. (1966). *Acta Biochim. Polon.* **13**, 251.
Szer W. & Shugar D. (1961). *Acta Biochim. Polon.* **8**, 235.
Szer W. & Shugar D. (1966). *J. Mol. Biol.* **17**, 174.
Szer W., Świerkowski M. & Shugar D. (1963). *Acta Biochim. Polon.* **10**, 87.
Świerkowski M. & Shugar D. (1969a). *J. Mol. Biol.*, in press.
Świerkowski M. & Shugar D. (1969b). *J. Med. Chem.* **12**, 533.
Świerkowski M., Szer W. & Shugar D. (1965). *Biochem. Z.* **342**, 429.
Tener G. M. (1961). *J. Amer. Chem. Soc.* **83**, 159.
Tramer Z., Wierzchowski K. L. & Shugar D. (1969). *Acta Biochim. Polon.* **16**, 83.
Ts'o P.O.P. (1968). In *Molecular Associations in Biology*, p. 39. Academic Press, N.Y.
Wierzchowski K.L., Litońska E. & Shugar D. (1965). *J. Amer. Chem. Soc.* **87**, 4621.

NOWY ANALOG TYMINY, 5-ETYLOURACYL; 5-ETYLOURYDYNO-5'-PIROFOSFORAN ORAZ KWAS POLI-5-ETYLOURYDYLOWY

Streszczenie

1. Przeprowadzono syntezę 5-etylourydyny, 5-etylourydyno-5'-pirofosforanu oraz szeregu związków pośrednich. Opisano również syntezę 5-hexylourydyny. Przedstawiono widma w nadfiolecie i stałe dysocjacji dla 5-etylouracylu, 5-etylourydyny i 5-hexylourydyny.

2. 5-Etylourydyno-5'-pirofosforan jest substratem dla fosforylazy polinukleotydowej z *Escherichia coli*, wykazuje bardzo słabe powinowactwo do enzymu z *Azotobacter vinelandi*, natomiast jest całkowicie odporny na działanie fosforylazy polinukleotydowej z *Micrococcus lysodeikticus*.

3. Opisano enzymatyczną syntezę oraz pewne właściwości kwasu poli-5-etylourydylowego; kwas ten z łatwością ulega hydrolizie pod wpływem rybonukleazy trzustkowej. W temperaturze pokojowej polimer ten istnieje w formie nieuporządkowanego kłębka; poniżej 10° przy wysokich stężeniach NaCl lub w 0,01 M-$MgCl_2$ tworzy strukturę spiralną o T_m około —2°. Porównano właściwości kwasu poli-5-etylourydylowego z właściwościami kwasu poliurydylowego i poli-5-metylourydylowego oraz omówiono struktury tych polimerów.

Received 18 February, 1969.

ACTA BIOCHIMICA POLONICA
Vol. 16 1969 No. 3

INA BUCHOWICZ, HALINA GOCH and K. ZAKRZEWSKI

EQUINE IMMUNOGLOBULINS: A COMPARISON OF MOLECULAR PROPERTIES

Serum and Vaccine Research Laboratory, ul. Chełmska 30/34, Warszawa 36

1. Immunoglobulin fractions, IgG and IgG(T), were isolated from horse hyperimmune anti-diphtheria serum by salt fractionation and chromatography on DEAE-Sephadex. 2. Properties of IgG and IgG(T) isolated from serum of the same horse were compared. Antibody activity was found only in IgG(T) fraction. 3. IgG and IgG(T) were found to differ in their carbohydrate content and in the susceptibility to urea-induced unfolding. 4. IgG and IgG(T) were found to be nearly identical in their antigenic properties, amino acid composition, molecular weight, tyrosyl ionization, optical rotatory parameters, and the accessibility of tyrosyl and tryptophyl residues to solvents.

Repeated immunization of horses brings about an increase in the amount of serum protein fraction the electrophoretic mobility of which is between γ-globulins and β-globulins. This fraction has been designated T-component by Van der Scheer & Wycoff (1940), and β_2-globulin by Kekwick & Record (1941). During the immunization, antibodies appear first in the γ_1-globulin fraction, but at the later stages almost exclusively in the T-component, although exceptions have been noted (Raynaud & Relyveld, 1959).

The antibody-rich fraction from horse hyperimmune sera, T-component, was isolated by a variety of methods. The classical salt fractionation, although still succesfully used alone (Allen, Sirisinha & Vaughan, 1965) or supplemented with acridine dye fractionation (Hořejši & Smetana, 1956; Schultze, Haupt, Heide, Heimburger & Schwick, 1965) has been largely superseded by ion-exchange chromatography (Raynaud, Iscaki & Mangalo, 1965; Klinman, Rockey & Karush, 1965; Klinman, Rockey, Fraunberger & Karush, 1966; Weir & Porter, 1966) or by ion--exchange chromatography combined with salt fractionation (Stefani & Kulberg, 1964; Stefani, Kulberg & Schachanina, 1965).

The ionic exchanger commonly used is diethylaminoethylated cellulose or Sephadex. The fraction of immunoglobulins which is not retained

on DEAE resin at about neutral pH and in low ionic strength buffers exhibits the electrophoretic mobility of γ_1-globulins, and is usually devoid of antibodies. According to the current nomenclature (World Health Organization, 1964) this fraction is classified as IgG. The other immunoglobulin fraction which is eluted from DEAE resin at an elevated ionic strength, exhibits the electrophoretic mobility of β_2-globulins; it is usually an antibody-rich fraction, and it contains essentially all antibodies in the case of anti-diphtheria serum.

The properties of the two fractions of immunoglobulins derived from horse hyperimmune anti-diphtheria serum were compared by Weir & Porter (1966) who showed the differences in their electrophoretic mobility, carbohydrate content, and antibody activity, but found a close similarity in their amino acid composition and molecular weight. Since the antigenic properties of the two fractions of immunoglobulins appeared very similar but not identical, Weir, Porter & Givol (1966) suggested that the antibody-rich fraction from horse hyperimmune sera was a subclass of IgG, and should therefore be designated as IgG(T) in preference to the previously used IgA (Raynaud *et al.*, 1965) or IgA(T) (Weir & Porter, 1966). The term IgG(T) will be used throughout this paper.

A very close similarity between the molecular properties of IgG and IgG(T) sharply contrasts with the difference in their antibody activity. Consequently, it appeared that the isolation of both immunoglobulins from the same immune animal could provide an opportunity to study the features which distinguish immunologically active from non-active proteins. This paper describes the simultaneous isolation of both subclasses of immunoglobulins, and presents a comparative study of some of their molecular parameters.

MATERIALS AND METHODS

Immune sera. Horse hyperimmune sera against diphtheria toxin were purchased from Biomed, Serum & Vaccine Manufacturers, Lublin, Poland. Sera were supplied under sterile conditions with no preservative added, at 4°, and were processed within a week of the date of the bleeding. Rabbit antiserum against horse serum proteins was purchased from Biomed, Serum & Vaccine Manufacturers, Kraków, Poland.

Rabbit antiserum against equine IgG(T) was obtained by immunization of rabbits with chromatographically purified IgG(T). A total of 20 mg. of the antigen was injected intramuscularly together with incomplete Freund's adjuvant at weekly intervals over a period of four weeks. IgG proved to be a much weaker antigen. In order to obtain a good precipitating serum, rabbits had to be injected with 40 mg. of purified IgG also with the incomplete Freund's adjuvant, followed by

15 mg. intravenously; the injections were done at weekly intervals; each dose contained 5 mg. of the antigen.

Enriched immunoglobulin preparation. Horse serum was fractionated with sodium sulphate, essentially as described by Kekwick & Record (1941). The final precipitate, at 12% (w/v) of sodium sulphate, was dissolved in a minimum quantity of 0.07 M-phosphate buffer, pH 8.0, and dialysed against 0.15 M-sodium chloride, pH 7.0, until the sulphate ions disappeared. The solution was then freed of insoluble matter by centrifugation, and freeze-dried. The enriched immunoglobulin preparation was used as starting material for chromatographic isolation of IgG and IgG(T) in most of the experiments. As it will be described in the text, immunoglobulins obtained from the enriched preparation were more homogeneous with respect to the antibody content than, and otherwise identical with, the immunoglobulins isolated by chromatography of the whole serum.

Chromatography. Glass columns (3 × 60 cm.) were filled with Sephadex A-50 (purchased from Pharmacia, Uppsala, Sweden) equilibrated with 17.5 mM-sodium phosphate buffer, pH 6.3, or 15 mM-potassium phosphate, pH 8.4, as indicated. Samples containing up to 6 g. protein, in the case of whole serum, or no less than 0.3 g., in the case of the enriched immunoglobulin preparation, were eluted with the same buffer but in which the content of sodium chloride was increased either linearly or stepwise. Fractions of 5 ml. were collected at a rate of about 50 ml./hr. The elution was monitored at about 260 mμ using the LKB Uvicord (Stockholm, Sweden) instrument, but the concentration of protein in each fraction was determined from the extinction at 280 mμ.

Protein concentration. The following factors were used to convert extinctions into protein concentration: $E^{1\%}_{280\,m\mu} = 13.0$ for IgG, and $E^{1\%}_{280\,m\mu} = 12.8$ for IgG(T). The conversion factors have been determined from the measurement of the extinction of isolated immunoglobulin fractions and of human pooled immunoglobulins for which $E^{1\%}_{280\,m\mu} = 13.6$ was assumed (Crumpton & Wilkinson, 1963). The concentration of protein in equine immunoglobulins and in human immunoglobulins, used as the reference preparation, was determined using the biuret methods (Hinsberg & Lang, 1957). The conversion factors employed in this paper are lower by about 10% than those used by other authors in their studies on equine immunoglobulins (Allen *et al.*, 1965; Klinman *et al.*, 1966; Genco, Karush & Tenenhouse, 1968).

pH *measurement.* Radiometer TTT-1 Titrator (Copenhagen, Denmark) with a glass electrode was used for pH measurements; sodium ion correction was introduced whenever required using the manufacturer's nomogram.

Electrophoretic methods. Paper electrophoresis was performed on Whatman no. 1 paper strips in veronal buffer, pH 8.6, using an apparatus

similar to that described by Durrum (1950). Starch gel electrophoresis was performed in borate buffer, pH 8.6, as described by Smithies (1959). Immunoelectrophoresis was performed according to Scheidegger (1955) in veronal buffer, pH 8.6.

Amino acid analysis. Proteins were hydrolysed with 6 N-hydrochloric acid in sealed ampoules placed in the boiling toluene bath (b.p. 110°) for 48 hr. The analysis was performed in the Beckmann-Spinco Automatic Amino Acid Analyser.

Carbohydrate determination. The content of carbohydrates was estimated by orcinol method (Winzler, 1955) using mannose as a standard.

Ultracentrifugation. Sedimentation coefficients were determined in Beckmann-Spinco Model E ultracentrifuge employing two standard 12 mm. cells in one rotor. One of the cells was equipped with 1° wedge window thus permitting simultaneous observation of both cells. In order to increase the precision of the comparison of molecular weights, one of the cells was filled with IgG and the other with IgG(T), both solutions being at equal concentrations. The temperature of the rotor was maintained at $20 \pm 0.5°$. Sedimentation coefficients were calculated after extrapolation to zero concentration.

Immunodiffusion. Glass slides (5×13 cm.) were covered with 1.5% (w/v) agar in veronal buffer, pH 8.6. The central trough (6.0×11 cm.) was filled with the immune reactant (antiserum or toxin, as indicated in the text), and the wells, 0.5 cm. diameter, contained samples of the investigated immunoglobulins. The slides were incubated in a wet chamber at room temperature for at least 24 hr. After the precipitation lines have clearly formed, the agar layer was washed in 0.15 M-sodium chloride, stained with Amido Black, and dried in air current.

Spectroscopy. Extinctions were measured on Unicam SP-500 spectrophotometer. Absorption spectra were registered on Unicam SP-700 instrument at ambient temperature. For the difference spectra, two pairs of matched 1 cm. cells were used. The pair in the sample beam of the spectrophotometer contained protein dissolved in an appropriate solvent, and a blank. The pair in the reference beam contained protein in 0.15 M-sodium chloride, pH 7.0, and the solvent. All concentrations were adjusted so that the contributions to the difference extinction from solutes and solvents were cancelled. The concentration of the protein was kept so low that the slit width in the 280 mμ range was below 0.8 mm. Spectrophotometric titration of tyrosyl residues was carried out in 0.1 M-glycine - NaOH buffers.

Polarimetry. Optical rotatory dispersion parameters (ORD) were measured on Rudolph Model 80 spectropolarimeter equipped with oscillating polarizer. The symmetrical angle was set at 1.5°. The measurements were taken at ambient temperature at 365, 405, 435, 546 and 578 mμ bands of the mercury-arc AH-4 lamp using the filters

supplied by manufacturers. ORD parameters a_0 and b_0 were computed using the least square method from the equation elaborated by Moffitt & Yang (1956). $\lambda_0 = 212°$ was assumed for computations.

Antibody assay. Both flocculating and protective antibodies were assayed. The flocculation test was carried out in water bath set at 42° in 10×80 mm. tubes observed with a magnifying glass. The tubes contained 20, 35 or 50 Lf units of diphtheria toxin, as required, in 1 ml. of 0.15 M-sodium chloride, pH 7.0. Properly diluted immunoglobulin was added to each tube, its volume increasing from 0.1 to 1.0 ml. in 0.1 ml. increments; in the vicinity of the optimum flocculation ratio the increments were 0.05 ml. The titre of the sample was defined as that amount of immunoglobulin which was the first to flocculate under the conditions of the assay, and expressed as limit of flocculation (Lf) units.

Protective antibodies were assayed by skin erythema protection test. Female albino rabbits, 3 to 4 kg. body weight, were depilated and injected in the dorsal area with 0.1 ml. of a mixture composed of 1 test-dose of diphtheria toxin and 0.05 ml. of immunoglobulin sample. One test-dose was defined as the smallest amount of toxin which caused skin erythema when mixed with 1/500 of one international unit of diphtheria antitoxin. The reagents used in the flocculation and in the skin erythema assays were standardized against the International Reference Preparation of Diphtheria Antitoxin kindly supplied by WHO International Laboratory for Biological Standards, Statens Serum Institute, Copenhagen, Denmark.

RESULTS AND DISCUSSION

Isolation of immunoglobulins

A typical chromatographic pattern of the whole anti-diphtheria serum is shown in Fig. 1. Three prominent peaks are apparent on the elution profile, but for the determination of electrophoretic mobilities, antibody activity, and of antigenic composition, the fractions were pooled in eight groups, as indicated in Fig. 1. Fraction I (marked IgG in Fig. 1 in anticipation of further discussion) was not retained on Sephadex A-50. On paper electrophoresis in veronal buffer, pH 8.6, peak I was found to consist only of proteins exhibiting a slight cathodic mobility with no apparent contaminations. The second major peak, II, marked IgG(T) consisted of proteins moving slightly towards anode on paper electrophoresis at pH 8.6. Other fractions showed under the same conditions variable amounts of α- and β-globulins, except for fraction VII which consisted almost entirely of proteins with the electrophoretic mobility of serum albumin. Immunodiffusion patterns (Fig. 5) indicated

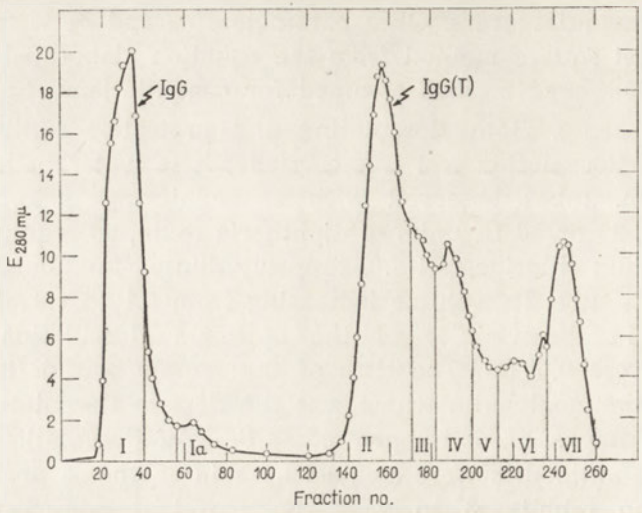

Fig. 1. Chromatography of whole equine anti-diphtheria serum on Sephadex A-50 column. Sephadex A-50 equilibrated with 17.5 mM-phosphate buffer, pH 6.3. Column charged with 68 ml. serum containing 500 Lf/ml. Fractions, 4.5 ml., collected at a rate of 35 to 45 ml./hr. Elution with 17.5 mM-phosphate buffer, pH 6.3; sodium chloride gradient linearly rising from fraction no. 54

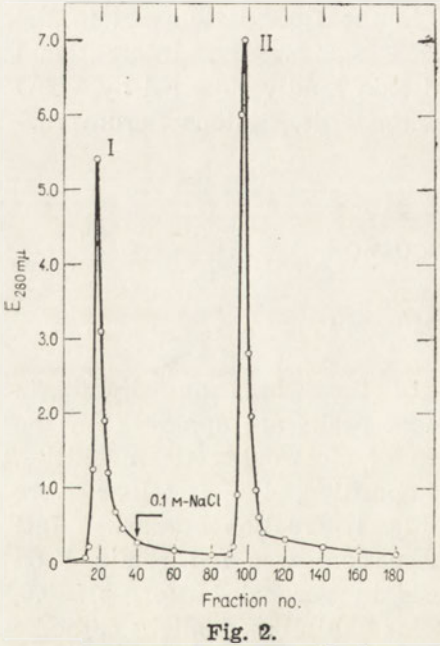

Fig. 2.

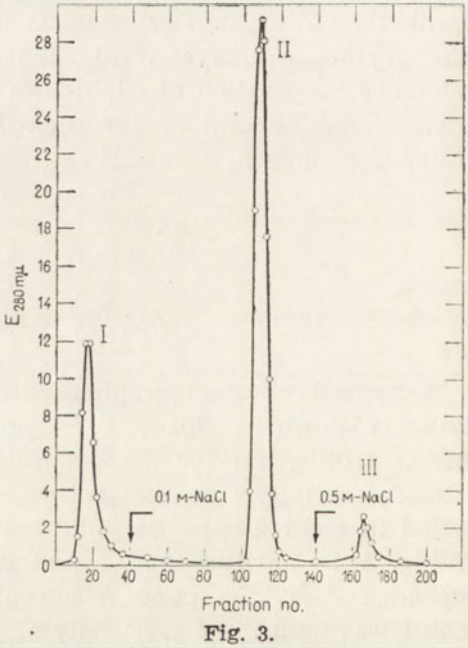

Fig. 3.

Fig. 2. Chromatography of the enriched immunoglobulin preparation obtained from non-immune horse serum. Conditions as in Fig. 1, but a stepwise elution used instead of the gradient elution. Column charged with 0.318 g. protein

Fig. 3. Chromatography of the enriched immunoglobulin preparation obtained from hyperimmune anti-diphtheria serum. Conditions as in Fig. 2. Column charged with 1.54 g. protein containing 6700 Lf/g., i.e. a total of 10 318 Lf units of diphtheria antibodies

Table 1

Distribution of anti-diphtheria antibodies among the fractions obtained by chromatography on Sephadex A-50, pH 6.3, of the whole hyperimmune serum

For the designation of fractions see Fig. 1.

Preparation	Recovery (%)		Lf units/g. protein
	protein	Lf units	
Whole serum	100	100	4 950
Fraction I	17.1	0	0
Fraction II	22.8	71	15 000
Fraction III	5.2	13.5	12 300
Fraction IV	6.4	8.4	5 950
Fractions V, VI and VII	43.0	0	0

that fraction III, and possibly also fraction IV, contained a protein component which was identical with one of the proteins present in fraction II.

Most of the diphtheria antibodies were found in fraction II (see Table 1), i.e. in IgG(T), but about 20% of the antibody activity was spread towards fractions emerging from the column in the buffer of higher ionic strength; no antibody activity was found in fraction I.

Chromatographic partitioning of the enriched immunoglobulin preparation, whether obtained from a non-immune horse (Fig. 2) or from a hyperimmunized animal (Fig. 3) yielded two major peaks, cleanly separated; the separation was much improved when stepwise elution was used instead of gradient elution. Only one additional protein peak emerged from the column at about 0.5 M-sodium chloride: it was a blue coloured protein fraction consisting mainly of ceruloplasmin with an admixture of some β-globulins.

Immunoglobulins recovered chromatographically from hyperimmune sera as well as from non-immune sera exhibited a typical electrophoretic behaviour on paper electrophoresis in pH 8.6 buffer, the unretained fraction moving towards cathode and the retained fraction towards anode. The distribution of antibodies is presented in Table 2. About 90% of anti-diphtheria activity contained in the enriched immunoglobulin preparation was recovered in the effluent. Essentially all antibodies were found in IgG(T) (fraction II), but about 2% was present in IgG (fraction I), as assayed by the skin erythema protection test. The over-all recovery of anti-diphtheria antibodies with respect to the initial whole serum was about 50%, which is of the order expected if the serum was chromatographed twice in order to achieve a comparable degree of purity of IgG(T).

The chromatography as described above differs from that used by Weir & Porter (1966) in that these authors used Sephadex A-50 column at pH 8.4 for rechromatography of immunoglobulins initially isolated

on pH 6.3 column. The enriched immunoglobulin preparation run on pH 8.4 column yielded in our hands essentially the same results as on pH 6.3 column with a similar recovery of the antibodies.

Comparison of IgG and IgG(T) isolated from the whole serum and from the enriched immunoglobulin preparation

The data presented in the preceding section show that the pattern of chromatographic partitioning of immunoglobulins is practically independent of whether the enriched preparation or the whole serum are run through the column. In each case two main immunoglobulin fractions are obtained, exhibiting typical properties of IgG (fraction I) and of IgG(T) (fraction II). However, the IgG/IgG(T) ratio depends on the nature of the starting material: the average ratio, calculated from a number of experiments was 1:1.4 in the case of the whole hyperimmune serum, and 1:2.7 in the case of the enriched immunoglobulin preparation. (The enriched preparation obtained from non-immunized horse yielded the IgG/IgG(T) ratio of 1:1.2 only). It appears that some of the IgG is lost during salt fractionation resulting in a preferential concentration of IgG(T). A similar observation was reported by Raynaud & Relyveld (1959) for a horse serum fractionated by salting out with ammonium sulphate.

Irrespective of the source and of the method of fractionation, IgG(T) was the only fraction in which the antibodies against diphtheria toxin were concentrated; it remains obscure whether a trace amount of the antibodies found in IgG (cf. Table 2) represented a different molecular entity or an incidental contamination with IgG(T). The specific activity of antidiphtheria antibodies in IgG(T) ranged from 12 000 to 19 000 Lf units per gram of protein; the difference appears rather insignificant in view of the limited precision of the flocculation test.

IgG and IgG(T) fractions exhibited characteristic mobilities on paper

Table 2

Distribution of anti-diphtheria antibodies among the fractions obtained by chromatography on Sephadex A-50, pH 6.3, from the enriched immunoglobulin preparation

For the designation of fractions see Fig. 3. AU, antitoxic units, skin erythema protection test.

Preparation	Recovery (%)		Lf/g. protein	AU/g. protein
	protein	Lf units		
Enriched immunoglobulin preparation	100	100	6 700	not done
Fraction I	26.6	0	0	350
Fraction II	59.0	91	12 200	12 400
Fraction III	4	0	0	0

electrophoresis, i.e. slightly cathodic and slightly anodic, respectively. Also, in the starch-gel electrophoresis (Fig. 4), IgG moved towards cathode from the point of application, and IgG(T) towards anode; no difference was found in the apparent homogeneity and contamination with other serum protein fractions between the immunoglobulin isolated from the whole serum and from the enriched immunoglobulin preparation.

In the agar precipitation test (Fig. 5), IgG formed a single line, and IgG(T) two lines with the rabbit serum against horse serum protein. The line formed by IgG appears confluent with the weaker of the two lines formed by IgG(T). The same pattern was obtained with immunoglobulins isolated from the whole serum and from the enriched preparation.

When instead of anti-horse serum, the trough was filled with diphtheria toxin, the precipitation line appeared only at the well containing IgG(T) (Fig. 6). The lines are sharp with immunoglobulins obtained from the whole serum as well as from the enriched preparation, despite the questionable purity of the toxin. In Fig. 6 the precipitation patterns are shown of the fractions which emerged from the column after IgG(T) when the latter was isolated chromatographically from the whole serum (cf. Fig. 1). Fraction III gave a multiple zone of precipitation, in which one or more lines were confluent with the line formed between IgG(T) and the toxin, but others were not. It seems likely that in fraction III, and possibly also in fraction IV, the antibodies are present which, although directed against diphtheria toxin preparation, are not identical with those present in IgG(T). Such antibodies were found in equine anti-hapten serum by Rockey, Klinman & Karush (1964). In the case of anti-diphtheria serum, the activity present in fractions III and IV are evidently lost during salt fractionation, thus increasing the purity of immunoglobulins if chromatography has been carried out after preliminary isolation of the enriched preparation.

The content of carbohydrate was markedly different in IgG and IgG(T) (Table 3). The amount of carbohydrates, calculated as hexose,

Table 3

Carbohydrate content of equine immunoglobulins

The content of hexose was determined by the orcinol method (Winzler, 1955).

Fraction derived from	IgG	IgG(T)
	hexose (moles/mole protein)	
Whole serum	5.7	13.9
Enriched immunoglobulin preparation	5.6	15.0

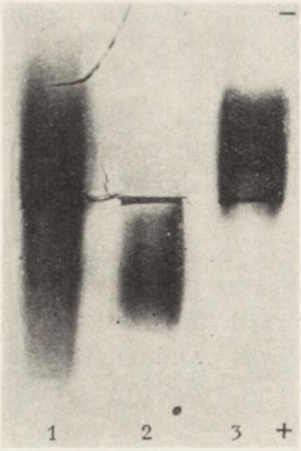

Fig. 4. Starch-gel electrophoresis of equine immunoglobulins. Borate buffer, pH 8.6, migration from top to bottom. From left to right: enriched preparation of immunoglobulins, IgG(T), IgG

in IgG was only about 30% of that in IgG(T), in agreement with the findings reported by Rockey *et al.* (1964) for equine anti-hapten serum, and by Weir & Porter (1966) for equine anti-diphtheria serum. Again, no difference was found between the respective immunoglobulins isolated from the whole serum and from the enriched preparation.

Comparison of the structural parameters of IgG and IgG(T)

All experiments aimed at the comparison of structural parameters of IgG and IgG(T) were carried out with the proteins isolated by Sephadex A-50 chromatography from the enriched immunoglobulin preparation, since, as shown in the previous section, no difference has been found between the immunoglobulin fractions isolated from these preparations and from the whole serum. The data presented below were always obtained from the proteins isolated from the same sample of serum, but various preparations yielded practically identical results.

Antigenic specificity of IgG and IgG(T) was studied using rabbit sera against fractions isolated by Sephadex A-50 chromatography. Immunodiffusion patterns with anti-IgG(T) sera are presented in Fig. 7, and those with anti-IgG sera in Fig. 8. Anti-IgG(T) serum forms a single line with IgG, and a single line with IgG(T), but at higher concentration of IgG(T) a second, more diffuse, line can be seen (cf. bottom row, Fig. 7). Neither of the lines formed between anti-IgG(T) serum and IgG(T) is confluent with the line formed between this serum and IgG. Anti-IgG serum yields a different pattern. A single line forms between IgG and anti-IgG serum, but distinct two lines appear with IgG(T). One of these two lines (further from the trough, cf. Fig. 8) seems confluent with the precipitation line characteristic of IgG. It may, therefore, be concluded that in the IgG(T) preparation at least two components are present, one of which is antigenically very similar to IgG. In order to

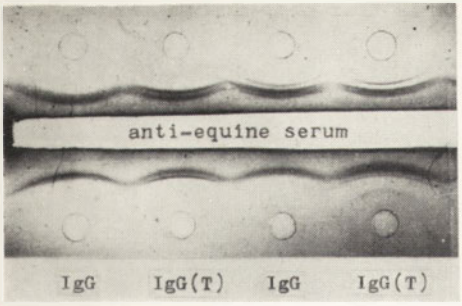

Fig. 5

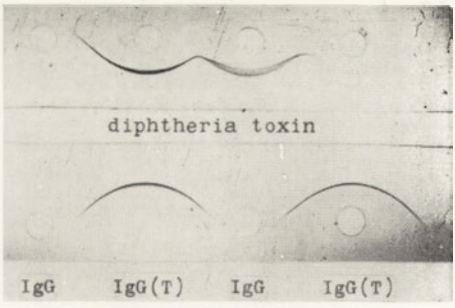

Fig. 6

Fig. 5. Immunodiffusion analysis of equine immunoglobulins. Centre trough: anti-equine serum. Wells, from left to right, top row: Fraction I, Fraction II, Fraction III, Fraction IV obtained by chromatography of the whole anti-diphtheria serum (cf. Fig. 1); bottom row: IgG, 5.5 mg./ml.; IgG(T), 8.1 mg./ml.; IgG, 6.8 mg./ml.; IgG(T), 16.5 mg./ml. IgG and IgG(T) obtained by chromatography of the enriched immunoglobulin preparation (cf. Fig. 3)

Fig. 6. Immunodiffusion analysis of equine anti-diphtheria immunoglobulins. Centre trough: diphtheria toxin. Wells: as in Fig. 5

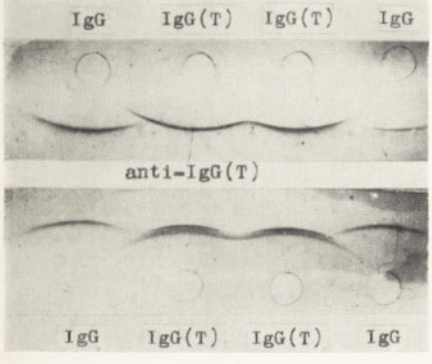

Fig. 7.

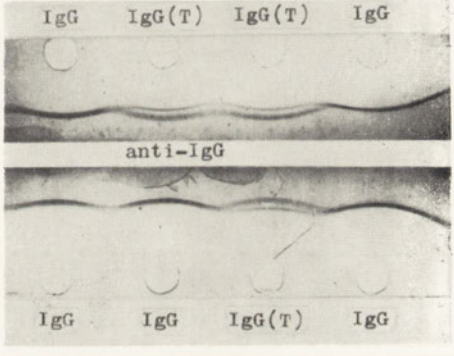

Fig. 8.

Fig. 7. Agar precipitation of equine immunoglobulins with anti-IgG(T) serum. Top row: 0.18 mg. protein per well; bottom row: 0.36 mg. protein per well

Fig. 8. Agar precipitation of equine immunoglobulins with anti-IgG serum. Top row: 0.18 mg. protein per well; bottom row: 0.36 mg. protein per well

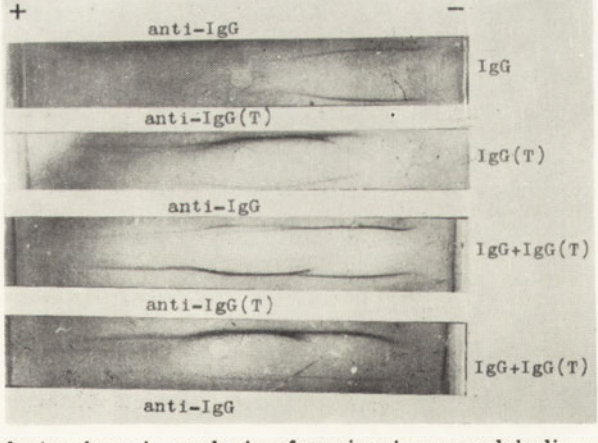

Fig. 9. Immunoelectrophoresis analysis of equine immunoglobulins. Veronal buffer, pH 8.6; 2.5 mA/cm; 2.5 hr.

study this relationship in more detail, immunoelectrophoresis was carried out using both antisera and both fractions, alone or mixed. The results are presented in Fig. 9. Anti-IgG(T) serum forms a sharp precipitation line in the far cathodic region with IgG, and an irregular precipitation line, close to the application point, with IgG(T). The latter is composed of two superimposed lines as evidenced by a short forking seen, in particular, on its cathodic end. When IgG(T) was applied to the agar layer together with IgG, the pattern obtained with anti-IgG(T) serum changed in that one of the arms of the forking was extended far towards cathode. A third precipitation line, such as should be expected in case of non-identity between IgG and IgG(T) was completely absent. Thus, the conclusion drawn from the agar precipitation tests was supported by immunoelectrophoresis, namely that in IgG(T) a component is present which is antigenically indistinguishable from IgG. The only difference between IgG and its counterpart in IgG(T) appears to consist in their widely different electrophoretic mobility: IgG moves towards cathode, being presumably transported by endosmotic current, while in IgG(T) a slightly higher charge, possibly due to anionic carbohydrate component, makes it move towards anode, or stay close to the application point as in the case of agar electrophoresis.

Despite the inhomogeneity of IgG(T) revealed by immunochemical procedures, the protein appears homogeneous in gel filtration (Fig. 10). Gel filtration patterns of IgG and IgG(T) were analysed by the probabilistic plot according to Tracey & Winzor (1966). A straight line

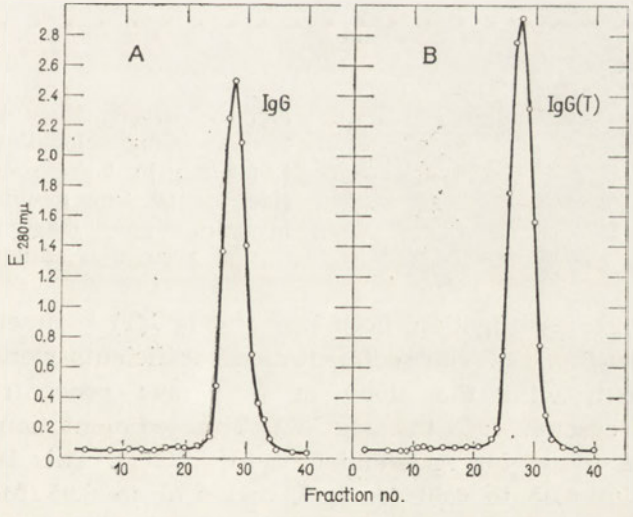

Fig. 10. Gel-filtration of equine immunoglobulins. Sephadex G-200 equilibrated and eluted with 0.15 M-sodium chloride, pH 7.0; column 2 × 115 cm.; elution rate 28 ml./hr., fractions 4.7 ml.

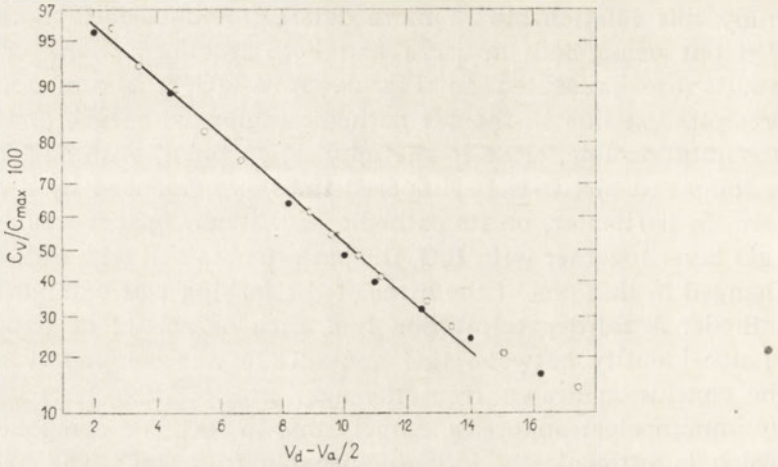

Fig. 11. Analysis of homogeneity of gel-filtration patterns of equine immunoglobulins. Full circles: IgG; empty circles: IgG(T). Data from Fig. 10. C_{max}: maximum concentration; C_v: concentration at a given elution volume; V denotes the elution volume on the descending (d) or on the ascending (a) arm of the elution curve

(Fig. 11) obtained by Tracey & Winzor procedure shows that both immunoglobulins are essentially homogeneous, and a slight deviation from the linearity, seen at the highest values on the abscissae, results from insufficient precision of the concentration measurement rather than from a true inhomogeneity.

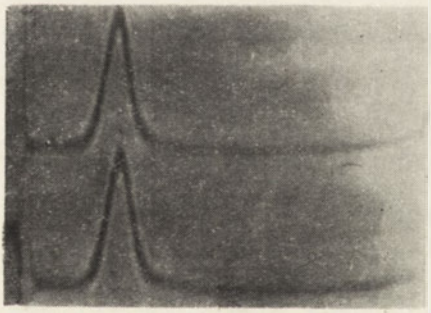

Fig. 12. Ultracentrifuge diagrams of equine immunoglobulins. Protein 4.6 mg./ml. in 0.15 M-sodium chloride, pH 7.0. 28th minute at 59 870 rev./min. Upper diagram: IgG(T); lower diagram: IgG

During ultracentrifugation, both IgG and IgG(T) sediment as homogeneous peaks (Fig. 12). The sedimentation coefficients were concentration dependent, with the slope of S *versus* concentration more steep in the case of IgG than IgG(T). The sedimentation coefficient $S^0_{20} = 6.70$ was found for IgG, and 6.92 for IgG(T). This is in a close agreement with 6.55 to 6.80 for IgG, and 6.75 to 6.95 for IgA from equine anti-hapten sera reported by Klinman *et al.* (1966), and by Rockey *et al.* (1964). Using the equation developed by Halsall (1967), molecular weight was estimated 146 000 for IgG, and 153 000 for IgG(T). Pain (1965)

found the molecular weight of horse serum IgG to be 148 000 from sedimentation equilibrium and diffusion measurements.

In order to compare the amino acid composition of IgG and IgG(T), the proteins were hydrolysed as described under Methods. Since only the comparison was aimed at but not the absolute amounts, no correction was introduced into the results for hydrolytic destruction (Table 4). The comparison between the amino acid composition of IgG and IgG(T) was made on the basis of null hypothesis, that is to say that no difference existed. For calculations, the number of amino acid residues per mole protein was determined and converted into mole-fractions, i.e. the number of residues of a given amino acid divided by the total number of amino acyl residues per mole protein. Then the ratio of mole-fractions in IgG to that in IgG(T) for each residue was calculated. The results are listed in Table 4 together with the data of Weir & Porter (1966) similarly treated. The mean ratio found for our preparations is 1.02 ± 0.093, and for the Weir & Porter's preparations 1.01 ± 0.072. The similarity of the amino acid composition of IgG and of IgG(T) is quite striking: all ratios

Table 4

Amino acid composition of equine immunoglobulins

Amino acid	IgG		IgG(T)		mole-fraction IgG / mole-fraction IgG(T)	
	moles per mole protein	mole-fraction (%)	moles per mole protein	mole-fraction (%)	this paper	Weir & Porter (1966)
Aspartic acid	76	5.40	86	6.45	0.84	0.93
Threonine	112	8.00	101	7.20	1.11	1.11
Serine	158	11.30	145	10.35	1.09	0.98
Glutamic acid	127	9.10	125	8.90	1.02	1.04
Proline	104	7.42	133	9.50	0.78	0.86
Glycine	100	7.12	96	6.85	1.04	0.97
Alanine	82	5.85	78	5.56	1.05	1.04
Valine	135	9.63	127	9.06	1.06	1.00
Isoleucine	51	3.62	48	3.43	1.06	1.16
Leucine	119	8.50	114	8.15	1.04	0.95
Tyrosine	45	3.20	45	3.22	0.99	1.09
Phenylalanine	33	2.36	33	2.36	1.00	1.05
Lysine	139	9.90	150	10.70	0.93	0.98
Histidine	37	2.64	32	2.28	1.16	0.95
Arginine	36	2.54	38	2.71	0.94	1.04
Tryptophan *	20	1.43	20	1.43	1.00	1.00
Cysteine	31	2.20	28	2.00	1.10	1.04
Total	1405	100.21	1399	100.15	—	—
Mean	—	—	—	—	1.02 ± 0.09	1.01 ± 0.07

* Spectrophotometric method (Beaven & Holiday, 1952).

of mole-fractions are within three standard deviations of the mean. It is, however, worth noting that in Weir & Porter's preparation as well as in that of ours, the prolyl residue seems slightly less abundant in IgG(T) than in IgG, since the ratio of mole-fractions is outside the two standard deviations. In view of the fact that IgG(T) is composed of two proteins differing in their antigenic specificity, it has to be concluded that the similarity of amino acid composition extends over IgG, its counterpart in IgG(T) and a third, distinct component in IgG(T), possibly IgA.

The gross conformation of immunoglobulins was studied by measurement of optical rotatory dispersion. The ORD parameters are shown in Table 5 from which it will be seen that both IgG and IgG(T) are characterized by low a_0 and very low b_0. Low values of these parameters appear to be a typical feature of immunoglobulins isolated from a variety of animals (Fleischman, 1966; Okulov, Troitsky & Gordeev, 1966). It is not known whether the low b_0 parameter reflects a low helicity of immunoglobulins or the presence of compensating conformations.

Table 5

Optical rotatory dispersion parameters of equine immunoglobulins

Fraction	a_0	b_0
IgG	—305	+3
IgG(T)	—302	+5

Whatever the conformation of equine immunoglobulins, there is little doubt that they do possess a considerable degree of molecular organization. Spectrophotometric titration of tyrosyl residues (Fig. 13) showed that the mid-point of phenolic ionization was at pH 11.9 when the proteins were titrated from neutral towards alkaline pH. The reverse titration, i.e. carried towards neutral pH after 24 hr. in 0.1 N-sodium hydroxide at room temperature, demonstrated a marked normalization of phenolic ionization: the mid-point was shifted to 10.5 which is close to the intrinsic pK of tyrosyl hydroxyl. No difference was found between the titration of tyrosyl residues in IgG and IgG(T).

In search of a more subtle sounding of tyrosyl residues in immunoglobulins, attempts have been made to apply the solvent perturbation method (Herskovits & Laskowski, 1962). With 30% (v/v) methanol as perturbant, about 60% of tyrosyl residues in IgG(T) were found solvent-accessible, but IgG could not be so investigated because a strong turbidity developed immediately on addition of methanol. With 20% (v/v) glycerol, only a faint and indistinct difference spectrum was generated, not amenable to quantitative interpretation. A qualitative conclusion which may be drawn from these experiments is that the

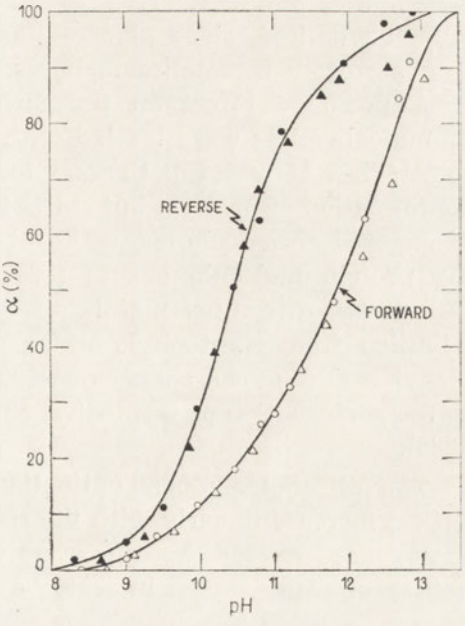

Fig. 13. Spectrophotometric titration of tyrosyl residues in equine immunoglobulins. Triangles: IgG; circles: IgG(T). Empty symbols: forward titration; full symbols: reverse titration

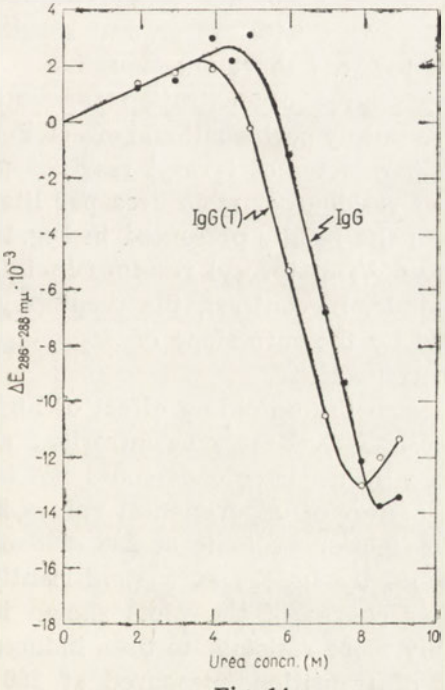

Fig. 14.

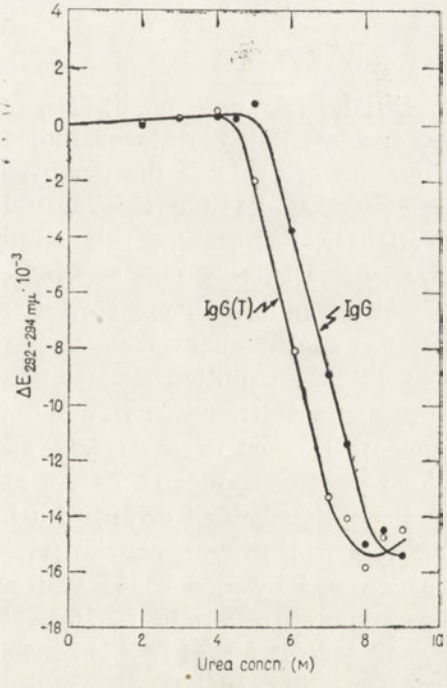

Fig. 15.

Fig. 14. The effect of urea on the exposure of tyrosyl residues in equine immunoglobulins. Full circles: IgG; empty circles: IgG(T).

Fig. 15. The effect of urea on the exposure of tryptophyl residues in equine immunoglobulins. Full circles: IgG; empty circles: IgG(T)

accessibility of tyrosyl residues to solvents in equine immunoglobulins must be limited, since glycerol, a relatively bulky perturbant, cannot gain access to these chromophores.

A profound effect on the difference spectra was observed when immunoglobulins were treated with urea solutions of various concentrations (Figs. 14 and 15). Urea at low concentrations does not affect the conformation of proteins but shifts the position of the absorption maxima of exposed chromophores, while at high concentrations an unfolding of the protein molecule ensues (Wetlaufer, 1962). The changes at 286 - 288 mμ are attributed mainly to tyrosyl perturbation, with some contribution from tryptophyls and a negligible contribution from phenylalanyl residues. The changes at 292 - 294 mμ are almost exclusively due to the perturbation of tryptophyl chromophores (Herskovits & Soerensen, 1968).

The difference molar extinction coefficients, $\Delta\varepsilon$, of both IgG and IgG(T) increase linearly with the rising urea concentration, but at higher urea concentrations the difference extinctions rapidly decrease. Positive values of $\Delta\varepsilon_{286-288m\mu}$ indicate a perturbation of tyrosyl chromophores resulting in a long wavelength shift of the absorption maximum. Negative values of $\Delta\varepsilon_{286-288m\mu}$ indicate the unfolding of the protein which brings tyrosyl chromophores in contact with water and results in a shift of the absorption maximum towards short wavelengths.

The initial slope of $\Delta\varepsilon_{286-288m\mu}$ *versus* urea concentration for both IgG and IgG(T) is rectilinear, indicating a purely perturbational effect. For a number of proteins the perturbation parameter of tyrosyl residues in urea was found 18.5 extinction units per residue per mole urea per liter (Wetlaufer, 1962). Using this value, from the results presented in Fig. 14 it can be calculated that approximately 65% of tyrosyl residues in IgG as well as in IgG(T) are solvent accessible. In contrast, the tryptophyl residues appear all masked, as indicated by the zero slope of $\Delta\varepsilon_{292-294m\mu}$ (Fig. 15) when plotted against urea concentration.

A distinct difference was found between the unfolding effect of high concentration of urea on IgG and on IgG(T). Urea concentrations at which positive values of $\Delta\varepsilon$ are replaced by negative ones could not be precisely determined owing to a large scatter of experimental values in this region. However, zero difference extinction ordinate at 286 - 288 mμ was crossed by IgG at 6.0 M-urea, while by IgG(T) at a significantly lower concentration of 5.2 M. A further course of the plots shown in Fig. 14 indicates that IgG is considerably more resistant to urea induced unfolding than IgG(T): the mid-point of transition, measured at 286 - 288 mμ, is at 6.8 M-urea in the case of IgG, but at 6.0 M in the case of IgG(T). Essentially the same mid-points are observed when the unfolding is followed at 292 - 294 mμ (Fig. 15). Thus it may be concluded that the unfolding of tyrosyl containing regions of both immunoglobulins is in

phase with the unfolding of tryptophyl containing regions. The difference in the susceptibility to unfolding by urea appears, so far, to be the only conformational difference between IgG and IgG(T).

REFERENCES

Allen P. Z., Sirisinha S. & Vaughan J. H. (1965). *J. Immunol.* **95**, 918.
Beaven G. H. & Holiday E. R. (1952). *Advanc. Protein Chem.* **7**, 373.
Crumpton M. J. & Wilkinson J. M. (1963). *Biochem. J.* **88**, 228.
Durrum E. L. (1950). *J. Amer. Chem. Soc.* **72**, 2943.
Fleischman J. B. (1966). *Ann. Rev. Biochem.* **35**, 835.
Genco R. J., Karush F. & Tenenhouse H. S. (1968). *Biochemistry* **7**, 2462.
Halsall H. B. (1967). *Nature* **215**, 880.
Herskovits T.T. & Laskowski M., Jr. (1962). *J. Biol. Chem.* **237**, 2481.
Herskovits T. T. & Soerensen Sr. M. (1968). *Biochemistry* **7**, 2523.
Hinsberg K. & Lang K. (1957). *Medizinische Chemie.* Urban & Schwarzenberg, Wien.
Hořejši J. & Smetana R. (1956). *Acta Med. Scand.* **155**, 65.
Kekwick R. A. & Record B. R. (1941). *Brit. J. Exp. Path.* **22**, 2944.
Klinman N. R., Rockey J. H., Fraunberger G. & Karush F. (1966). *J. Immunol.* **96**, 587.
Klinman N. R., Rockey J. H. & Karush F. (1965). *Immunochemistry* **2**, 51.
Moffitt W. & Yang J. F. (1956). *Proc. Natl. Acad. Sci. U.S.* **42**, 596.
Okulov V. I., Troitsky G. V. & Gordeev Ju. N. (1966). *Biokhimiya (U.S.R.R.)* **31**, 768.
Pain R. H. (1965). *Biochim. Biophys. Acta* **94**, 183.
Raynaud M., Iscaki S. & Mangalo R. (1965). *Ann. Inst. Pasteur* **109**, 525.
Raynaud M. & Relyveld E. H. (1959). *Ann Inst. Pasteur* **97**, 636.
Rockey J. H., Klinman N. R. & Karush F. (1964). *J. Exp. Med.* **120**, 589.
Scheidegger J. J. (1955). *Int. Arch. Allergy* **7**, 103.
Schultze H. E., Haupt H., Heide K., Heimburger N. & Schwick H. G. (1965). *Immunochemistry* **2**, 273.
Smithies O. (1959). *Biochem. J.* **71**, 585.
Stefani D. W. & Kulberg A. Ja. (1964). *Wopr. Med. Chimji (U.S.R.R.)* **10**, 279.
Stefani D. W., Kulberg A. Ja. & Schachanina K. L. (1965). *Wopr. Med. Chimji (U.S.R.R.)* **11**, 34.
Tracey M. V. & Winzor D. J. (1966). *Arch. Biochem. Biophys.* **117**, 184.
Van der Scheer J. & Wycoff R.W.G. (1940). *J. Immunol.* 39, 65.
Weir R. C. & Porter R. R. (1966). *Biochem. J.* **100**, 63.
Weir R. C., Porter R. R. & Givol D. (1966). *Nature* **212**, 205.
Wetlaufer D. B. (1962). *Advanc. Protein Chem.* **17**, 304.
Winzler R. J. (1955). *Methods in Biochemical Analysis* (D. Glick, ed.) vol. 2, p. 279.
World Health Organization (1964). *Bull. World Hlth. Org.* **30**, 447.

IMMUNOGLOBULINY KOŃSKIE: PORÓWNANIE WŁAŚCIWOŚCI MOLEKULARNYCH

Streszczenie

1. Z surowicy koni hiperimmunizowanych w stosunku do błonicy izolowano frakcje immunoglobulin IgG i IgG(T) przy pomocy wysalania i chromatografii na Sephadex'ie DEAE.

2. Właściwości IgG i IgG(T) izolowanych z surowicy tego samego konia były przedmiotem porównania. Aktywność przeciwciał stwierdzono jedynie w IgG(T).

3. IgG i IgG(T) różniły się zawartością węglowodanów i podatnością na rozfałdowanie przez mocznik.

4. IgG i IgG(T) były nieomal identyczne co do właściwości antygenowych, składu aminokwasowego, ciężaru molekularnego, jonizacji tyrozyn, parametrów skręcalności optycznej, oraz dostępności tyrozyn i tryptofanów dla rozpuszczalnika.

Received 8 March, 1969.

ACTA BIOCHIMICA POLONICA
Vol. 16 1969 No. 3

LIDIA PAŚŚ and KONSTANCJA RACZYŃSKA-BOJANOWSKA

ARGININE IN VIOMYCIN BIOSYNTHESIS

Biochemical Laboratory, Institute of Antibiotics, ul. Starościńska 5, Warszawa

1. Arginine in contrast to citrulline, ornithine, urea, lysine, leucine, glycine and serine constitutes almost as good nitrogen source for viomycin biosynthesis as $(NH_4)_2SO_4$. 2. Inhibition of antibiotic production is associated, except in stationary cultures, with accumulation of arginine in mycelium. 3. The activities of arginase and serine dehydratase (deaminating) are not related to the process of antibiotic formation, or to concentration of the respective amino acids in mycelium. 4. A non-specific X-amidinotransferase transferring amidino group to an unknown acceptor was found in *Streptomyces* strain producing viomycin. The enzyme was repressed by inorganic phosphate, oxygen deficiency and canavanine, factors inhibiting viomycin biosynthesis. 5. The role of transamidination in a general mechanism of biosynthesis of guanidino group in antibiotics is discussed.

Viomycin, a strongly basic peptide of tuberculostatic properties, belongs to a large group of guanidine antibiotics (Szylagyi, Valyi-Nagy, Szabo & Keresztes, 1964). Its structure has recently been extensively studied (Kitagawa, Sawada, Miura, Ozasa & Tanyiama, 1968; Bycroft *et al.*, 1968a,b; Takita & Maeda, 1968; Lechowski, 1969), but no attempt has been made to elucidate the biosynthetic mechanism of amino acid sequence and the origin of guanidino group.

Complete acid hydrolysis of viomycin yields: viomycidine, considered as an artifact of the natural guanidine component, L-α,β-diaminopropionic acid, L-β-lysine, L-serine, urea, and carbon dioxide (Bowie, Cox, Johnson & Thomas, 1964).

The presence of the ureido and guanidino groups in viomycin prompted us to investigate the effect of arginine on biosynthesis of this antibiotic. Two types of enzymes have been taken into consideration: arginase responsible for catabolic breakdown of arginine and supply of urea, and the amidino group transferring system demonstrated by Walker (1958) in *Streptomyces griseus* and called X-amidinotransferase (Walker & Hnilica, 1964) to indicate the unknown receptor of the amidino group.

MATERIALS AND METHODS

Reagents. Lactate dehydrogenase (5 mg./ml.), glutamate dehydrogenase (20 mg./ml.), aspartate transaminase (2 mg./ml.), malate dehydrogenase (0.5 mg./ml.), NAD^+ and NADH were products of C. F. Boehringer & Soehne (Mannheim, G.F.R.). L-Serine and L-ornithine were obtained from Reanal (Budapest, Hungary); L-aspartic acid, L-citrulline, L-glutamic acid, and L-canavanine · H_2SO_4 were from Calbiochem. (Lucerne, Switzerland). L-Arginine · HCl was from Sojuzchimexport (U.S.S.R.), tris(hydro-xymethyl)aminomethane was a product of Loba Chemie (Wien-Fischamend, Austria), β-mercaptoethanol of Light & Co. Ltd. (Colnbrook, Bucks., England), lysozyme (from egg white, 25 units/mg.) of Sigma Chemical Co. (St. Louis, Mo., U.S.A.), Difco yeast extract of Difco Laboratories (Detroit, Mich., U.S.A.), bovine blood serum albumin cryst., of Serva (Heidelberg, G.F.R.) and sodium nitroferricyanide of Merck (U.S.A.). Other reagents were from Fabryka Odczynników Chemicznych (Gliwice, Poland). Arginase from rat liver was prepared according to Schimke (1964) and contained 250 units/ml. Pentacyanoammoniumferrate trisodium was obtained from sodium nitroferricyanide according to Breuer (1962). The purified arginase inhibitor from sunflower seeds was a gift of Dr. Grażyna Muszyńska (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa) prepared as described by Morawska-Muszyńska & Reifer (1965).

Organisms. Streptomyces sp. strain producing viomycin and *Streptomyces erythreus* were obtained from Drs. M. Tyc and Barbara Ostrowska-Krysiak of the Division of Microbiology, and the other strains: *Streptomyces griseus, Streptomyces nursei, Bacillus subtilis* and *Bacillus licheniformis* were from the Strain Collection, Institute of Antibiotics, Warsaw.

Culture conditions and the complex soluble basic medium were the same as used previously (Paśś & Raczyńska-Bojanowska, 1968). In the experiments on the effect of various nitrogen sources on viomycin biosynthesis ammonium sulphate was replaced by amino acids or urea supplying the equivalent amount of nitrogen. The basic medium was supplemented in some experiments with canavanine (1.5 µmole/ml.), or vetch flour extract was used instead of the soya flour extract because of the unusually high canavanine content in the former plant (Bell, 1965). The vetch flour employed (*Vicia sativa*, variety Sielecka) contained per 1 gram 96 µmoles of canavanine and 3.5 µmoles of arginine, whereas soya flour contained 2.6 and 8.4 µmoles, respectively. The specific arginase inhibitor from sunflower seeds (10 µg./ml.) and phosphorus (150 and 1000 µg./ml.) in the form of KH_2PO_4-NaOH, pH 7.0, were added to the basic complex soluble medium in the experiments indicated in the text.

Preparation of mycelium extracts and spheroplasts, and assay of free and bound amino acids were as described previously (Paśś & Raczyńska-Bojanowska, 1968).

Enzyme assay. Arginase was determined according to Greenberg (1955). The incubation mixture contained: L-arginine·HCl, 5 μmoles; $MnCl_2$, 0.05 μmole; and mycelium extract (0.8 - 1.0 mg. protein) in 1.0 ml. of 0.25 M-glycine buffer, pH 9.5; after 10 min. incubation at 37° the reaction was stopped by adding 1 ml. of 6 N-phosphoric acid, and ornithine formed was measured after Chinard (1952).

Determination of serine dehydratase (deaminating) was coupled with reduction of formed pyruvate in the presence of NADH and lactate dehydrogenase according to Freedland & Avery (1964).

The activities of arginase and serine dehydratase were expressed in μmoles/min./g. wet wt. of mycelium; the wet weight referred to mycelium obtained on centrifuging the culture at 2000 rev./min. for 15 min. The activity of X-amidinotransferase was determined according to Walker (1958) using hydroxylamine as an acceptor of the amidino group. The reaction mixture containing arginine·HCl, 20 μmoles; hydroxylamine·HCl, 400 μmoles; and mycelium extract (8 - 10 mg. protein) in 1.0 ml. of 0.2 M-phosphate buffer, pH 7.0, was incubated for 2 hr. at 37° and deproteinized with 1.0 ml. of a mixture of 30% trichloroacetic acid--acetone (1:1, v/v). Hydroxyguanidine formed in the reaction was estimated colorimetrically with Na_3-pentacyanoammoniumferrate at 480 mμ. Arginine in some experiments was replaced by other guanidine compounds: α-amino-β-guanidinopropionate, α-amino-γ-guanidinobutyrate, creatine and creatinine in the same molar concentration. One unit of enzyme activity was defined as that amount of enzyme which increased the extinction at 480 mμ by 0.01, and the activity was expressed in units/mg. protein/2 hr.

Determination of protein and nitrogen. Protein in the extract was assayed according to Lowry *et al.* (Layne, 1955), and nitrogen in the medium by the Kjeldahl method.

Determination of wet and dry weight of mycelium and the amount of viomycin were performed as described previously (Paśś & Raczyńska-Bojanowska, 1968).

RESULTS

Effect of nitrogen source and inhibitors on viomycin biosynthesis. Utilization of arginine for growth and viomycin production by the investigated *Streptomyces* strain was compared with that of ammonium sulphate and compounds related to arginine: urea, L-ornithine and L-citrulline. In addition L-leucine, L-lysine, glycine and L-serine, a component of viomycin, were included in the experiments. Data given in Table 1 show that the amount of nitrogen present in the medium with-

out ammonium sulphate was insufficient for antibiotic production, growth of the mycelium being also markedly reduced. The addition of ammonium sulphate, amino acids or urea resulted in pronounced differences in viomycin yield, while the mycelium crop varied insignificantly. The amount of antibiotic produced on the medium containing arginine was only 13% lower than that obtained on control medium with ammonium sulphate and so was the number of antibiotic units per 1 μg. of nitrogen taken up by the mycelium (0.91 and 1.01, respectively). The nitrogen uptake was calculated from the difference between the content of nitrogen in the initial medium and the medium from which a 120-hr. mycelium was centrifuged off. On media containing other nitrogen sources viomycin biosynthesis was reduced to 20 - 50% with a 2 - 5-fold lower viomycin:nitrogen ratio. On ornithine and citrulline this ratio was 1/2, and on urea 1/5 that on control medium. Utilization of lysine and serine as the main nitrogen source in viomycin biosynthesis was similar to that of ornithine and citrulline, while that of leucine was about 30% higher. Glycine was a nitrogen source for antibiotic production as poor as urea.

Viomycin biosynthesis could be inhibited specifically by canavanine, an antimetabolite of arginine, or inhibited non-specifically by inorganic phosphate or oxygen deficiency in stationary culture. As can be seen from the results presented in Table 2, the addition of canavanine (262 μg./ml.) *in substantia* or in the vetch flour extract had little effect on growth of the mycelium but prevented biosynthesis of viomycin. In the stationary culture, growth was reduced by 50% and viomycin yield by 75%. On medium containing 186 μg.P_i/ml., growth was stimulated by 20% and antibiotic formation inhibited by 90%; at about 5 times higher P_i concentration, production of the antibiotic was totally abolished and dry weight of mycelium reduced to 40% of that produced on control medium containing 36 μg. P_i/ml.

Changes in amino acid pool. The time course of changes in arginine concentration in mycelium under various conditions of viomycin biosynthesis illustrates the relation of arginine metabolism to the process of antibiotic formation. These changes were confronted with the size of amino acid pool and differences in concentration of other amino acids: serine, a component of viomycin, aspartate, a precursor of the imino group of arginine, and glutamate, a constituent of one of the most active transamination systems.

The composition of amino acid pool was studied in mycelium grown on the control, ammonium sulphate containing medium, and on media with arginine or serine, nitrogen sources of different value in viomycin biosynthesis (cf. Table 1). The analysis was extended to conditions of viomycin biosynthesis inhibited by inorganic phosphate, oxygen shortage or canavanine.

Table 1

Effect of nitrogen source on mycelium growth and viomycin synthesis

The medium contained: water, 100 ml.; glucose, 5.0 g.; Difco yeast extract, 0.5 g.; soya flour extract, 1.5 g.; $CaCO_3$, 0.5 g.; soya oil, 2.0 g. As nitrogen source, 0.6 g. of $(NH_4)_2SO_4$ (control) or appropriate amounts of the indicated compounds were added. After 120 hr. of growth, the mycelium was separated by centrifuging, in the medium viomycin and nitrogen were determined, and the uptake of nitrogen was calculated. The values are means of 2 - 3 experiments.

Nitrogen source added	Mycelium dry wt.		Viomycin		Viomycin: nitrogen ratio (units/μg.)
	mg./ml.	% of control	units/ml.	% of control	
No addition	16.1	58	0	0	—
Ammonium sulphate (control)	27.9	100	800	100	1.01
L-Arginine	23.2	83	700	87	0.91
L-Citrulline	32.2	115	425	53	0.41
L-Ornithine	29.9	107	400	50	0.40
Urea	23.9	86	230	29	0.18
L-Serine	24.7	88	280	35	0.38
Glycine	25.1	90	220	27	0.17
L-Leucine	24.0	86	320	40	0.68
L-Lysine	21.8	78	190	24	0.32

Table 2

Effect of inorganic phosphate, canavanine and oxygen deficiency on mycelium growth and viomycin biosynthesis

To the control medium the indicated compounds were added.

Factors	Growth (%)	Viomycin yield (%)
Control	100	100
Inorganic phosphate		
186 μg./ml.	120	10
1036 μg./ml.	39	0
Canavanine (262 μg./ml.)		
in substantia	112	5
vetch flour extract	98	0
Stationary culture	48	25

Concentration of free and bound amino acids in mycelium grown on arginine was increased during growth in the same way as that observed in control cultures, the size of the pool was, however, slightly lower (Fig. 1). Similar accumulation of amino acids was also observed in

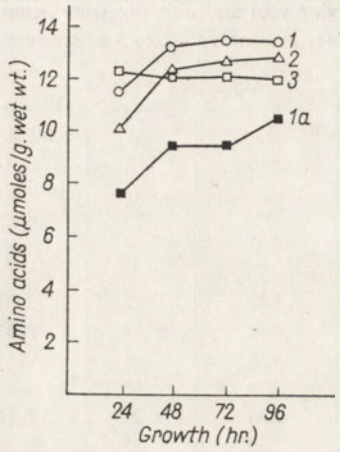

Fig. 1. Amino acid pool in mycelium grown on media containing: (*1*), ammonium sulphate in control and in (*1a*), stationary cultures; (*2*), arginine; and (*3*), serine

stationary culture, with a concomitant about 30% decrease in the total size of the pool. In mycelium grown on serine containing medium the pool remained practically constant during growth.

Concentration of arginine, serine+threonine, aspartate and glutamate in mycelium was practically unaffected when ammonium sulphate was replaced in the medium by arginine (Fig. 2). Low concentration of arginine in mycelium grown on arginine containing medium should be emphasized since the concentration of serine + threonine in mycelium grown on serine was elevated about 5 times in 24-hr. cultures, and at the 96th hour of growth still remained about 1.5-fold higher than in control. On the medium with serine, arginine tended to accumulate during the last stage of growth. The concentration of aspartate and glutamate in mycelium was not changed on supplying arginine or serine instead of ammonium sulphate to the medium.

As found previously (Paśś & Raczyńska-Bojanowska, 1968) the addition of inorganic phosphate decreased the amino acid pool especially in the stationary phase of growth, and resulted in accumulation of all the investigated amino acids in mycelium.

In mycelium grown in the stationary culture the amount of serine+ +threonine was decreased by about 40% and that of glutamate by about 30% as compared with the normally aerated cultures; the concentration of arginine and aspartate was practically unchanged.

On medium with canavanine (vetch flour extract), the concentration

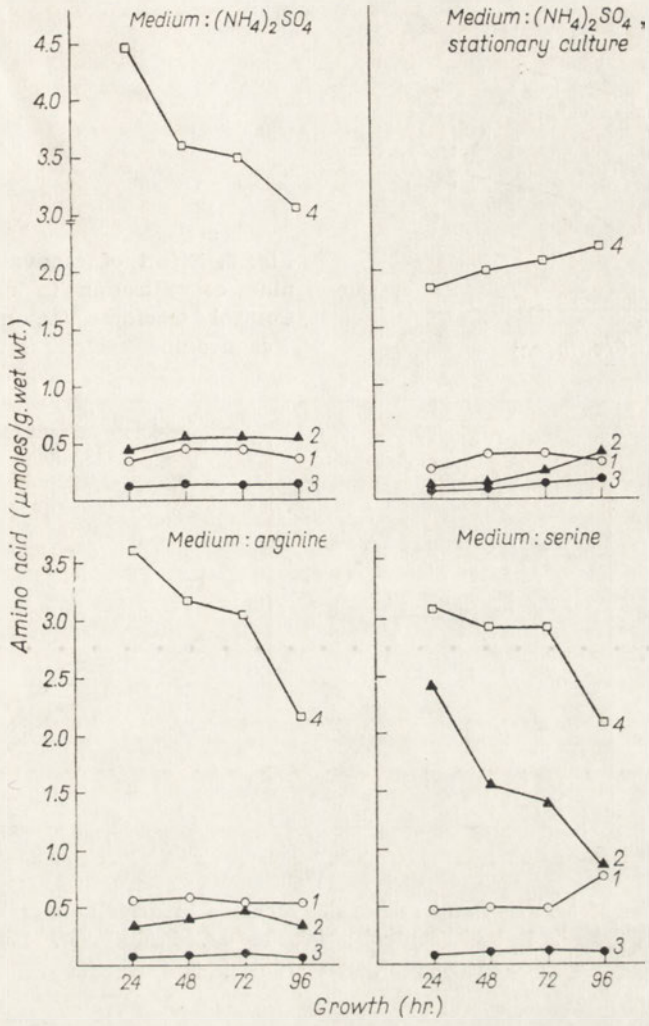

Fig. 2. Concentration of (*1*), arginine; (*2*), serine+threonine; (*3*), aspartate; and (*4*), glutamate, in mycelium grown on media containing various nitrogen sources, as indicated in the Figure

of arginine in mycelium was generally lower (Fig. 3) and a steady accumulation of this amino acid was found during growth, the regression coefficient being +0.040. Low concentration of arginine in mycelium and high viomycin yield obtained on arginine containing medium suggest high activity of the enzymes responsible for the metabolism of this amino acid.

Catabolizing enzymes: arginase versus *serine dehydratase.* The activity of arginase and hence its role in viomycin biosynthesis was compared with that of serine dehydratase, the enzyme responsible for metabolic breakdown of serine.

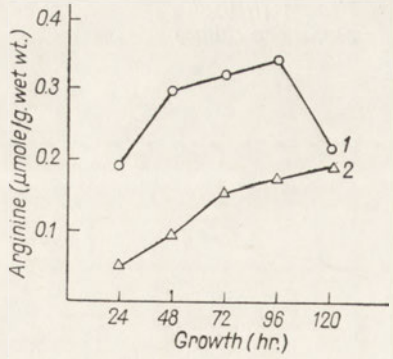

Fig. 3. Effect of canavanine on arginine concentration in mycelium. (*1*), Control medium; (*2*), medium with canavanine (vetch flour extract)

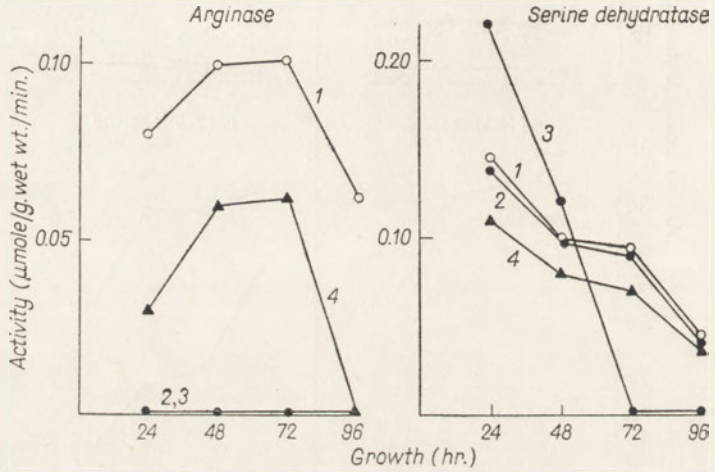

Fig. 4. Activities of arginase and serine dehydratase in mycelium grown on media containing: (*1*), 36 µg. P_i/ml. (control); (*2*), 186 µg. P_i/ml.; (*3*), 1036 µg. P_i/ml.; (*4*), 36 µ. P_i/ml. in stationary culture

In mycelium grown on control medium the activity of arginase was increasing up to the 48th hour of growth, it remained unaltered to the 72th hour, and decreased only in the last stage of antibiotic production (Fig. 4). Deficiency of oxygen in the stationary cultures resulted in a decrease in arginase activity, but did not affect the time course of changes in this enzymic activity during growth. The addition to the medium of inorganic phosphate, an inhibitor of viomycin biosynthesis, resulted in a total inhibition of arginase.

Serine dehydratase (Fig. 4), in contrast to arginase, was most active in the logarithmic phase of growth, whereas in the stationary phase connected with a high production rate of the antibiotic its activity was gradually decreasing. Under conditions of oxygen deficiency this enzymic activity was less affected than that of arginase and the total viomycin yield (cf. Table 2). Inorganic phosphate added in the amount inhibiting

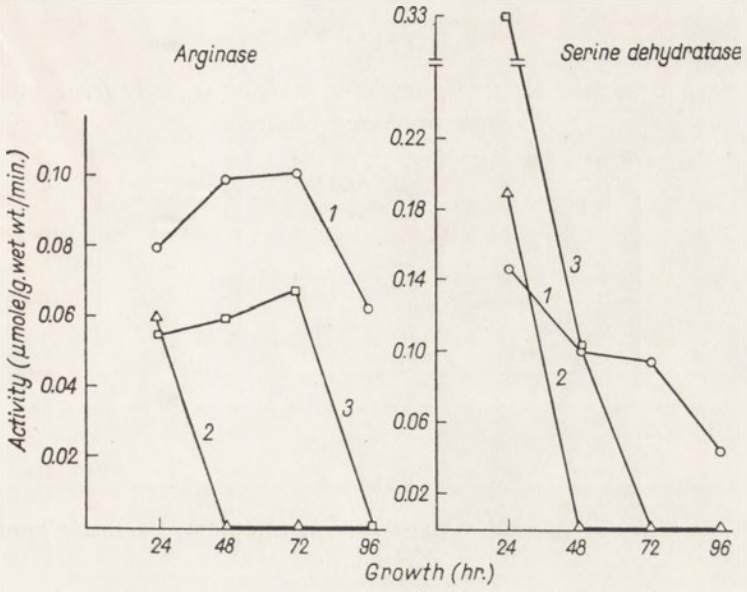

Fig. 5. Activities of arginase and serine dehydratase in mycelium grown on media containing: (*1*), ammonium sulphate; (*2*), arginine; (*3*), serine

antibiotic synthesis by 90% had no effect on serine deaminating enzyme, whereas in mycelium grown on media containing 1036 µg. P_i/ml. the activity was markedly increased in the 24-hr. cultures and dropped rapidly thereafter: in the 48-hr. mycelium it was still higher than in control cultures but no activity was observed in the 72-hr. and 96-hr. mycelium.

Arginine used as the main nitrogen source reduced the activities of the investigated enzymes by 20 - 30% in the 24-hr. cultures and since the 48th hour of growth total inhibition of both enzymes was noted (Fig. 5) despite a high viomycin yield obtained under these conditions (cf. Table 1). On serine containing medium the activity of arginase was diminished by about 30% and a substrate induction of serine dehydratase was found in the 24-hr. mycelium followed by a steep decrease of the activity in the 48-hr. and 72-hr. mycelium.

To obtain more direct evidence concerning participation of arginase in viomycin biosynthesis, a natural inhibitor of this enzyme from sunflower seeds was used. The inhibitor in concentration of 50 µg./ml. produced *in vitro* a 77% inhibition of arginase activity (Table 3) and when added to the growth medium (10 µg./ml.) it decreased this activity by 40, 80, 87% and totally in the four successive stages of mycelium growth (Fig. 6). Despite this distinct reduction of arginase activity, viomycin biosynthesis was but slightly affected.

X-Amidinotransferase. The enzyme transferring the amidino group of arginine to an unknown acceptor was found in *Streptomyces* producing

Table 3

Effect of the natural inhibitor from sunflower seeds on arginase from Streptomyces *strain producing viomycin*

Inhibitor concn. (μg./ml.)	Arginase activity (μmole/g./min.)	Inhibition (%)
None	0.086	—
10	0.042	52
50	0.020	77

Table 4

Substrate specificity of X-amidinotransferase in Streptomyces *strain producing viomycin*

The extract from 72-hr. mycelium grown on control medium was used.

Donors	Activity	
	units/mg. protein/2 hr.	%
Arginine	15.4	100
L-α-Amino-β-guanidinopropionate	11.4	74
L-α-Amino-γ-guanidinobutyrate	9.8	64
Creatine	7.7	50
Creatinine	0.0	0

Table 5

Localization of arginase, serine dehydratase and X-amidinotransferase in mycelium of Streptomyces *strain producing viomycin*

Spheroplasts and the "cell wall" fraction were isolated after treatment of mycelium with lysozyme. The activities of arginase and serine dehydratase are expressed in μmoles/mg. protein/min. and that of X-amidinotransferase in units/mg. protein/2 hr.

Enzyme	Expt. no.	Activity		
		Whole cell sonicated	"Cell wall" fraction	Spheroplasts
X-Amidinotransferase	I	0.594	0.632	0.000
	II	0.680	0.660	0.000
Arginase	I	0.115	0.047	0.075
	II	0.107	0.045	0.053
Serine dehydratase	I	0.175	0.090	0.094

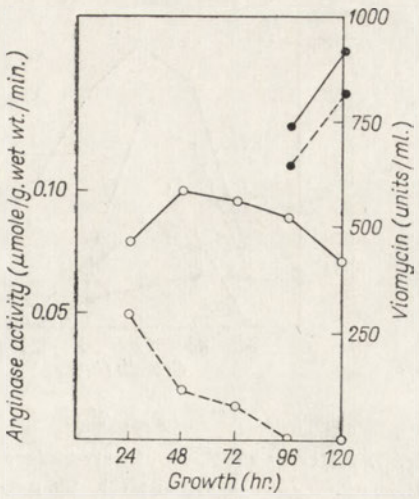

Fig. 6. Effect of the inhibitor from sunflower seeds on (○), arginase activity, and (●), viomycin biosynthesis. Full line, mycelium grown on control medium; broken line, mycelium grown on medium supplemented with the inhibitor (10 µg./ml.)

viomycin; in the assay hydroxylamine was used as non-physiological acceptor. The activity was of the same order as that in *Streptomyces griseus* producing streptomycin, i.e. about 35 units/mg. protein/2 hr. in the 48-hr. cultures, whereas no X-amidinotransferase activity was detected in *Streptomyces nursei*, *Bacillus subtilis* or *Bacillus licheniformis* synthesizing non-guanidine antibiotics.

The enzyme occurring in the strain producing viomycin was not specific for arginine: the L-α-amino-β-guanidinopropionate, L-α-amino-γ--guanidinobutyrate and creatine could be donors of the amidino group in the investigated system although 30 - 50% less effectively (Table 4). This holds especially for creatine since the reaction with this substrate was about twice as low as with arginine; L-α-amino-β-guanidinopropionate was a better donor in the transferase system than its higher analogue. Creatinine of a cyclic guanidine structure did not serve as an amidino group donor.

The time course of changes in X-amidinotransferase activity is presented in Fig. 7. In mycelium grown on control medium this activity was increased reaching the maximum in the 72-hr. cultures; in the later stages it was decreased but in the 120-hr. mycelium was still higher than in the 24-hr. mycelium.

Under conditions of viomycin biosynthesis inhibited by inorganic phosphate or oxygen deficiency in the stationary cultures (cf. Table 2) the activity of X-amidinotransferase was significantly reduced: in the 72-hr. mycelium in the stationary culture this reduction amounted to 60%; on addition of inorganic phosphate the activity was inhibited by 30% on medium containing 186 µg. P_i/ml. and in the medium with 1036 µg. P_i/ml. a 90% decrease in the activity was noted.

A very pronounced 70% lowering of the X-amidinotransferase activity was found in mycelium grown on medium with the vetch flour

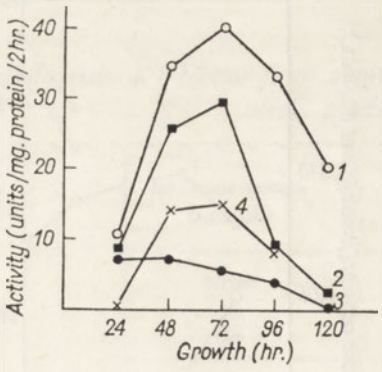

Fig. 7

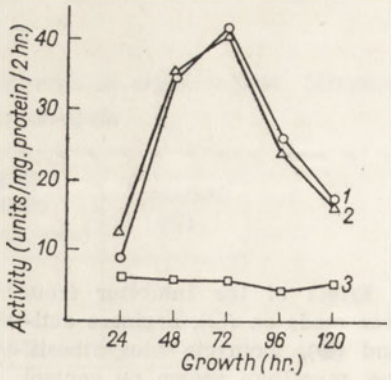

Fig. 8

Fig. 7. Activity of X-amidinotransferase in mycelium grown on media containing: (*1*), 36 µg. P_i/ml. (control); (*2*), 186 µg. P_i/ml.; (*3*), 1036 µg. P_i/ml.; (*4*), 36 µg. P_i/ml. in stationary culture

Fig. 8. Effect of arginine and canavanine on the activity of X-amidinotransferase. (*1*), Control medium; (*2*), medium with arginine added instead of ammonium sulphate; (*3*), medium with ammonium sulphate and canavanine (vetch flour extract)

extract containing canavanine; the enzyme activity did not show a characteristic maximum in the 72-hr. mycelium and remained at the same level during growth (Fig. 8). It is noteworthy that practically no differences in the X-amidinotransferase activity were found in mycelia grown on the control and arginine containing media, which is in good agreement with practically the same yield of the antibiotic obtained under these conditions.

Localization of the enzymes. As can be seen from Table 5, X-amidinotransferase was totally released upon lysozyme treatment of mycelium indicating localization of this enzyme in the cell wall or on the inner spheroplast membrane. Arginase and serine dehydratase were partially retained in spheroplasts.

DISCUSSION

The role of arginine in biosynthesis of guanidine antibiotics was investigated in connection with streptomycin biosynthesis. The isotopic studies with ^{14}C- and ^{15}N-labelled arginine (Horner, 1964; Tovarova *et. al.*, 1966) showed incorporation of the amidino group of arginine to streptidine, a diguanidine component of streptomycin. It has been proved recently that this process consists in a two-step transamidination reaction with scyllo-inozamine phosphate and *N*-amidinostreptamine phosphate acting as acceptors of the amidino group derived from arginine (Walker & Walker, 1967a). On the basis of a large-scale

screening of this enzymic activity in various micro-organisms, Walker & Hnilica (1964) and Walker & Walker (1967b) claimed that this type of transamidination reaction is limited to biogenesis of streptomycins and a closely related bluensomycin, antibiotics possessing free guanidino groups.

The results of our studies proved participation of arginine in biosynthesis of viomycin in which guanidino group is built into the antibiotic moiety in probably a cyclic ring structure (Bycroft *et al.*, 1968b; Takita & Maeda, 1968). High viomycin yield and low concentration of arginine in mycelium grown on medium containing arginine as the main nitrogen source emphasized the importance of arginine in the metabolism of *Streptomyces* strain producing viomycin. Other amino acids studied including serine, a constituent of viomycin, were distinctly less effective in antibiotic production.

The addition of canavanine to the growth medium resulted in an almost total suppression of viomycin biosynthesis and accumulation of arginine in mycelium. The increase in arginine concentration was observed also in mycelium grown under unfavourable conditions of viomycin biosynthesis on medium containing serine. As it was found previously, accumulation of arginine accompanied the reduction of antibiotic synthesis by inorganic phosphate (Paśś & Raczyńska-Bojanowska, 1968) and a steady increase in concentration of this amino acid during growth was found in the non-productive mutants (Raczyńska-Bojanowska, Tyc, Paśś & Kotula, 1969). The correlation between utilization of arginine and intensity of antibiotic synthesis did not hold for stationary cultures which indicates that under conditions of oxygen deficiency other mechanisms are responsible for the inhibition of antibiotic biosynthesis.

Participation of arginine in viomycin biosynthesis may be connected with biogenesis of both the ureido and guanidino group of the antibiotic. It may be presumed that arginase is involved in generation of the ureido group and rapid mobilization of arginine in viomycin biosynthesis. Low utilization of urea does not contradict this suggestion since urea in high concentration may inhibit urease (Varner, 1960) and consequently lower utilization of this compound as a nitrogen source. Arginase was active in the stationary phase of growth when the bulk of antibiotic was produced, and was repressed by inorganic phosphate, but it was absent from mycelium grown on arginine or on medium containing a natural arginase inhibitor, i.e. conditions of intensive viomycin synthesis. This excludes direct connection between arginase activity and antibiotic production. Similarly, the activity of another catabolizing enzyme, serine dehydratase, was not related to viomycin biosynthesis.

It is noteworthy that X-amidinotransferase of a similar type to that present in *S. griseus* was found in *Streptomyces* producing viomycin. The enzyme did not occur in *Bacillus subtilis* or *Bacillus licheniformis*, which

synthesize a non-guanidine peptide, bacitracin, and are known for their intensive arginine metabolism (De Hauwer, Lavalle & Wiame, 1964; Laishley & Bernlohr, 1968). The activity of X-amidinotransferase in the strain producing viomycin was 4-fold higher in stationary phase than in logarithmic phase of growth, and was repressed under conditions of viomycin biosynthesis inhibited by inorganic phosphate, oxygen deficiency or canavanine, but remained highly active in mycelium grown on arginine. Isolation of non-productive mutants which were depleted of this activity (Raczyńska-Bojanowska *et al.*, 1969) confirms our suggestion concerning participation of transamidination in viomycin biosynthesis. The possible action of alkaline phosphatase in this process in the strain producing viomycin (Paśś & Raczyńska-Bojanowska, 1968) should be mentioned since Walker & Walker (1967a) include co-operation of the phosphotransferase and dephosphorylating enzyme in generation of guanidino group in streptomycin. The evidence obtained implies therefore a general mechanism of biosynthesis of guanidine antibiotics.

X-Amidinotransferase was completely set free upon lysozyme treatment of mycelium. This means localization of the enzyme in the cell wall or the outer surface of the inner membrane surrounding the spheroplast (cf. Salton, 1964). Since alkaline phosphatase remained tightly bound to spheroplasts under these conditions (Paśś & Raczyńska-Bojanowska, 1968) the compartmentalization of the enzymes involved in transamidination might contribute to regulation of the antibiotic synthesis.

Our thanks are due to Dr. M. Tyc. for supplying the *Str.*strain producing viomycin and Prof. Dr. I. Reifer and Dr. Grażyna Muszyńska for preparation of the natural arginase inhibitor. The authors wish to acknowledge the skillful technical assistance of Mrs. M. Girdwoyń.

REFERENCES

Bell E. A. (1965). *In Ciba Found. Symp.: Comparative Biochemistry of Arginine and Derivatives* (J. Roche, ed.) p. 20. I. & A. Churchill Ltd. London.

Bowie J. H., Cox D. A., Johnson A. W. & Thomas G. (1964). *Tetrahedron Letters* **45**, 3305.

Breuer G. (1962). *Handbuch der Präparativen Anorganischen Chemie*, vol. 2, p. 1317. Ferdinand Enke Verlag, Stuttgard.

Bycroft B. W., Cameron D., Croft R. L., Johnson A. W. & Webb T. (1968a). *Tetrahedron Letters* **25**, 2925.

Bycroft B. W., Cameron D., Croft R. L., Hassanali-Walji A., Johnson A. W. & Webb T. (1968b). *Tetrahedron Letters* **56**, 5901.

Chinard F. P. (1952). *J. Biol. Chem.* **199**, 91.

Freedland R. A. & Avery E. A. (1964). *J. Biol. Chem.* **239**, 3357.

Greenberg D. M. (1955). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 2, p. 368. Academic Press, New York.
De Hauwer G., Lavalle R. & Wiame J. M. (1964). *Biochim. Biophys. Acta* **81**, 257.
Horner V. H. (1964). *J. Biol. Chem.* **239**, 2256.
Kitagawa T., Sawada Y., Miura T., Ozasa T. & Tanyiama T. (1968). *Tetrahedron Letters* **1**, 109.
Laishley E. J. & Bernlohr R. W. (1968). *J. Bacteriol.* **96**, 322.
Layne E. (1955). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 3, p. 448. Academic Press, New York.
Lechowski L. (1969). *Tetrahedron Letters* **6**, 479.
Morawska-Muszyńska G. & Reifer I. (1965). *Acta Biochim. Polon.* **12**, 187.
Paśś L. & Raczyńska-Bojanowska K. (1968). *Acta Biochim. Polon.* **15**, 355.
Raczyńska-Bojanowska K., Tyc M., Paśś L. & Kotula Z. (1969). *Bull. Acad. Polon. Sci. Ser. Sci. Biol.* **17**, 203.
Salton M. R. J. (1964). *The Bacterial Cell Wall.* Elsevier Publ. Co., Amsterdam.
Schimke R. T. (1964). *J. Biol. Chem.* **239**, 3808.
Szylagyi I., Valyi-Nagy T., Szabo I. & Keresztes T. (1964). *Nature* **201**, 81.
Takita Y. & Maeda K. (1968). *J. Antibiotics* **8**, 512.
Tovarova I. I., Kornitskaya E. Y., Pliner S. A., Puchkov V. A., Wulfson N. S. & Khokhlov A. S. (1966). *Izw. Akad. Nauk SSSR ser. biol.* **31**, 911.
Varner J. E. (1960). In *The Enzymes* (P. D. Boyer, H. Lardy & K. Myrbäck, eds.) vol. IV, p. 247. Academic Press, New York, London.
Walker J. B. (1958). *J. Biol. Chem.* **231**, 1.
Walker J. B. & Hnilica V. S. (1964). *Biochim. Biophys. Acta* **89**, 473.
Walker J. B. & Walker M. S. (1967a). *Biochem. Biophys. Res. Commun.* **26**, 278.
Walker M. S. & Walker J. B. (1967b). *Biochim. Biophys. Acta* **136**, 272.

ROLA ARGININY W SYNTEZIE WIOMYCYNY

Streszczenie

1. Arginina w przeciwieństwie do cytruliny, ornityny, mocznika, lizyny, leucyny, glicyny i seryny jest wykorzystywana w równym prawie stopniu jak siarczan amonu w biosyntezie wiomycyny.

2. Stwierdzono nagromadzanie argininy w grzybni w warunkach zahamowanej biosyntezy antybiotyku; wyjątek stanowią hodowle stacjonarne.

3. Aktywność arginazy i dehydratazy serynowej (dezaminującej) nie jest związana z procesem syntezy antybiotyku ani ze stężeniem metabolizowanych aminokwasów.

4. W grzybni *Streptomyces* sp. wytwarzającego wiomycynę stwierdzono występowanie nieswoistej X-amidynotransferazy, enzymu przenoszącego grupę amidynową na nieznany akceptor; czynność tego enzymu jest hamowana przez nieorganiczny fosforan, niedobór tlenu i kanawaninę — czynniki hamujące jednocześnie syntezę antybiotyku.

5. Omówiono udział transamidynacji w ogólnym mechanizmie syntezy ugrupowania guanidynowego w antybiotykach.

Received 12 March, 1969.