Large Plasmids in Different *Rhizobium* Species

By M. P. NUTI,* A. M. LEDEBOER, A. A. LEPIDI* AND
R. A. SCHILPEROORT

*Department of Biochemistry, State University of Leiden, Wassenaarseweg 64,
Leiden, The Netherlands*

(Received 11 October 1976; revised 3 December 1976)

SUMMARY

Large plasmids were detected in *Rhizobium* species by sedimentation analysis of lysates on alkaline sucrose gradients and by dye buoyant density centrifugation. Mitomycin C treatment did not increase the amount of plasmid DNA in the bacteria. Renaturation kinetics were used to confirm that these large DNA molecules had the molecular complexity of plasmids and to estimate their molecular weights. These ranged from 0.7×10^8 to 4.0×10^8 daltons. The maximum yield of isolated plasmid DNA relative to chromosomal DNA was 3.8 %.

INTRODUCTION

The Gram negative bacterium *Rhizobium* can form root nodules in a complex symbiosis with legumes (Dixon & Cannon, 1975; Dilworth, 1969). The triggers for the initiation of this symbiosis are still unknown (Libbenga & Bogers, 1974; Brill, 1975). Possibly plasmid genes are involved in its establishment since rhizobia treated with acridine dyes or chemicals known to eliminate extrachromosomal DNA lose their ability to form nodules (Higashi, 1967; Dunican & Cannon, 1971; Zurkowski, Hoffman & Lorkiewicz, 1973). The isolation of plasmid DNA from rhizobia by dye buoyant density centrifugation of 'cleared' lysates has recently been reported (Tshitenge *et al.*, 1975; Luyindula *et al.*, 1975; Dunican, O'Gara & Tierney, 1975); but only Tshitenge *et al.* (1975) gave an estimation of its molecular weight (about 28×10^6 daltons). No findings correlating nodule formation with the presence of plasmid have yet been published. It has been suggested that nitrogen fixation genes might be plasmid borne in *Rhizobium* (Dunican & Tierney, 1974), although no additional evidence to support this idea has been reported.

In this paper, we report conditions suitable for the reproducible recovery of large plasmids from *Rhizobium* species, which are not detected when using 'cleared' lysates. Estimates of the molecular weight of the plasmids based on renaturation kinetics data are presented and the possible significance of such large extrachromosomal DNA in *Rhizobium* is discussed.

METHODS

Bacterial strains and their symbiotic or biological properties are listed in Table 1.

Cultivation of bacteria. The strains of *Rhizobium* were maintained by weekly subculturing on slants of yeast/mannitol/agar (YMA) containing (g l⁻¹): K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.2; NaCl, 0.1; yeast extract (Difco), 0.4; mannitol, 10.0; and agar (Difco), 15.0; the pH was adjusted to 6.9 before sterilization.

* Present address: Istituto di Microbiologia Agraria, Università di Pisa, Via del Borghetto 80, Pisa, Italy.

Table 1. *Symbiotic and biological properties of bacterial strains*

Species	Strain no.	Source	Symbiotic properties*		
			Inf	Eff	Test plant
<i>Rhizobium leguminosarum</i>	1001	Rothamsted (Harpender)	+	+	<i>Vicia hirsuta</i>
<i>R. leguminosarum</i>	1016	Rothamsted (Harpender)	+	+	<i>V. faba</i>
			—	—	<i>Vicia hirsuta</i>
			—	—	<i>V. faba</i>
<i>R. trifolii</i>	5	Rothamsted (Harpender)	+	+	<i>Trifolium pratense</i>
			+	—	<i>T. parviflorum</i>
<i>R. trifolii</i>	0402	Rothamsted (Harpender)	—	—	<i>T. pratense</i>
<i>R. japonicum</i>	3407	Rothamsted (Harpender)	+	+	<i>Glycine max</i>
<i>R. 'dolichos'</i>	RDI	Nodules of <i>Dolichos lab lab</i> (Agoi, Somaliland)	+	+	<i>Dolichos lab lab</i>
<i>Agrobacterium tumefaciens</i>	LBA601	Dept of Biochemistry, Leiden, Holland	Tumour-forming on dicotyledonous plants (Hooykaas <i>et al.</i> , 1977)		<i>Kalanchoë daigremontiana</i> , <i>Nicotiana tabacum</i> , <i>Helianthus annuus</i>

* Inf, infectiveness, i.e. ability to form nodules; Eff, effectiveness, i.e. ability to fix nitrogen.

Liquid cultures were grown in yeast/mannitol/broth (YMB) which had the same composition as YMA without agar. Unless otherwise indicated, 25 ml cultures were inoculated from slants and shaken at 29 °C on a rotary shaker at 250 rev. min⁻¹. All *Rhizobium* strains used for plasmid DNA analysis were able to nodulate the appropriate host.

Agrobacterium tumefaciens strain LBA601 was cultured as described by Ledebøer *et al.* (1976) and its purity was checked as described by Hooykaas *et al.* (1977).

Nodulation tests. Enclosed cultures on seedling agar were carried out according to Vincent (1970). Each *R. trifolii* strain was tested on 12 plants of *Trifolium pratense* cv. 5123 and *T. parviflorum* (kindly provided by Dr P. S. Nutman), each *R. leguminosarum* strain on eight *Vicia faba* and *V. hirsuta*, and the *R. 'dolichos'* strain on eight *Dolichos lab lab*. Acetylene reduction by intact plants was measured in air containing 10 % (v/v) acetylene (Planqué & van Brussel, 1976); effectiveness for *Dolichos* nodules was assumed on the basis of their inner pigmentation. Gas chromatography was performed as described by Citeresi *et al.* (1976).

Mitomycin C treatment. Mitomycin C was added to a final concentration of 1 µg ml⁻¹ to mid-exponential phase cultures in YMB at a density of about 2 × 10⁸ bacteria ml⁻¹. Bacteria were sedimented after 90 s as described by Pariiskaya (1973).

Labelling procedure. Rhizobia were grown in YMB to a density of 1 × 10⁸ bacteria ml⁻¹. To 0.9 ml of this bacterial culture, 0.1 mCi [³H]thymidine (30 Ci mm⁻¹; The Radiochemical Centre, Amersham) was then added slowly and continuously by means of a peristaltic pump during a growth period of 3 to 4 h, depending on the strain used. Bacteria were grown to a density of 4 × 10⁸ to 6 × 10⁸ bacteria ml⁻¹. Deoxyadenosine was not added since it did not improve labelling efficiency. After a further 30 min growth without addition of [³H]thymidine, bacteria were harvested by centrifugation at 12000 g for 20 min and plasmid DNA was isolated. *Agrobacterium tumefaciens* was labelled as described by Ledebøer *et al.* (1976).

Plasmid isolation and purification. (i) Lysate preparation. *Rhizobium* cells were lysed as described by Ledebøer *et al.* (1976) with the following modifications: cells were resuspended

in 0.45 vol. sterile TES buffer (containing 0.01 M-Tris pH 8.0, 0.05 M-NaCl and 0.05 M-EDTA) and added to 0.45 vol. lysozyme (400 $\mu\text{g ml}^{-1}$; eggwhite, grade I, Sigma) in TES buffer saturated with n-dodecylamine. After 30 min incubation on ice without stirring, 0.1 vol. 10 % (w/v) Sarkosyl NL97 (Ciba Geigy, Switzerland) in TES was added, followed by 0.1 vol. 3 M-NaOH plus 1 M-NaCl in TES. This procedure always gave clear lysates within a few minutes. In some experiments, a 3 to 7 min incubation at 37 °C with pronase (250 $\mu\text{g ml}^{-1}$; B grade, Calbiochem), autodigested for 1.5 h at 37 °C in prewarmed TES buffer, was used before addition of Sarkosyl. Alternatively, the procedure reported by Zaenen *et al.* (1974) was used, which gave almost clear lysates.

(ii) Alkaline sucrose gradients. The lysate [0.2 ml prepared by the Zaenen *et al.* (1974) procedure or 1 to 3 ml by our procedure] was sheared for 1.5 min in a test tube (1.6 cm diam.) using a Vortex mixer at maximum speed (Vortex Genie, Wilten, Holland). Sheared lysate (0.1 to 1 ml) was layered on a 5 to 20 % linear sucrose gradient containing 1 M-NaCl and 0.3 M-NaOH in TES buffer. Autoclaved sucrose stock solutions were used. Gradients were centrifuged, unless stated otherwise, for 18 min at 4 °C and 37 000 rev. min⁻¹ using a SW41 rotor in a Beckman Spinco LIIB preparative ultracentrifuge. Alternatively, 3 ml lysate was layered on top of a 36 ml 5 to 20 % alkaline sucrose gradient and centrifuged for 55 min at 4 °C and 25 000 rev. min⁻¹ in a SW27 rotor. The gradients were fractionated, precipitated and radioactivity was measured as described by Ledebøer *et al.* (1976).

(iii) Dye buoyant density equilibrium centrifugation. Using neutral lysates, propidium di-iodide (A grade, Calbiochem) buoyant density centrifugation was performed as described by Ledebøer *et al.* (1976).

(iv) Alkaline large scale isolation. A combination of the alkaline large-scale isolation procedure adopted by Ledebøer *et al.* (1976) and of the alkaline lysis procedure of Zaenen *et al.* (1974) was chosen. Cultures (250 ml) were grown to a density of 5×10^8 bacteria ml⁻¹ under the same conditions as described for 25 ml samples. The bacteria were harvested and washed with the same volume of washing mixture, then resuspended carefully in 115 ml lysis buffer (Zaenen *et al.*, 1974); 35 ml lysing mixture (containing 0.8 M-NaOH and 5 % Sarkosyl) were added in 3 min with slow stirring. After shearing for 1 min with a Vibromixer (A. G. für Chemie Apparatenbau, Switzerland), the lysate was processed as described by Ledebøer *et al.* (1976). Alkaline sucrose gradients (10 to 20 %) containing 1 M-NaCl were run for 70 min at 25 000 rev. min⁻¹ in a SW27 rotor at 4 °C.

DNA renaturation kinetics. DNA renaturation kinetics and DNA concentrations were determined as described by Ledebøer *et al.* (1976).

RESULTS

Lysate preparation and plasmid isolation

The carbenicillin lysis procedure developed by Ledebøer *et al.* (1976), for agrobacteria was not suitable for the recovery of large plasmids from the *Rhizobium* species. Furthermore lysozyme/EDTA incubation followed by neutral Sarkosyl lysis was only suitable for isolating plasmid from *R. trifolii*. However, the procedure described in Methods resulted in clear lysates for all the strains studied. From these lysates, large plasmids could be isolated reproducibly in alkaline sucrose gradients, both in small (Fig. 1) and large scale experiments. We also studied whether the use of pronase B or increased concentrations of lysozyme before addition of alkali was beneficial, but the isolation of large plasmids on alkaline sucrose gradients from such lysates was often unsuccessful.

Usually plasmids could only be isolated when alkaline conditions for lysis were used,

Fig. 1. The isolation of *Rhizobium* plasmids on alkaline sucrose gradients: (a) 17 min run of sheared lysates from *R. trifolii* strain 5 (○) and *R. leguminosarum* strain 1001 (●); (b) 20 min run of sheared lysates of *A. tumefaciens* LBA601 (○), resulting in separation of the TI plasmid and RP4, and of *R. leguminosarum* strain 1016 (●).

Fig. 2. CsCl /propidium di-iodide buoyant density profile of a neutral lysate of *R. trifolii* strain 5: ●, ^3H radioactivity; ▲, density.

though that from *R. trifolii* strain 5 could be isolated from neutral lysates by propidium di-iodide buoyant density centrifugation (Fig. 2).

A 250 ml culture of *R. leguminosarum* strain 1001 at a concentration of 5×10^8 to 6×10^8 bacteria ml^{-1} yielded 9 μg plasmid DNA. Assuming one plasmid copy per cell and taking into account the molecular weights determined by renaturation kinetics, the recovery is

about 25 % of the theoretical value. By renaturation kinetics the purity of the plasmid preparations was estimated to be over 90 % (Ledeboer *et al.*, 1976).

Plasmid characterization

The sedimentation profiles of clear lysates of rhizobia in alkaline sucrose gradients are shown in Fig. 1. *Agrobacterium tumefaciens* strain LBA601, lysed as described by Ledeboer *et al.* (1976), was used as an internal standard because it carries both the large (1.1×10^8 daltons) TI plasmid (Zaenen *et al.*, 1974) and RP4 which has a molecular weight of 40×10^6 daltons (Datta *et al.*, 1971; O'Gara & Dunican, 1973). The large *Rhizobium* plasmids were invariably lost when alkaline sucrose gradients were run for longer than 22 min using a SW41 rotor in a Beckman Spinco LIIB preparative ultracentrifuge. For most strains good separation of plasmid and chromosomal DNA occurred in runs of 17 to 20 min.

The nature of the peaks in the alkaline sucrose gradients was established by determination of the molecular complexities using renaturation kinetics (Ledeboer *et al.*, 1976). As standards, the molecular weights of the *A. tumefaciens* TI plasmid and chromosomal DNA were used [1.1×10^8 and 3.7×10^9 daltons respectively (Zaenen *et al.*, 1974)]. The satellite peaks in alkaline sucrose gradients appear to represent plasmid DNA (Fig. 3) and their molecular complexity was 10 to 25 times lower than that of the chromosomal DNA of *A. tumefaciens* strain LBA601.

The molecular weights of the *Rhizobium* plasmids studied were generally higher than that of the *Agrobacterium* TI plasmid (Table 2). The plasmid from *R. trifolii* strain 5 had the highest molecular weight, but although it also had the highest sedimentation value, the latter was not consistent with the high molecular complexity.

To determine whether the number of plasmid copies per cell could be increased, bacteria were treated with mitomycin C. No effect on copy number was observed.

DISCUSSION

Other workers (Tshitenge *et al.*, 1975; Luyindula *et al.*, 1975; Dunican *et al.*, 1974) using 'cleared' lysates were unable to demonstrate the presence of large plasmids in *Rhizobium* species. This was probably because the initial step in their preparation involved the removal of chromosomal DNA as part of a membrane complex and so large plasmids which are tightly associated with the membrane or the chromosomal DNA, such as the *Agrobacterium* TI plasmid (Ledeboer *et al.*, 1976), are lost. This phenomenon has been encountered with other plasmids (Kline & Miller, 1975; Palchaudhuri & Chakrabarty, 1976). Neutral lysates were always less clear than the alkaline ones and, except for *R. trifolii* strain 5, it was not possible to isolate the large *Rhizobium* plasmids using neutral conditions. To make the neutral lysates clearer we used *Rhizobium* grown in the presence of carbenicillin, as described for *Agrobacterium* (Ledeboer *et al.*, 1976). Probably due to the much lower growth rate of rhizobia, carbenicillin did not sufficiently alter wall structure to promote lysis by lysozyme.

The presence of a very large plasmid in *R. trifolii* strain 5 is suggested by the renaturation kinetics data. The sedimentation profile (Fig. 1*a*) shows that this plasmid is somewhat larger than that from *R. leguminosarum* 1001, but its molecular weight is different when calculated from renaturation data. It is possible that two different plasmids of comparable molecular weight sediment at the same position. This would be consistent with the very high molecular complexity of the *R. trifolii* strain 5 plasmid. A similar discrepancy caused by the presence of two different plasmids has recently been observed for *Agrobacterium* strain LBA2 (A. M. Ledeboer, unpublished data).

Fig. 3. C_0t curves of plasmid and chromosomal DNA isolated from *R. trifolii* and *A. tumefaciens*: plasmid (●) and chromosomal (○) DNA from *A. tumefaciens* LBA601; plasmid (▲) and chromosomal (△) DNA from *R. trifolii* strain 5; plasmid (■) and chromosomal (□) DNA from *R. trifolii* strain 0402. Curves have been drawn on the basis of the theoretical sigmoid curves as described by Ledebøer *et al.* (1976).

Table 2. *Characterization of large plasmids in different Rhizobium species*

Species	Strain no.	Symbiotic properties*	C_0t_i	Calculated mol. wt (daltons)	Yield (%)†
<i>R. leguminosarum</i>	1001	Inf ⁺ Eff ⁺	0.070	1.3×10^8 (3 expts)	1.6 (max. 2.4)
<i>R. leguminosarum</i>	1016	Inf ⁻	0.040	$0.7-1.0 \times 10^8$ (3 expts)	2.8 (max. 3.2)
<i>R. trifolii</i>	5	Inf ⁺ Eff ⁺	0.200	$3.5-4.0 \times 10^8$ (5 expts)	2.4 (max. 3.8)
<i>R. trifolii</i>	0402	Inf ⁻	0.080	1.4×10^8 (3 expts)	1.4 (max. 2.0)
<i>R. japonicum</i>	3407	Inf ⁺ Eff ⁺	—	—	1.3 (max. 1.8)
<i>R. 'dolichos'</i>	RDI	Inf ⁺ Eff ⁺	0.090	1.6×10^8 (1 expt)	2.7 (max. 2.7)
<i>A. tumefaciens</i>	LBA601	Tumour-forming	0.065	1.1×10^8 (6 expts)	0.91 (max. 1.3)

* See Table 1.

† The ratio of plasmid to chromosomal DNA has been calculated according to Zaenen *et al.* (1974).

The four nodule-forming effective strains of *Rhizobium* harboured large plasmids having molecular weights in the range of 1.3×10^8 to 4.0×10^8 daltons. The two non-infective strains carried large plasmids with lower molecular weights. These were not genetically-marked isogenic strains and so it is not possible to draw any conclusion from these data about the involvement of plasmid genes in nodulation. However, a large plasmid controls the interaction between *A. tumefaciens* and the plant cell in crown gall formation (van Larebeke *et al.*, 1974, 1975). It is possible that something similar occurs in nodule formation

by *Rhizobium* as agrobacteria and rhizobia are taxonomically closely related (Gibbins & Gregory, 1972; White, 1972). Nodulating effective rhizobia, able to induce tumours, have been produced by transferring the TI plasmid from *Agrobacterium* (Hooykaas *et al.*, 1977). Therefore, it seems worthwhile to investigate whether the large plasmids of *Rhizobium* are involved in the establishment of the association between plant and bacterium.

The authors gratefully acknowledge the technical assistance of Mrs A. den Dulk-Ras and Mr G. Ooms. Professor P. S. Nutman generously provided the *Rhizobium* strains used in this study. The authors thank Dr J. E. Beringer for his critical reading of the manuscript. This investigation was partly supported by E.E.C. grant 0441. M.P.N. was on leave from the University of Pisa with a NATO-CNR fellowship.

REFERENCES

- BRILL, W. J. (1975). Regulation and genetics of bacterial nitrogen fixation. *Annual Review of Microbiology* **29**, 109-129.
- CITERNESI, U., NEGLIA, R., SERITTI, A., LEPIDI, A. A., FILIPPI, C., BAGNOLI, G. & NUTI, M. P. (1976). Nitrogen fixing bacteria in the gastroenteric cavity of soil animals. *Soil Biology and Biochemistry* (in the Press).
- DATTA, N., HEDGES, R. W., SHAW, E. J., SYKES, R. B. & RICHMOND, M. (1971). Properties of an R factor from *Pseudomonas aeruginosa*. *Journal of Bacteriology* **108**, 1244-1249.
- DILWORTH, M. J. (1969). The plant as the genetic determinant of leghaemoglobin production in the legume root nodule. *Biochimica et biophysica acta* **184**, 432-441.
- DIXON, R. A. & CANNON, F. C. (1975). Recent advances in the genetics of nitrogen fixation. In *Symbiotic Nitrogen Fixation in Plants*, pp. 3-24. Edited by P. S. Nutman. Cambridge: Cambridge University Press.
- DUNICAN, L. K. & CANNON, F. C. (1971). The genetic control of symbiotic properties in *Rhizobium*: evidence for plasmid control. *Plant and Soil*, special volume 73-79.
- DUNICAN, L. K. & TIERNEY, A. B. (1974). Genetic transfer of nitrogen fixation from *R. trifolii* to *K. aerogenes*. *Biochemical and Biophysical Research Communications* **57**, 62-72.
- DUNICAN, L. K., O'GARA, F. & TIERNEY, A. B. (1975). Plasmid control of effectiveness in *Rhizobium*: transfer of nitrogen-fixing genes on a plasmid from *Rhizobium trifolii* to *Klebsiella aerogenes*. In *Symbiotic Nitrogen Fixation in Plants*, pp. 77-90. Edited by P. S. Nutman. Cambridge: Cambridge University Press.
- GIBBINS, A. M. & GREGORY, K. F. (1972). Relatedness among *Rhizobium* and *Agrobacterium* species determined by three methods of nucleic acid hybridization. *Journal of Bacteriology* **111**, 129-141.
- HIGASHI, S. (1967). Transfer of clover infectivity of *R. trifolii* to *R. phaseoli* as mediated by an episomic factor. *Journal of General and Applied Microbiology* **13**, 391-403.
- HOOPYKAAS, P. J. J., KLAPWIJK, P. M., NUTI, M. P., SCHILPEROORT, R. A. & RÖRSCH, A. (1977). Transfer of the *Agrobacterium tumefaciens* TI plasmid to avirulent *Agrobacteria* and to *Rhizobium ex planta*. *Journal of General Microbiology* **98**, 477-484.
- KLINE, B. C. & MILLER, J. R. (1975). Detection of nonintegrated plasmid DNA in the folded chromosome of *E. coli*. *Journal of Bacteriology* **121**, 165-172.
- LAREBEKE, N. VAN, ENGLER, G., HOLSTERS, M., VAN DEN ELSACKER, S., ZAENEN, I., SCHILPEROORT, R. A. & SCHELL, J. (1974). Large plasmid in *Agrobacterium tumefaciens* essential for crown gall inducing ability. *Nature, London* **252**, 169-170.
- LAREBEKE, N. VAN, GENETELLO, CH., SCHELL, J., SCHILPEROORT, R. A., HERMANS, A. K., HERNALSTEENS, J. P. & VAN MONTAGU, M. (1975). Acquisition of tumour inducing ability by nononcogenic *Agrobacteria* as a result of plasmid transfer. *Nature, London* **255**, 742-743.
- LEDEBOER, A. M., KROL, A. J. M., DONS, J. J. M., SPIER, F., SCHILPEROORT, R. A., ZAENEN, I., VAN LAREBEKE, N. & SCHELL, J. (1976). On the isolation of TI plasmid from *A. tumefaciens*. *Nucleic Acids Research* **3**, 449-463.
- LIBBENGA, K. R. & BOGERS, R. J. (1974). Root-nodule morphogenesis. In *The Biology of Nitrogen Fixation*, pp. 430-472. Edited by A. Quispel. Amsterdam: North Holland Publishing Company.
- LUYINDULA, N., TSHITENGE, G., LURQUIN, P. & LEDOUX, L. (1975). Étude des plasmides de *Rhizobium japonicum*. *Archives internationales de physiologie et de biochimie* **83**, 199-200.
- O'GARA, F. & DUNICAN, L. K. (1973). Transformation and physical properties of R factor RP4 transferred from *Escherichia coli* to *Rhizobium trifolii*. *Journal of Bacteriology* **116**, 1177-1180.
- PALCHAUDHURI, S. & CHAKRABARTY, A. (1976). Isolation of plasmid DNA from *Pseudomonas putida*. *Journal of Bacteriology* **126**, 410-416.

- PARIISKAYA, A. N. (1973). The effect of acridine orange and mitomycin C on the symbiotic properties of *R. meliloti*. *Mikrobiologiya* **42**, 119–121.
- PLANQUÉ, K. & VAN BRUSSEL, A. A. N. (1976). An improved large scale isolation procedure for bacteroids of *R. leguminosarum* Frank from *Pisum sativum*. *Plant and Soil* **45**, 309–315.
- TSHITENGE, G., LUYINDULA, N., LURQUIN, P. F. & LEDOUX, L. (1975). Plasmid DNA in *R. vigna* and *R. trifolii*. *Biochimica et biophysica acta* **414**, 357–361.
- VINCENT, J. M. (1970). *A Manual for the Practical Study of Root-nodule Bacteria*, pp. 75–82. Oxford: Blackwell Scientific Publications.
- WHITE, L. O. (1972). The taxonomy of the crown gall organism *Agrobacterium tumefaciens* and its relationship to Rhizobia and other Agrobacteria. *Journal of General Microbiology* **72**, 565–574.
- ZAENEN, I., VAN LAREBEKE, N., TEUCHY, H., VAN MONTAGU, M. & SCHELL, J. (1974). Supercoiled circular DNA in crown gall inducing *Agrobacterium* strains. *Journal of Molecular Biology* **86**, 109–127.
- ZURKOWSKI, W., HOFFMAN, M. & LORKIEWICZ, Z. (1973). Effect of acriflavine and sodium dodecyl sulphate on infectiveness of *R. trifolii*. *Acta microbiologia polonica, Series A* **5**, 55–60.