Proc. Indian Acad. Sci. (Plant Sci.), Vol. 94, Nos 4-6, June 1985, pp. 575-582

Immunogenic analysis of extra-cellular polysaccharide of rhizobia collected from South Indian soils

R KASTURI BAI* and P N RAJU

Centre for Advanced Studies in Botany, University of Madras, Madras 600 005, India

* Present address: School of Energy, Environment and Natural Resources, Madurai Kamaraj University, Madurai 625 021, India

MS received 23 November 1983; revised 26 March 1985

Abstract. Immunogenic analyses were carried out for the rhizobial isolates collected from South Indian soils using the extracellular polysaccharide as the antigen. They are immunogenic eventhough their immunogenic nature was not established unequivocally in the previous studies. There was wide variation in their monosaccharide composition which could not be correlated with serological reactions. IR studies of these exopolysaccharides reveal that there are two major functional groups, amide and carboxyl in most of the isolates. The immunodiffusion analysis revealed that there are four distinct bands common to all the isolates. The antisera of the isolates D_2, D_9, V_2, V_3, Sa_1 and P_1 showed an additional hazy line.

Keywords. Rhizobial isolates; monosaccharide composition; infrared analysis; immunogenicity; extra-cellular polysaccharides.

1. Introduction

The capsular and extra-cellular polysaccharides constitute important immunological determinants in bacteria (Wilkinson 1958; Liu 1961; Sutton and Williams 1970). The noteworthy discovery of Heidelberger and Avery (1962) that carbo-hydrates are indeed of no less significance than proteins for the immunological specificity of bacteria and of bacterial types indicates that the existence of a great number of specific polysaccharides is after all as intelligible as the enormous multiplicity of proteins. The antigenic sites in serological reactions depend upon the chemical composition and properties of the component sugars mainly aminosugars and 2-acetamido-2-deoxy D-Glucose (Wilkinson 1958; Wagner *et al* 1973).

There is no unequivocal evidence for the immunogenicity of purified exopolysaccharides of rhizobia (Vincent 1977). Humphrey and Vincent (1959, 1965) on the basis of their studies on the exopolysaccharide of *R. trifolii* concluded that it is not antigenic. However, Dudman's (1964) studies on the extra-cellular soluble antigens of some of the strains of *R. melitoti* have proved the antigenicity of the extra-cellular polysaccharide fraction. There is practically little mention in literature on serological studies on rhizobia using the extra-cellular polysaccharide as the immunogen. On the basis of the IR spectral studies of the important functional groups of carbohydrates, Gouldon and Sharpe (1958) have reported serological and physiological differences among the species of the genus *Lactobacillus*.

The analysis of the monosaccharide pattern of extracellular polysaccharides of the rhizobial isolates under study indicates there is a wide variation. The present investigation was undertaken to prove the immunogenicity of the extra-cellular

polysaccharides, to see whether any correlation if at all, exists between the monomers, functional groups and the specificity of serological reactions.

2. Material and Methods

The history of the collection of the rhizobial isolates and authentication against standard hosts are given in table 1. The special synthetic medium of Zevenhuizen (1971) was used for the exopolysaccharide production of these isolates. Isolation and purification of the extra-cellular polysaccharides was done following the method of Bailey *et al* (1971) and deproteinized several times by the method of Sevag *et al* (1938) to exclude the protein completely from the polysaccharide.

2.1 *Chromatographic analysis of exopolysaccharides for their monomers*

Known quantity of the polysaccharide was dried, hydrolysed at 121°C for 1 hr with 6N HCl and then evaporated to dryness *in vacuo*. The residue was dissolved in water and was desalted (Block *et al* 1963) using anionic exchange resin (Amberlate IRA-410) and a cationic exchange resin (Amberlate IRC-50) and the clear supernatant was used for chromatographic analysis which was carried out according to the method of Zweig and Whitaker (1971) and Hepper (1972). Unidimensional chromatograms were run with ethylacetate: pyridine: water (12:5:4 v/v) as the irrigating solvents and sprayed with aniline pthalate reagent for producing a colour. The spots were identified with co-chromatograms of the respective sugars with standards. Quantitative estimation of sugars was carried out by eluting the coloured spots with 50% acetone and read at 490 nm with the respective sugars as the standard.

2.2 *Sample preparation for* IR *analysis*

About 2 mg calculated as glucose equivalents of polysaccharides was dried for 1 hr and powdered. It was then made into a fine thin film with potassium bromide (IR grade) by grinding them together. The spectra were taken in a Beckman IR-20 IR spectrophotometer.

Table 1. Details of the rhizobial isolates selected for study.

Isolate name and original host	Place of collection	Test host showing best nodulation
D_1 *Dolichos lab-lab*, Linn.	Kanjangad	*Vigna sinensis*, (L) Savi
D_2 *Dolichos lab-lab*, Linn.	Bijapur	*Dolichos lab-lab*, Linn.
D_9 *Dolichos lab-lab*, Linn.	Coimbatore	*Dolichos lab-lab*, Linn.
A_1 *Arachis hypogaea*, Willd.	Cuddalore	*Vigna sinensis*, (L) Savi
A_2 *Arachis hypogaea*, Willd.	Ponneri	*Vigna sinensis*, (L) Savi
V_2 *Vigna sinensis*, (L) Savi	Mettupalayam	*Vigna sinensis*, (L) Savi
V_3 *Vigna sinensis*, (L) Savi	Salem	*Vigna sinensis*, (L) Savi
Sa_1 *Sesbania aegyptiaca*, Pers.	Maduravoyal	*Vigna sinensis*, (L) Savi
Sa_2 *Sesbania aegyptiaca*, Pers.	Tirunelveli	*Vigna sinensis*, (L) Savi
Cg_1 *Canavalia gladiata*, DC	Shenkottai	*Vigna sinensis*, (L) Savi
P_1 *Phsaeolus trilobus*, Air.	Chepauk (Univ. Campus)	*Phaseolus mungo*, Linn

2.3 *Immunization schedule*

Before starting the injection schedule, the test animals were bled and the serum was tested with the exopolysaccharide antigens of the rhizobial isolates and found to be free of antibodies. Known quantity of the purified exopolysaccharide was dried, ground well in a mortar and dissolved in 2 ml of physiological saline to get a homogeneous solution to the final concentration of 2 mg/ml. It was mixed with an equal volume of incomplete adjuvant Arlacil, and the mixture was made into an emulsion and injected into the rabbits intramuscularly at 5-day intervals up to the 40th day when a maximum titre could be got. The schedule was started with 5 mg of exopolysaccharide, gradually increased to 7·5 mg and 10 mg in the subsequent injections and in total, 72·5 mg of exopolysaccharide was injected for each isolate. For control, the animals were given only the physiological saline and the adjuvant. The animals were bled for antisera 5 days after the last injection though the marginal earveins and the antisera were stored at $-10^{\circ}C$.

2.3a *Immunodiffusion analysis on agar gel:* Agar gel was prepared using 0·9 % of Agar-Agar serva in 0·01 M sodium phosphate buffer, pH 7·0 and 0·85 % of sodium chloride. Sodium azide was aded as a preservative and the immunodiffusion studies were carried out in transparent plastic petri plates. Each antiserum was tested against its homologous polysaccharide antigen and also against antigen A and B simultaneously. The lower three walls all contain the same antiserum of the respective isolate (figure 3).

2.3b *Antigen A*: Rhizobial cells collected from 72 hr cultures were washed with sodium phosphate buffer repeatedly to remove the gum and flagella. They were machanically disrupted in a mortar in 0·85 % physiological saline. This suspension with appropriate dilution was used as antigen A and prepared for all the isolates of the present study.

2.3c *Antigen B*: Cells collected from 72 hr old liquid cultures were washed thoroughly with the buffer to remove the gum and the flagella and then ground well with a acid washed sand and the physiological saline. The suspension was centrifuged at 5000 g for 20 min and the supernatant was used as antigen B and prepared for all the isolates of the present study.

3. Results

3.1 *Monomers of extra-cellular polysaccharides*

There is qualitative and quantitative variation in the monosaccharide pattern of the extra-cellular polysaccharides. In general, on acid hydrolysis, galactose and glucose are the predominant sugars in all the rhizobial isolates occurring in varying concentrations (figure 1, table 2) except the isolate Sa_2. Only three isolates had uronic acids in their polysaccharides. In general, the concentration of glucose was higher than that of galactose in all the isolates (table 2). Methyl pentose was present in five of the isolates namely A_1, V_3, Sa_2, Cg_1, and P_1.

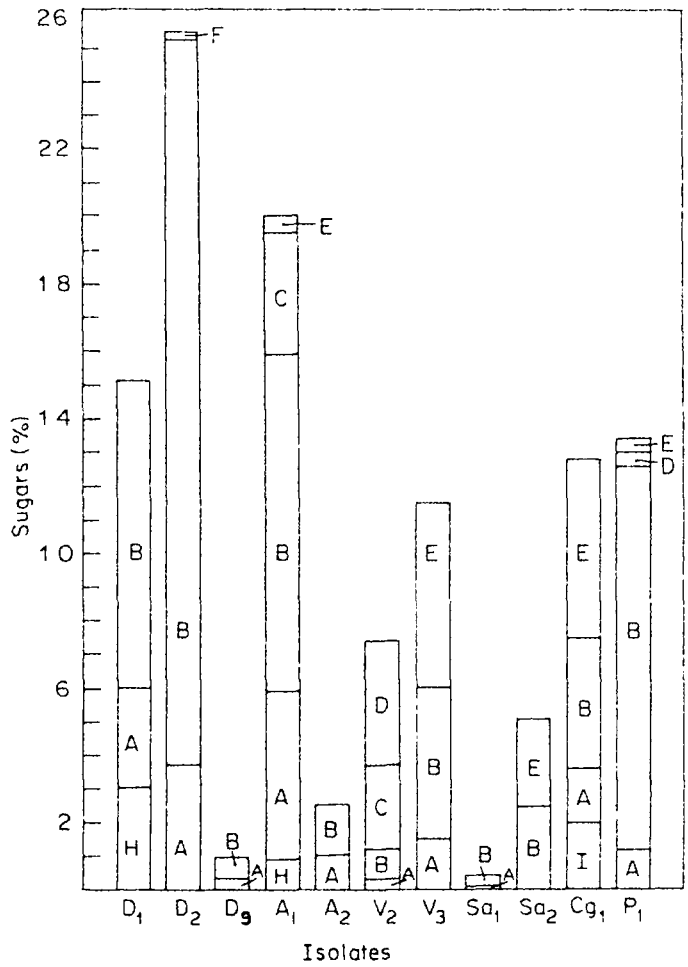

Figure 1. Quantitative analysis of the monosaccharide composition of extracellular polysaccharides of rhizobial isolates. A—Galactose. B—Glucose. C—Arabinose. D—Xylose. E—Rhamnose. F—Mannose. H—Galacturonic acid. I—Glucuronic acid.

3.2 *IR studies*

The exopolysaccharide of all the isolates except P_1 yielded two similar peaks at wave numbers 1570 cm^{-1} (6·45 μ) and 1420 cm^{-1} (7·1 μ) (figure 2) showing the presence of amide and carboxyl groups respectively. The isolates D_9, A_1 and V_2 have different type of spectrum above 8·5 μ indicating their dissimilarity in chemical structure (Levine *et al* 1953). The peaks at 6·45 μ and 7·1 μ were not seen in the isolate P_1 which is unique in having the characteristic absorption bands beyond 8·5 μ.

3.3 *Immunodiffusion reactions*

The exopolysaccharide of all the isolates were immunogenic as evidenced by the induction of antibodies in rabbits. Most of the isolates showed four distinct precipitin

Table 2. Monosaccharide composition of extra-cellular polysaccharides in μg/10 mg of polysaccharide.

Isolates	Galactose	Glucose	Calacturonic acid	Glucoronic acid	Xylose	Arabinose	Rhamnose	Mannose	Total
D_1	300	909	300	–	–	–	–	–	1509
D_2	370	2000	–	–	–	–	–	20	2390
D_9	27	55	–	–	–	–	–	–	82
A_1	500	1000	90	–	–	360	50	–	2000
A_2	100	150	–	–	–	–	–	–	250
V_2	28	90	–	–	370	250	–	–	738
V_3	150	450	–	–	–	–	550	–	1150
Sa_1	5	30	–	–	–	–	–	–	35
Sa_2	–	250	–	–	–	–	226·6	–	516·6
Cg_1	155	390	–	200	–	–	533·3	–	1278·3
P_1	120	1140	–	–	48	–	30	–	1338

bands against the homologous antigen indicating the presence of similar antigenic sites of all these polysaccharides (figure 3). In general, bands 1 and 3 were very thick and 2 and 4 were thin and formed the precipitin lines. In some of the isolates, the precipitin lines 2 and 4 were distinct but in others one or other was merged with the adjacent thick

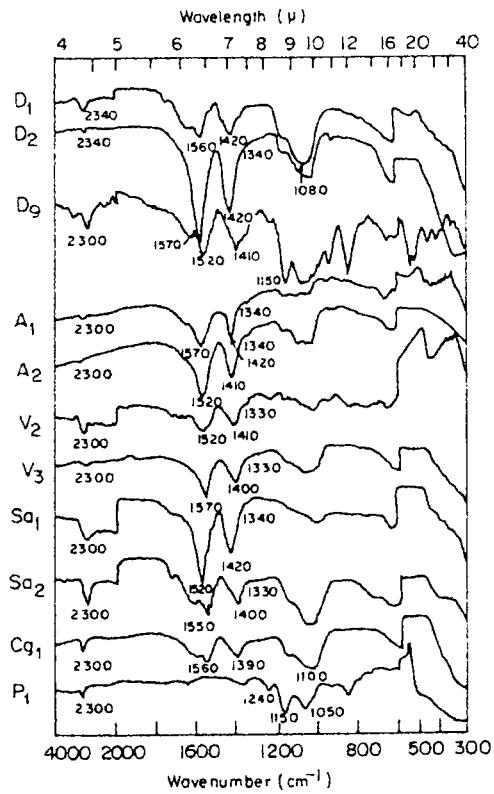

Figure 2. Infrared spectra of the purified extra-cellular polysaccharides of rhizobial isolates.

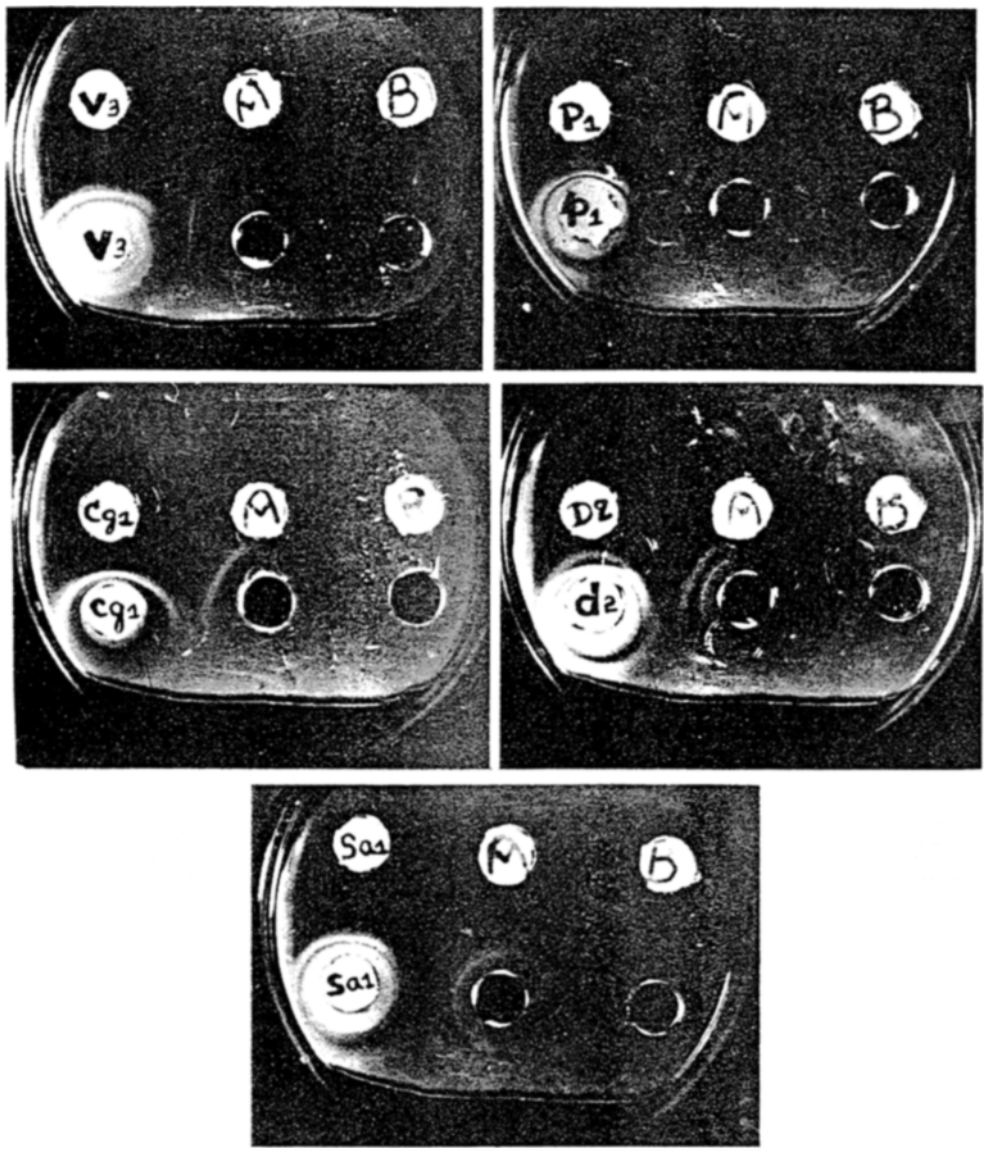

Figure 3. Demonstration of the immunogenicity of the extra-cellular polysaccharides of the different rhizobial isolates (P_1, Sa_1, V_3, d_2 and Cg_1) **A.** Broken cells (Antigen A). **B.** Cell-free extract (Antigen B).

band. With antisera V_2, Sa_2 and P_1, the second precipitin line appeared to merge with band 1. In the isolates D_2, D_9, V_2, V_3, Sa_1 and P_1 there was also an additional hazy line. In Sa_1 and V_3, the fourth line is absent.

Antigen A and B from each isolate when tested along with the polysaccharide antigen against the respective homologous antiserum, do not produce any precipitin lines ruling out the possibility of contamination from cell wall polysaccharides or the cell contents, in the induction of antibodies in rabbits. It is also evident from the above

results that the extra-cellular polysaccharides under study are not analogous with the cell wall polysaccharide.

4. Discussion

It is evident from the results that the purified exopolysaccharides of all the isolates were immunogenic, although only the antigenic nature of the polysaccharide was reported earlier (Dudman 1964). The possibility of contaminating cellular components released by lysis of cells or cell wall lipopolysaccharides in producing the antibodies in rabbits is ruled out as shown by the negative reactions of the antigen A and B (Humphrey and Vincent 1969) (figure 3). It is convincing from the above results that the purified exopolysaccharide used in immunizing the rabbits were not analogous with any of the above mentioned antigens.

In spite of the wide variation in the component sugars which constitute the specificity of the antigenic sites (Wilkinson *et al* 1954; Wilkinson 1958), the polysaccharide of all the isolates showed identical antigenic sites as shown by the similar immunodiffusion reaction. The same type of serological reactions found with the polysacchride antigens could be also due to the presence of the identical functional groups. The occurrence of similar spectra in the longer wavelengths of IR, inspite of the dissimilarities in the shorter wavelengths reflects the inherent structural similarities of these exopolysaccharide.

There was dissimilarity only in the functional group of P_1 isolate. However, this was not reflected in the immunological reactions. The differences in the spectra of the isolates D_9, A_1 and V_2 beyond 8·5 μ might indicate their chemical dissimilarity rather than their functional dissimilarity. Eventhough, the differences in the chemical constitution is responsible for the immunological specificity, serological diversity of chemically similar substances and similarity of quite different polysaccharides were reported (Landsteiner 1962).

The presence of uronic acids as reported in the exopolysaccharide of the isolates D_1, A_1 and Cg_1 does not contribute to chemical differences and the cross reactions depend upon the presence of related or identical constituents.

In conclusion, it may be stated that the rhizobial polysaccharides are very different from those of most of the bacteria where the bacterial slime is made of acid polysaccharides or acid mucopolysaccharides (Clausen 1969). These acidic polysaccharides are not antigenic as is shown in the case of capsules of *Escherichia coli* (Orskov *et al* 1963). In some bacteria, the polysaccharides show much a highly specific reaction that it is possible to use the antigen-antibody reaction for identification of carbohydrates and glycosidic bonds in different carbohydrate antigens (Clausen 1969). Thus the presence of glucose and galactose as monomers, amide and carboxyl as functional groups and the formation of the four characteristic precipitin bands with homologous antisera might well be distinctive characteristics of the polysaccharides of rhizobia. These could possibly be utilised in conjunction with other cultural, physiological and biochemical characteristics to identify rhizobial isolates.

Acknowledgement

One of the authors (RKB) is grateful to UGC for the fellowship and to the Director, University of Madras for providing necessary facilities. Special thanks are due to Dr R

Balasubramanian Lecturer, CAS in Botany and Dr V G Malathi, Scientist, CTCRI, Trivandrum. This paper is part of the Ph.D. thesis approved by the University of Madras.

References

Bailey R W, Greenwood R M and Graig A 1971 Extracellular polysaccharides of *Rhizobium* strains associated with Lotus species; *J. Gen. Microbiol.* **65** 315–322

Block R J, Durram B L and Zweig G 1963 *A manual of paper chromatography and paper electrophoresis* (New York: Academic Press)

Clausen J 1969 *Immunochemical techniques for the identification and estimation of macromolecules.* (Amsterdam, London: North Holland) pp. 571

Dudman W F 1964 Immune diffusion analysis of the extracellular soluble antigens of two strains of *Rhizobium meliloti; J. Bacteriol.* **88** 782–794

Goulden J D S and Sharpe M E 1958 The infrared absorption of Lactobacilli; *J. Gen. Microbiol.* **19** 76

Heidelberger and Avery 1962 in *The Specificity of serological reactions* by Karl Landsteiner (New York: Dover Publications Inc.) 218–219

Hepper M C 1972 Composition of extra cellular polysaccharides of *Rhizobium trifolii; Antonie van Leeuwenhoek* **38** 437–445

Humphrey B A and Vincent J M 1959 Extra-cellular polysaccharides of rhizobium; *J. Gen. Microbiol.* **21** 477–484

Humphrey B A and Vincent J M 1965 The effect of calcium nutrition on the production of diffusible antigens by *Rhizobium trifolii; J. Gen. Microbiol.* **41** 109–118

Humphrey B A and Vincent J M 1969 The somatic antigens of two strains of *Rhizobium trifolii; J. Gen. Microbiol.* **59** 411–425

Landsteiner K 1962 *The specificity of serological reactions* (New York: Dover Publications Inc.)

Levine S, Stevenson H J R and Kablar P W 1953 Quantitative studies of Pneumococcal polysaccharides by infrared spectrophotometry; *Arch. Biochem. Biophys.* **45** 65

Liu P V 1961 Identification of pathogenic *Pseudomonas* by extra cellular antigens: *J. Bacteriol.* **81** 28–30

Orskov I, Orskov F, Jann B and Jann K 1963 Acidic polysaccharide antigen of a new type from *E. coli* capsules; *Nature (London)* **200** 144–146

Sevag M G, Lackman D B and Smolens J 1938 The isolation of the components of streptococcal nucleoproteins in serologically active form; *J. Biol. Chem.* **124** 425–436

Sutton J V and Williams P G 1970 Comparison of extracellular polysaccharide of *Xanthomonas campestris* from culture and from infected cabage leaves: *Can. J. Bot.* **48** 645–651

Vincent J M 1977 Rhizobium—general microbiology; In *A treaties in dinitrogen fixation Section III Biology* (eds) R W F Hardy and W S Silver (New York, London: John Wiley) pp. 227–367

Wagner G H, Cooper F P and Bishop C T 1973 Extra cellular carbohydrate antigens from some non-pathogenic *Neisseria* spp; *Can. J. Microbiol.* **19** 703–708

Wilkinson J F, Duguid J F and Edmunds P N 1954 The distribution of polysaccharide production in *Aerobacter* and *Escherichia* strains and its relation to antigenic character; *J. Gen. Microbiol.* **11** 59–72

Wilkinson J F 1958 The extra-cellular polysaccharides of bacteria; *Bact. Rev.* **22** 46–73

Zevenhuizen L P T M 1971 Chemical composition of exopolysaccharides of *Rhizobium* and *Agrobacterium; J. Gen. Microbiol.* **68** 239–243

Zweig G and Whitaker J R 1971 *Paper chromatography* and electrophoresis (New York, London: Academic Press) Vol 2 pp. 152–169