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PEPTIDOGLYCAN-ASSOCIATED OUTER MEMBRANE PROTEINS IN GRAMNEGATIVE BACTERIA

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Summary

The peptidoglycan-associated protein fractions of various strains of *Escherichia coli* and of other gramnegative rod-shaped bacteria were isolated and compared. Peptidoglycan-associated proteins are always outer membrane proteins. All *E. coli* strains tested contain at least one peptidoglycan-associated protein.

Procedures are described for the isolation of the two peptidoglycan-associated proteins b and c of *E. coli* K12.

All nine Enterobacteriaceae tested contain one or more peptidoglycan-associated proteins. Two gramnegative rod-shaped bacteria that do not belong to the family of Enterobacteriaceae do not contain peptidoglycan-associated protein.

It is suggested that peptidoglycan-associated proteins form, or are part of, hydrophilic channels through the outer membrane.

Introduction

Using a variation on the procedure for the isolation of peptidoglycan sacculi, Rosenbusch [1] reported that a major outer membrane protein of various *Escherichia coli* strains is tightly associated with peptidoglycan. The complex is stable in 2% sodium dodecyl sulphate at 60°C, but is dissociated upon boiling. Using these properties, the peptidoglycan-associated protein of *E. coli* strain BE has been purified and characterized extensively. It consists of only one polypeptide from which approximately 10^5 copies per cell are arranged in a lattice structure with hexagonal symmetry on the outer face of the peptidoglycan [1]. The physiological function of this "matrix protein" is unresolved.

In this paper the major outer membrane proteins of *E. coli* are defined as outer membrane proteins with apparent molecular weights between 33 000 and 44 000, which correspond to the major outer membrane protein originally

described by Schnaitman [2]. By using sodium dodecyl sulphate-polyacrylamide gel electrophoresis systems with improved electrophoretic resolution, this major outer membrane protein of *E. coli* K12 can be separated into two [2,3] or even four [4] protein bands. In the latter case the bands are designated as a, b, c and d, from which a, as it is present in relatively low amounts, can hardly be considered as a major protein [4]. For a comparison of the nomenclature of protein bands used by various authors, the reader is referred to a previous paper [5].

We have reported that *E. coli* strains B and K12 differ in their "major outer membrane proteins". Whereas the peptidoglycan-associated protein of *E. coli* strains B and BE shows only one band on sodium dodecyl sulphate-polyacrylamide gels, the same fraction of *E. coli* K12 strains can be resolved into two bands designated as b and c [5]. In this paper we report the occurrence of peptidoglycan-associated proteins in various strains of *E. coli* and other Enterobacteriaceae. The results may contribute to the elucidation of the function of these proteins.

Materials and Methods

Strains and growth conditions. Relevant properties of the *E. coli* strains used as well as differences in their major outer membrane proteins have been described elsewhere [5,6]. *Citrobacter freundii* G38, *Enterobacter cloacae* H478, *Klebsiella aerogenes* S45, *K. edwardsii edwardsii* S15 and *Shigella sonnei* S81 were obtained from Dr. F.K. de Graaf; *Salmonella typhimurium* strain G30 (*galE*) was obtained from Dr. V. Lehmann. All other strains are from our laboratory collection. Unless otherwise indicated, bacteria were grown under aeration in yeast broth [5] at 37°C and harvested in the late exponential growth phase.

Membrane fractions. Cell envelopes were prepared after ultrasonic desintegration by differential centrifugation as described previously [4]. The modified [5] Triton X100-$MgCl_2$ extraction method of Schnaitman [7] was used to characterize which envelope proteins are located in the cytoplasmic and outer membrane respectively. In contrast to cytoplasmic membrane proteins outer membrane proteins are insoluble under these conditions [7,5]. After extraction of isolated cell envelopes at 60°C in sample buffer for gel electrophoresis (containing 2% sodium dodecyl sulphate [5]), peptidoglycan-associated proteins were isolated by centrifugation for 30 min at 225 000 × *g* at 23°C and once washed with distilled water.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Proteins of the various membrane fractions were solubilized in sample buffer [4] and boiled for 5 min. Slab gels were prepared with compositions according to Laemmli [8], Neville [9] or Lugtenberg et al. [4]. Approx. 20 μg of protein was applied per slot except for supernatant and pellet fractions obtained after extraction of cell envelopes at 60°C. In these cases amounts derived from 20 μg of envelope protein were applied. Electrophoresis was carried out as described previously [4] with a constant current of 30 mA per gel, except that for the Neville system a 20 mA current was used. Staining, destaining and scanning were carried

out as described previously [4]. The standard proteins used have been described previously [4].

Results

Peptidoglycan-associated proteins in various E. coli strains

As reported previously [5] various *E. coli* strains differ considerably with respect to number, electrophoretic mobility and relative amounts of their cell envelope proteins in the 33 000—44 000 molecular weight region (Table I). Cell envelopes were isolated of various *E. coli* strains. Band patterns of cell envelopes, the Triton-$MgCl_2$-insoluble cell envelope fraction and peptidoglycan-associated protein were compared. The major outer membrane protein could be resolved into up to four bands, from which at least one could be isolated complexed with peptidoglycan (Table I). Peptidoglycan-associated proteins are always outer membrane proteins in the 33 000—44 000 molecular weight region. A photograph of a gel showing the peptidoglycan-associated proteins of various *E. coli* strains is shown in Fig. 1A. In *E. coli* K12 two proteins, b and c, are peptidoglycan-associated. *E. coli* B, which lacks protein c, contains one peptidoglycan-associated protein [1] with the same electrophoretic mobility as protein b of *E. coli* K12 [5]. Usually at least 90% of a certain major outer membrane protein band was found either in the pellet or in the supernatant fraction obtained after incubation of cell envelopes at 60°C in sodium dodecyl sulphate and subsequent ultracentrifugation. Some batches of the pellet fraction contained small amounts of the free form of Braun's lipoprotein [10,4], protein d and of a 50 000 dalton protein, identified as pilin in *E. coli* K12 [5]. As other batches of the same strain were devoid of these bands, these proteins were not considered to be peptidoglycan-associated. If a strain possesses proteins with electrophoretic mobilities corresponding with proteins b and c of *E. coli* K12, these proteins are always peptidoglycan-associated except protein c of strain MRE 600, which is partly soluble in sodium dodecyl sulphate at 60°C (Table I

TABLE I

PEPTIDOGLYCAN-ASSOCIATED PROTEINS OF VARIOUS *E. COLI* STRAINS

Strain	Reference(s)	Major outer membrane proteins (percentage of total major outer membrane protein)				Peptidoglycan-associated protein(s) [b]
		a	b	c	d	
K12 PC1349	5	10	15	25	50	b, c
K12 AB1859	5, 11	10	10	35	45	b, c
BE	1, 5	2	73	0	25	b
C	5	5	5	45 [a]	45	b, c
K235	5	35	10 [a]	20	35	a *, b, c
J5	12, 5	5	25 [a]	30	40	b, c
MRE 600	5	7	3	35 [a]	55	(b), c *

[a] These bands have a slightly lower electrophoretic mobility than the corresponding bands of *E. coli* K12.

[b] Proteins indicated with an asterisk are not quantitatively bound to peptidoglycan. The protein given in parenthesis is present in too low an amount to decide whether part of it is not associated with peptidoglycan.

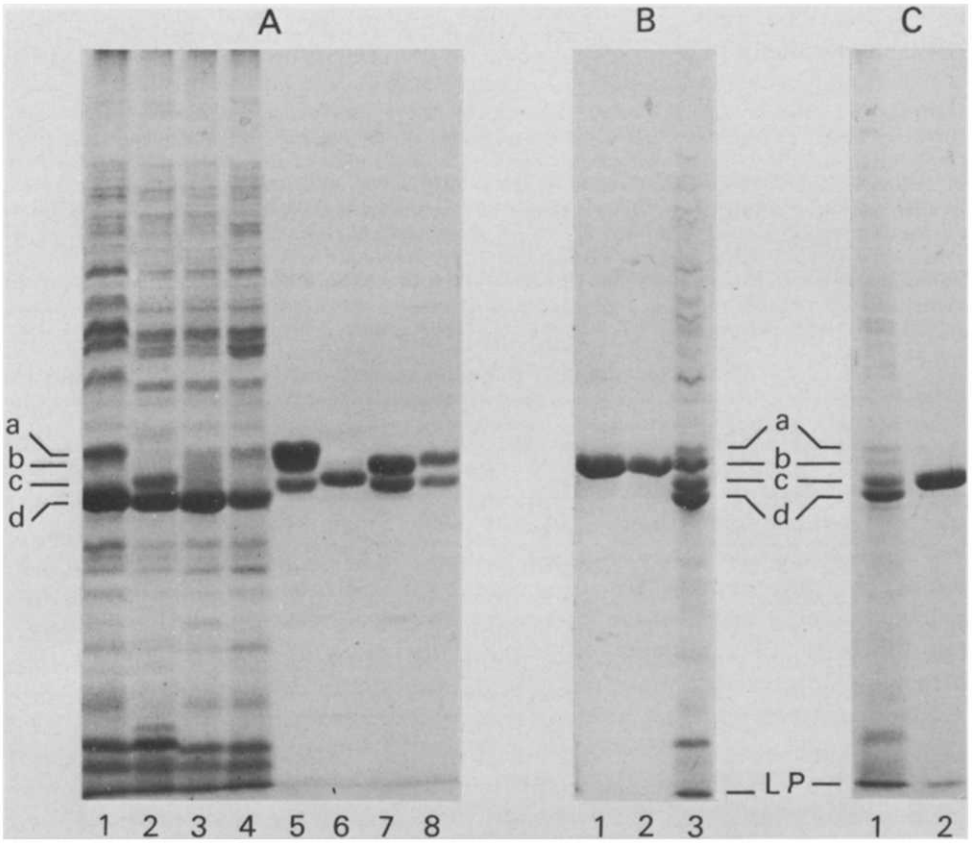

Fig. 1. A. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of fractions of various *E. coli* strains. 1—4, solubilized cell envelope material after heating at 60°C in sodium dodecyl sulphate; 5—8, insoluble cell envelope material after heating at 60°C in sodium dodecyl sulphate; 1 and 5: strain K235; 2 and 6, strain MRE 600; 3 and 7, K12 strain PC1349; 4 and 8, strain J5. The positions of the major outer membrane proteins a, b, c and d of *E. coli* K12 are indicated. B. Purification of protein b. Peptidoglycan-associated protein was isolated from cell envelopes of strain CE1041 after growth in yeast broth and chromatographed on a Sepharose 6B column. 1 and 2, 20 and 10 μg respectively of the peak fraction; 3, cell envelope proteins of strain PC1349 as a reference. C. Purification of protein c. 1, Cell envelope proteins of strain PC0221; 2, Peptidoglycan-associated protein of strain CE1034, grown in yeast broth supplemented with 0.3 M NaCl. The small amount of lipoprotein can be separated from protein c upon chromatography on a Sepharose 6B column. The gel system described by Lugtenberg et al. [4] was used. The position of the free form of Braun's lipoprotein is indicated (LP).

and Fig. 1A). The latter phenomenon has been observed earlier by Rosenbusch for some *E. coli* strains [1]. Protein a is present in high amounts in strain K235. Only in this strain it is peptidoglycan-associated to a large extent (Table I and Fig. 1A).

Purification of proteins b and c

The isolation of peptidoglycan-associated protein can be used as a crucial step in the purification of protein b of *E. coli* B [1]. Proteins b and c of *E. coli* K12 have not been separated yet. An attempt to separate these proteins by differential heating of cell envelopes in sodium dodecyl sulphate at temperatures between 60 and 100°C failed. Both proteins b and c are dissociated from pep-

tidoglycan between 70 and 80°C. Application of the procedure for the isolation of peptidoglycan-associated protein on *E. coli* mutant strain CE1041, which lacks proteins c and d but contains a high amount of protein b [6], results in a protein fraction that contains protein b (Fig. 1B) with a small amount of the free form of Braun's lipoprotein as the only protein contamination. "Accidently" we found that when 0.3 M NaCl is added to yeast broth, the envelopes of some strains are lacking protein b which is compensated for by increased amounts of protein c (Van Alphen, W. and Lugtenberg, B., unpublished). When strain CE1034, which lacks protein d and contains low amounts of b [6], is grown in the presence of 0.3 M NaCl, its peptidoglycan-associated proteins contain protein c with a small amount of Braun's lipoprotein as the only protein contamination (Fig. 1C). The contaminating lipoprotein can easily be separated from b or c by column chromatography on Sepharose 6B [1] or Biogel P-150 [13]. The procedures described above are used now in our laboratory for large scale purifications of proteins b and c.

Peptidoglycan-associated proteins in other gramnegative bacteria

The proteins of cell envelopes and of Triton-$MgCl_2$-insoluble envelopes from various gramnegative bacteria were analyzed in three gel systems. The system that resolved the highest number of major outer membrane protein bands in the molecular weight region between 33 000 and 44 000 was used for further analysis of the membrane proteins of a certain strain. Examples of comparisons between cell envelope proteins and outer membrane proteins of various strains are given in Fig. 2. The protein patterns of cell envelopes, Triton-$MgCl_2$-insoluble cell envelopes, and cell envelope fractions that were soluble and insoluble in 2% sodium dodecyl sulphate at 60°C were compared. The results are summarized in Table II. With the exception of *Alcaligenes faecalis* all strains tested contained one or more major cell envelope proteins bands (Fig. 2). Except for the minor 40 000 dalton band of *Salmonella typhimurium* these proteins are outer membrane proteins. The major outer membrane proteins and peptidoglycan-associated proteins detected in the various strains are listed in Table II. Only one major outer membrane protein band could be detected in *Pseudomonas aeruginosa* but it could not be recovered in the peptidoglycan pellet. *Alcaligenes faecalis* is the only bacterium tested that does not contain major

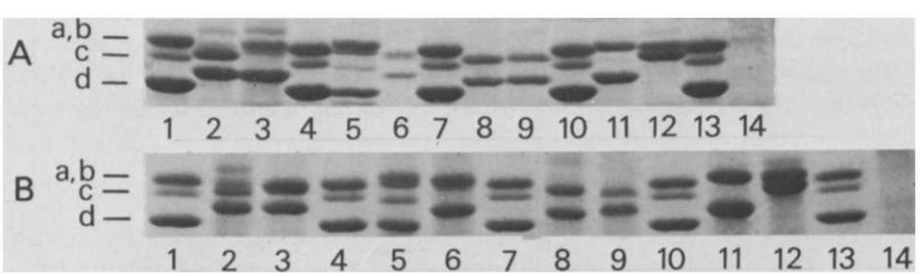

Fig. 2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of cell envelopes (A) and the Triton-$MgCl_2$-insoluble cell envelope fraction (B) of various gramnegative bacteria. The gel system described by Neville [9] was used. 1, 4, 7, 10, 13, *E. coli* K12; 2, *Citrobacter freundii*; 3, *S. typhimurium*; 5, *Shigella sonnei*; 6, *E. cloacae*; 8, *K. edwardsii edwardsii*; 9, *K. aerogenes*; 11, *P. vulgaris*; 12, *P. mirabilis*; 14, *Alcaligenes faecalis*.

TABLE II

PEPTIDOGLYCAN-ASSOCIATED PROTEINS IN GRAMNEGATIVE BACTERIA

Strain	Reference to gel electrophoresis system	Apparent molecular weight of major outer membrane proteins (percentage of total major outer membrane protein)	Apparent molecular weight of peptidoglycan-associated proteins
Alcaligenes faecalis (26)	9	No bands	None
Citrobacter freundii (G38)	9	41, 38.5, 38, 36 (10, 20, 30, 40)	41, 38.5, 38
Enterobacter cloacae (H478)	9	38a, 38b, 36 (15, 35, 50)	38a, 38b
Klebsiella aerogenes (S45)	9	38, 35 (45, 55)	38
Klebsiella edwardsii edwardsii (S15)	9	38, 35 (50, 50)	38
Proteus mirabilis (57)	9	39, 38 (50, 50)	38
Proteus vulgaris (32)	9	39, 35 (45, 55)	None [a]
Pseudomonas aeruginosa (S837)	4	37 (100)	None
Salmonella typhimurium (G30)	9	39, 38.5, 36 (15, 35, 50)	39, 38.5
Serratia marcescens (36)	4	42, 41, 37 (30, 35, 35)	42, 41
Shigella sonnei (S81)	9	40, 39, 37, 34, 33 (25, 15, 10, 45, 5)	40, 39, 37

[a] The 35 000 dalton protein was found to be associated with peptidoglycan when the treatment in sodium dodecyl sulphate was carried out at 40°C.

outer membrane protein and therefore no peptidoglycan-associated protein. All other bacteria listed in Table II belong to the family of Enterobacteriaceae. They all contain at least two major outer membrane proteins. All Enterobacteriaceae except *Proteus vulgaris* contain at least one peptidoglycan-associated protein. However, when cell envelopes of this strain were heated with sodium dodecyl sulphate at 40°C instead of at 60°C, the 35 000 dalton protein was found to be associated with peptidoglycan. We therefore suggest that this protein has the same function as the other peptidoglycan-associated proteins found in Enterobacteriaceae, but that the complex is easier dissociated by sodium dodecyl sulphate-heat treatment. Both *Proteus* strains differ from the other Enterobacteriaceae in that in *Proteus* the fastest moving major outer membrane protein is peptidoglycan-associated.

Discussion

Protein I of *E. coli* B (our protein b) consists of a single polypeptide [1,14]. Protein I of *E. coli* K12 can be resolved into two protein bands Ia and Ib (our bands b and c respectively). As judged by analysis of their cyanogen bromide fragments, Ia and Ib are almost identical. The difference between these polypeptides is located in part of the protein that does not correspond to the

C-terminal or N-terminal region [15]. Both proteins are peptidoglycan-associated [15,5]. It is not clear yet whether protein b is modified by *E. coli* K12 to protein c or if the cell has two genes, each coding for one of the proteins [15]. Strain K235 has three peptidoglycan-associated proteins (Table I, Fig. 1) which have not been characterized yet. The simplest explanation for the occurrence of a family of three peptidoglycan-associated proteins seems to be an evolutionary multiplication of the gene that originally coded for one peptidoglycan-associated protein, followed by modification (small deletions or insertions) of one of more of the resulting genes. Gene multiplication might have had an evolutionary advantage as much as about 10^5 copies of peptidoglycan-associated proteins are present per cell [1]. This is approximately ten times more than the number of individual ribosomal proteins in fast growing cells.

A number of gramnegative bacteria has been tested for the occurrence of another major constituent of the outer membrane, the murein-lipoprotein [10]. The murein-lipoprotein is present in *E. coli*, *Salmonella* and *Serratia marcescens* [16]. The two Proteeae tested, *Proteus vulgaris* and *Proteus mirabilis* were the only Enterobacteriaceae described that did not contain the murein-lipoprotein. Also in *Pseudomonas fluorescens* lipoprotein linked to the murein was absent [16]. Although the number of gramnegative bacteria tested for the presence of murein-lipoprotein and peptidoglycan-associated protein is rather small, the results suggest that Enterobacteriaceae have two ways to link peptidoglycan with the outer membrane, namely by lipoprotein covalently linked to peptidoglycan as well as by their peptidoglycan-associated protein(s). The only exceptions are found in the Enterobacteriaceae subgroup of Proteeae with respect to murein-lipoprotein. In the small number of gramnegative rods tested that do not belong to the family of Enterobacteriaceae neither protein could be detected. It can be concluded that the distribution of major outer membrane proteins and peptidoglycan-associated proteins among gramnegative bacteria corresponds well with their taxonomic classification.

In the last part of this paper we shall propose that the general function of peptidoglycan-associated proteins is the formation of hydrophilic pores through the outer membrane. The outer membrane of *S. typhimurium* contains such pores [17] with an exclusion limit between 600 and 800 daltons for oligosaccharides [18]. A pore-containing outer membrane of *S. typhimurium* can be simulated in vitro. Liposomes containing phospholipids and lipopolysaccharide, prepared in the presence of sucrose (342 daltons) and dextran (of 10 000 daltons), are not leaky for these sugars [19]. However, the addition of an outer membrane protein complex, containing only three protein species, induces the leakage of sucrose but not of dextran. The addition of the matrix protein of *E. coli* B, the only peptidoglycan-associated protein of this organism, to phospholipid-lipopolysaccharide liposomes, also induces leakage of sucrose but not of dextran [19]. Comparison of the molecular weights of the proteins in the active complex of *S. typhimurium* [19] with those described for the major outer membrane proteins of this organism [20], shows that all major outer membrane proteins, except the fastest moving, heat-modifiable protein, are present. Our results (Table II) show that the proteins in the active complex then must correspond with the peptidoglycan-associated proteins. It therefore appears that for both *S. typhymurium* and *E. coli* B the addition of peptidogly-

can-associated proteins to phospholipid-lipopolysaccharide liposomes induces aqueous channels.

Pore-forming proteins should span at least the lipid bilayer of the outer membrane. Strong evidence for such properties exists for the two peptidoglycan-associated proteins of *E. coli* K12. Protein b obviously interacts with the heptose-bound phosphate group of lipopolysaccharide [5], which is assumed to be present near the surface of the cell. Protein c is probably the receptor for various phages [15,21]. Phages of *S. typhimurium* have also been described which are specific for two of the three proteins present in the active complex [22]. We therefore propose that peptidoglycan-associated proteins of all Enterobacteriaceae span the outer membrane and form hydrophilic channels or play a role in their formation. The pores in which peptidoglycan-associated proteins participate are probably not the only hydrophilic pores present in Enterobacteriaceae as *E. coli* K12 mutants lacking both peptidoglycan-associated proteins are perfectly viable [23] and therefore should be able to take up components from the medium which should penetrate through aqueous pores.

The presence of lipopolysaccharide in liposomes in which channels are induced [19] might not be accidental. Experimental evidence indicates the existance of an interaction between protein b and lipopolysaccharide [5]. Moreover, protein c is absent in mutants which most likely have a changed lipopolysaccharide [15]. Therefore lipopolysaccharide and peptidoglycan-associated proteins possibly interact with each other in the outer membrane and both protein and lipopolysaccharide may be required for the formation of hydrophilic pores.

References

1 Rosenbusch, J.P. (1974) J. Biol. Chem. 249, 8019—8029
2 Schnaitman, C.A. (1970) J. Bacteriol. 104, 890—901
3 Henning, U., Höhn, B. and Sonntag, I. (1973) Eur. J. Biochem. 39, 27—36
4 Lugtenberg, B., Meijers, J., Peters, R., Van der Hoek, P. and Van Alphen, L. (1975) FEBS Lett. 58, 254—258
5 Lugtenberg, B., Peters, R., Bernheimer, H. and Berendsen, W. (1976) Molec. Gen. Genetics, 147, 251—262
6 Van Alphen, W., Lugtenberg, B. and Berendsen, W. (1976) Molec. Gen. Genetics, 147, 263—269
7 Schnaitman, C.A. (1971) J. Bacteriol. 108, 545—552
8 Laemmli, U.K. (1970) Nature (Lond.) 227, 680—685
9 Neville, D.M. (1971) J. Biol. Chem. 246, 6328—6334
10 Braun, V. and Rehn, K. (1969) Eur. J. Biochem. 10, 426—438
11 Schnaitman, C.A. (1974) J. Bacteriol. 118, 454—464
12 Schnaitman, C.A. (1970) J. Bacteriol. 104, 882—889
13 Hindennach, I. and Henning, U. (1975) Eur. J. Biochem. 59, 207—213
14 Garten, W., Hindennach, I. and Henning, U. (1975) Eur. J. Biochem. 60, 303—307
15 Schmitges, C.J. and Henning, U. (1976) Eur. J. Biochem. 63, 47—52
16 Braun, V. (1975) Biochem. Biophys. Acta 415, 335—377
17 Nikaido, H. (1976) Biochim. Biophys. Acta 433, 118—132
18 Nakae, T. and Nikaido, H. (1975) J. Biol. Chem. 250, 7359—7365
19 Nakae, T. (1976) J. Biol. Chem. 251, 2176—2178
20 Ames, G.F., Spudich, E.N. and Nikaido, H. (1974) J. Bacteriol. 117, 406—416
21 Verhoef, C., de Graaff, P.J. and Lugtenberg, E.J.J. (1977) Molec. Gen. Genetics, in the press
22 Nurminen, M. and Nakae, T. (1976) Abstracts 8th North West European Microbiological Group, Helsinki, p. 142
23 Henning, U. and Haller, I. (1975) FEBS Lett. 55, 161—164