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Isolation and purification of amyloid protein A by sodium dodecyl sulphate polyacrylamide gel electrophoresis and reversed-phase high-performance liquid chromatography

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ABSTRACT

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and reversed-phase high-performance liquid chromatography (HPLC) were used consecutively for the isolation of amyloid protein A (protein AA) from amyloid fibrils. Highly purified protein AA was obtained and determined by electrophoretic and amino acid analyses. The heterogeneity of protein AA was shown by HPLC. The isoforms of protein AA had different hydrophobicities, although they were equal in size and similar in amino acid composition. Compared with the conventional amyloid separation procedure (gel permeation chromatography), this technique is rapid, requires only small amounts of amyloid fibrils and may provide new information on amyloid proteins.

INTRODUCTION

Gel permeation chromatography (GPC) is commonly used for the fractionation of amyloid fibrils [1–4]. This technique has been successfully applied to the isolation of many amyloid proteins of various origins and structures. In some instances GPC is less effective, especially for the separation of amyloid components of similar molecular mass. In general, the fractionation of amyloid proteins by GPC (in 5 *M* guanidine hydrochloride) is followed by extensive dialysis of the separated proteins; it is thus a time-consuming procedure which requires considerable amounts of amyloid fibrils.

In a search for more rapid and effective methods, the possibility of applying reversed-phase high-performance liquid chromatography (HPLC) or sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for the preparative separation of amyloid proteins on a microgram scale was examined [5]. As the amyloid fibril may consist of different-sized proteins and proteins similar in size but different in structure, the combined use of SDS-PAGE and reversed-phase HPLC was considered very promising.

A procedure has been developed [6] for the preparative separation of proteins using consecutively SDS-PAGE and reversed-phase HPLC. The proteins (insulin, lysozyme, ribonuclease, β-lactoglobulin and bovine serum albumin) were

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analysed by SDS-PAGE, extracted from the gel, purified from the SDS and separated by HPLC. In this study, this procedure was applied to the isolation of amyloid protein A (protein AA) from amyloid fibrils.

EXPERIMENTAL

Materials

Acetonitrile (Bio-Lab, Jerusalem, Israel) was of HPLC grade. Trifluoroacetic acid (TFA), acrylamide, bromophenol blue, Coomassie brillant blue R (Coomassie blue), 2-mercaptoethanol, N,N,N′,N′-tetramethylenediamine and a kit of SDS molecular mass markers (M_r = 14 300–66 000) were purchased from Sigma (St. Louis, MO, USA). The amyloid fibrils were obtained from liver and spleen samples of patients with familial Mediterranean fever (samples CO and GAM) by the method reported previously [7]. The samples of protein AA were obtained from amyloid fibrils using GPC in 5 *M* guanidine hydrochloride on 860 mm × 24 mm I.D. Sephadex G-150 [2] and Fractogel TSK HW-55 (F) columns (Merck, Darmstadt, Germany).

SDS-PAGE

SDS–PAGE was performed on 17% polyacrylamide slab gels [8], 1.5 mm thick, with 5 mm wide sample wells. The amyloid fibrils were dissolved at 37°C for 2 h in a 0.0625 *M* Tris–HCl (pH 6.8) sample buffer containing 3% SDS, 5% 2-mercaptoethanol and 10% glycerol. The obtained solutions (15–20 mg/ml) were loaded into the wells (20 μl per well in the preparative separations). For the localization of the separated amyloid protein, the part of the gel containing the reference proteins (SDS molecular mass markers, protein AA and a sample of amyloid fibrils) was stained with Coomassie blue. The gel slices containing the localized unstained amyloid proteins were cut out and subjected to electro-elution. The gel slices were placed in a dialysis bag with an M_r cut-off of 1000 (Spectrum Medical Industries, Los Angeles, CA, USA). The electro-elution (120 V, 2 h) was carried out in a horizontal electrophoretic cell filled with 0.025 *M* Tris, 0.192 *M* glycine buffer (pH 8.3) and 0.1% SDS. The contents of the dialysis bag were centrifuged and the supernatant containing the electro-eluted proteins was collected.

Removal of SDS

The solution containing the electro-eluted proteins was acidified (0.1% TFA, final concentration), diluted with aqueous 55% acetonitrile (0.1% TFA) and separated on a Fractogel TSK HW-40 (F) column (Merck), as described previously [6,9]. The void volume material containing the proteins, free from SDS, was collected and lyophilized.

HPLC

The HPLC equipment consisted of a Spectra-Physics 8700 solvent delivery system, 8500 dynamic mixer and 8750 organizer, coupled to a Jasco Uvidec 100-IV spectrophotometer with an 8-μl cassette-type cell (10 mm pathway), a Hewlett-Packard 3390A integrator and an LKB 2211 Superrack fraction collector. A Vydac 218 TP (5 μm) (Alltech, Deerfield, IL, USA) column (250 mm × 4.6 mm I.D.) was used.

The samples of amyloid fibrils were suspended in aqueous 20% acetonitrile, containing 0.1% TFA, and stirred gently for 0.5–2 h at room temperature. The mixtures (1.3–2.5 mg/ml) were centrifuged with an Eppendorf 5415 centrifuge (Hamburg, Germany) at 13 700 *g* for 5 min. The obtained supernatants were applied to the column in 50-μl aliquots. HPLC was performed using a linear gradient from 20 to 47.5% acetonitrile in 0.1% TFA (6 min) followed by a linear increase of acetonitrile concentration to 75% (0.1% TFA) over 84 min. A flow-rate of 1.0 ml/min was maintained. The elution of the proteins was monitored by UV absorbance at 220 nm.

Amino acid analysis

The Hewlett-Packard HPLC 1090 system was adapted for amino acid analysis. A Pickering (Mountain View, CA, USA) column was used for the conventional (Stein and Moore) amino acid analysis procedure.

Recovery of amyloid proteins

The yield of the recovered proteins was determined using SDS-PAGE [5] by calculating the amount of stain (Coomassie blue) bound to the proteins.

RESULTS

Amyloid fibrils were dissolved in aqueous 20% acetonitrile containing 0.1% TFA and separated by HPLC. Several fractions [capacity factor (k') 3.6–5.4] were detected (Fig. 1). The electrophoretic analysis of these fractions revealed protein AA and small amounts of other proteins (Fig. 2).

Most protein AA isolated from the amyloid fibrils by GPC shows homogeneous bands (M_r about 9000) on SDS-PAGE (Fig. 3, tracks 2, 3, 5 and 7). However, HPLC separation of protein AA (Fig. 4) showed several UV-absorbing peaks (k' from 4.4 to 5.4). Very similar elution profiles were obtained when protein AA was run on SDS-PAGE, electro-eluted from the gel, purified from SDS (and buffer salts) and then subjected to HPLC (Fig. 5). The protein recovered by this procedure corresponded to 20–25% of its initial amount. The material separated by HPLC (peaks I – IV, Fig. 4c) were pooled and purified by several rechromatography steps. The proteins found in each of these obtained pools showed electrophoretic mobilities (Fig. 6) and amino acid compositions (Table I) typical of protein AA.

Amyloid fibrils were run on SDS-PAGE and the protein AA (localized in gel) was electro-eluted, purified from SDS and the buffer salts and separated by HPLC. The material eluted within 19.5–23 min (Fig. 7, k' 4.4–5.4) was pooled and checked by SDS-PAGE. Fig. 3 (tracks 6 and 7) shows the electrophoretic purity of protein AA obtained by this procedure. The main fractions observed in the chromatogram (Fig. 7a, peaks I–III) were collected. These fractions showed the electrophoretic mobility characteristic of protein AA. The amino acid compositions of these fractions varied only slightly and were also typical of protein AA (Table I).

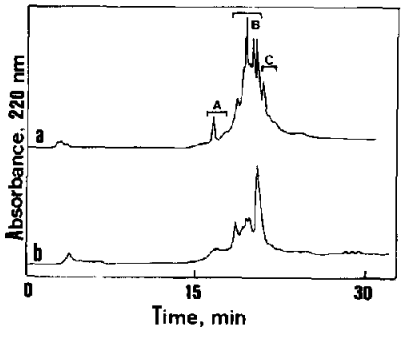

Fig. 1. Reversed-phase HPLC of amyloid samples. (a) CO (spleen) and (b) GAM (liver). Amyloids fibrils were dissolved in aqueous 20% acetonitrile solution containing 0.1% TFA. The elution was carried out using a linear gradient from 20 to 47.5% acetonitrile in 0.1% TFA (6 min) followed by a linear increase of acetonitrile concentration to 75% (0.1% TFA) over 84 min. A flow-rate of 1.0 ml/min was used. The effluent was monitored by measuring the UV absorbance at 220 nm, sensitivity 0.53 a.u.f.s.

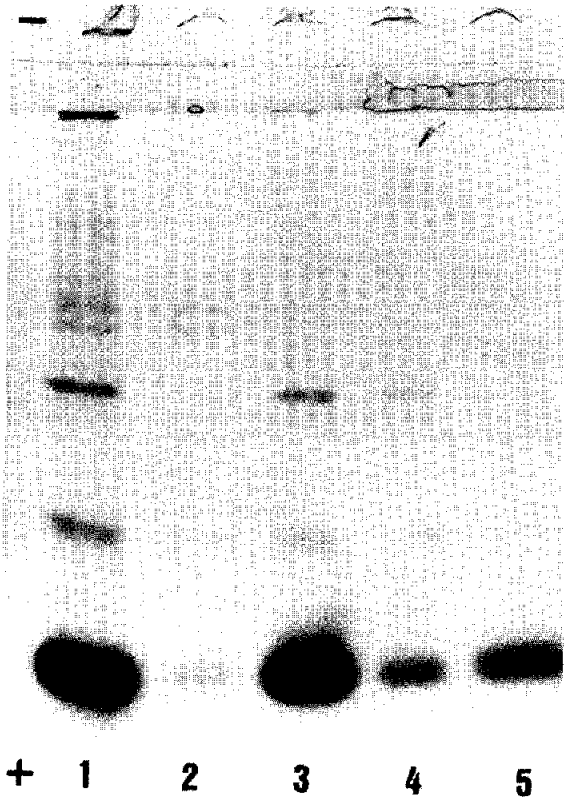

Fig. 2. SDS-PAGE of amyloid CO (spleen) fractions obtained by HPLC (Fig. 1a). 1 = Amyloid fibrils (prior to HPLC); 2 = fraction A; 3 = fraction B; 4 = fraction C; 5 = protein AA, isolated from amyloid CO by GPC (Sephadex G-150).

DISCUSSION

Amyloids are complex biological compounds, which are deposited in the form of fibrils in various organs. The main components of the different amyloid fibrils are proteins of various chemical types (AA, AL, AP and others); these can be isolated by GPC. As amyloid fibrils are not soluble in water, denaturants such as urea or guani-

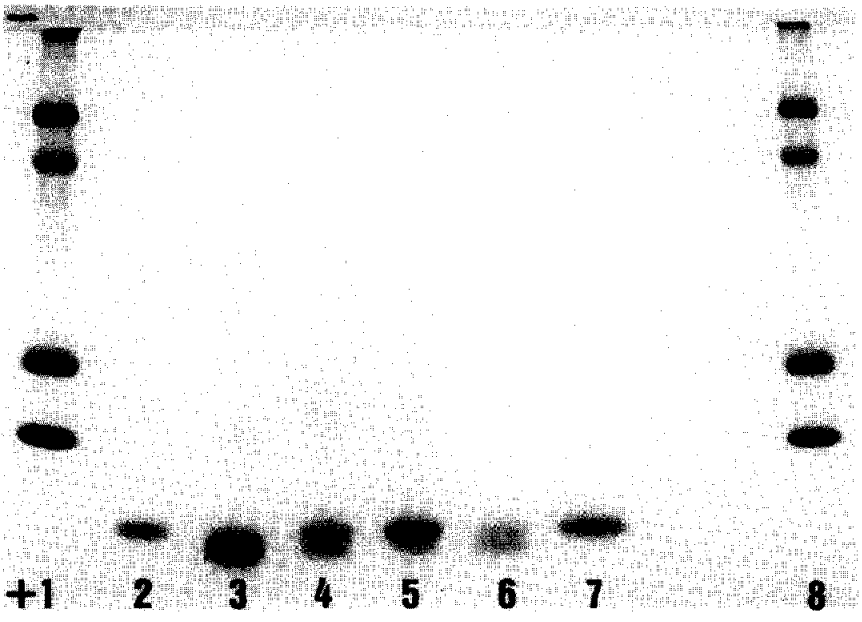

Fig. 3. SDS-PAGE of protein AA isolated from amyloid fibrils by GPC (2–5) and by consecutive SDS-PAGE and HPLC (6 and 7). 1 and 8 = molecular mass markers, from top to bottom bovine serum albumin, ovalbumin, β-lactoglobulin and lysozyme; 2 = protein AA isolated from amyloid CO (spleen) on Sephadex G-150; 3 = protein AA from GAM spleen (Sephadex G-150); 4 = protein AA from GAM liver (Fractogel TSK HW-55); 5 = protein AA from CO spleen (Fractogel TSK HW-55); 6 = protein AA from GAM liver (SDS-PAGE and HPLC, pool of fractions k' from 4.4 to 5.4, Fig. 7b); 7 = protein AA from CO spleen (SDS-PAGE and HPLC, pool of fractions k' from 4.4 to 5.4, Fig. 7a).

dine hydrochloride are used to dissolve the amyloid proteins for fractionation by GPC.

In a previous study the amyloid fibrils were found to be partially soluble in acidic aqueous acetonitrile solution; this made it possible to fractionate the amyloid proteins by reversed-phase HPLC [5]. A procedure has been developed for the separation of proteins by consecutive SDS-

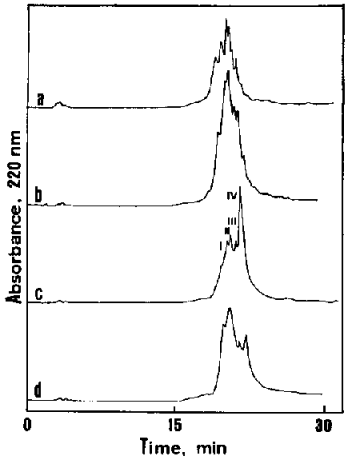

Fig. 4. Reversed-phase HPLC of protein AA isolated from amyloid fibrils by GPC. (a) CO spleen (GPC was performed on Sephadex G-150); (b) CO spleen (Fractogel TSK HW-55); (c) GAM spleen (Sephadex G-150); (d) GAM liver (Fractogel TSK HW-55). The HPLC separation was performed as described in Fig. 1.

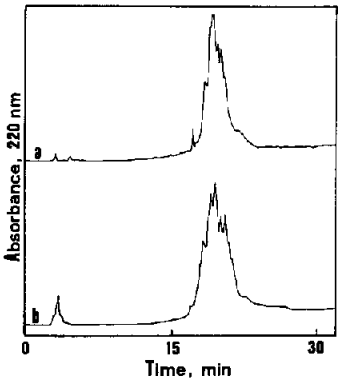

Fig. 5. Reversed-phase HPLC of protein AA recovered from SDS-PAGE. Protein AA was obtained from amyloid CO (spleen) by GPC (Fractogel TSK HW-55) and then subjected to SDS-PAGE, electro-eluted and purified from SDS and buffer salts. (a) HPLC profile of protein AA prior to the electrophoretic run; (b) HPLC profile of protein AA recovered from the polyacrylamide gel.

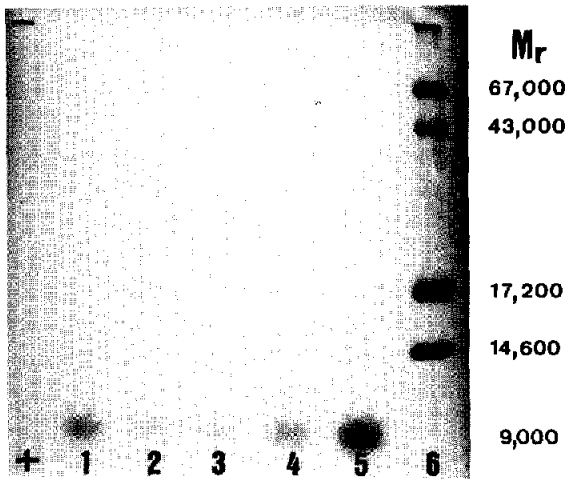

Fig. 6. SDS-PAGE of protein AA (GAM spleen), separated by HPLC. Protein AA was obtained from amyloid fibrils by GPC (Sephadex G-150) and then subjected to HPLC (Fig. 4c). 1 = Peak IV; 2 = peak III; 3 = peak II; 4 = peak I; 5 = protein AA (prior to HPLC); 6 = molecular mass markers, from top to bottom bovine serum albumin, ovalbumin, β-lactoglobulin and lysozyme.

PAGE and reversed-phase HPLC [6,10]. The proteins (insulin, lysozyme, ribonuclease, β-lactoglobulin and bovine serum albumin) were separated by SDS-PAGE, recovered from the gel and purified from SDS. The HPLC profile of the proteins obtained corresponded to that of standard proteins (not treated by this procedure). In this study it has been shown that protein AA can be eluted by SDS-PAGE and purified from SDS; the technique used did not affect the HPLC behaviour of this protein (Fig. 5). This procedure can therefore be applied to the isolation of protein AA from amyloid fibrils.

The consecutive use of SDS-PAGE and HPLC allowed highly purified protein AA to be obtained, as shown by electrophoretic and amino acid analyses. This isolation procedure is rapid (two to three days) and requires only a few milligrams of amyloid fibrils; 50–80 mg of amyloid were used for each chromatographic run in the traditional GPC procedure. For the isolation of

TABLE I

AMINO ACID COMPOSITION OF PROTEIN AA ISOFORMS OBTAINED BY REVERSED-PHASE HPLC

Amino acid	Residue per 100 residues							
	GAM spleen					CO spleen		
	Protein AA isolated by GPC	HPLC fraction (Fig. 4c)				Protein AA isolated by SDS-PAGE and HPLC (k' = 4.4–5.4)	HPLC fraction (Fig. 7a)		
		I	II	III	IV		I	II	III
Asp	14.5	14.4	13.8	14.7	13.4	12.4	14.0	13.4	14.3
Thr	0.5	0.7	0.5	0.4	0.6	1.9	1.1	1.2	1.5
Ser	6.9	7.3	7.0	7.3	7.1	6.7	6.8	6.7	7.3
Glu	7.1	7.8	7.5	7.9	7.6	10.4	10.3	9.6	9.4
Pro	1.9	2.2	3.1	1.8	2.7	2.9	2.7	2.0	1.5
Gly	11.2	12.0	11.8	11.7	11.7	11.8	12.1	12.1	12.5
Ala	16.8	17.0	16.8	17.5	17.1	15.4	16.0	16.2	16.3
Val	1.8	2.1	2.0	1.8	1.8	2.5	1.8	1.7	1.5
Met	3.7	1.5	2.3	2.1	2.6	2.0	1.3	1.6	1.5
Ile	4.0	3.7	3.8	3.7	3.8	4.3	3.9	4.0	4.2
Leu	3.2	2.7	3.0	3.0	2.7	4.6	4.0	3.8	3.8
Tyr	5.1	4.9	4.9	4.6	5.5	3.5	3.3	4.3	3.9
Phe	8.9	8.6	8.5	8.5	8.7	6.9	7.3	7.8	7.9
His	1.5	1.7	1.7	1.7	1.6	1.9	1.9	2.0	1.9
Lys	3.2	3.6	3.4	3.5	3.4	3.7	3.5	3.3	3.0
Arg	9.8	9.9	10.1	9.8	9.7	9.2	10.1	10.5	9.6

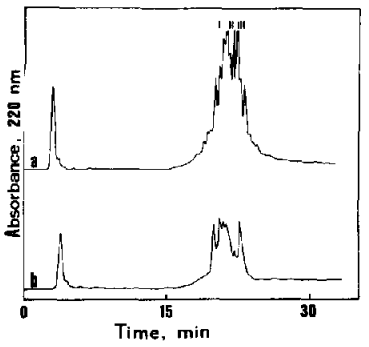

Fig. 7. Reversed-phase HPLC of protein AA isolated from amyloid fibrils by preparative SDS-PAGE. (a) Protein AA isolated from 1.5 mg of amyloid fibrils (CO spleen); (b) protein AA isolated from 2 mg of amyloid fibrils (GAM liver).

larger amounts of amyloid proteins, GPC is still essential. In this instance, Fractogel was preferable to Sephadex. Like Sephadex, Fractogel was efficient in the separation of protein AA; however, the mechanical and chemical stability of Fractogel was higher, and it was not degraded in the presence of solutions containing guanidine hydrochloride; Sephadex was partially destructed by guanidine hydrochloride [11–13].

When a Sephadex G-100 or G-150 column was washed with 5 *M* guanidine hydrochloride solution, water-soluble material was found in the effluent (28 mg per 100 ml) after dialysis against water (unpublished data). This material reacted strongly with anthrone and was intensely coloured with periodic acid–Schiff stain; only glucose was found in an analysis of monosaccharides (by thin-layer chromatography). These data, in addition to results from SDS-PAGE and GPC analyses, showed that this material is a high-molecular-mass polysaccharide (>200 000 daltons), obviously derived from the Sephadex gel.

In contrast to a previous study of amyloids by HPLC [5], a wide-pore (300 Å) octadecyl column (Vydac 218 TP, 5-μm packing) was used, which significantly increased the efficiency of the HPLC separation. Several forms of protein AA of various hydrophobicities, although very similar in size and in amino acid composition, were isolated. The existence of several isoforms of protein AA was suggested in an earlier amyloid study using the isoelectric focusing technique [14]. No additional information on these isoforms is available.

The heterogeneity of protein AA is obviously linked with the existence of several isoforms of its precursor molecule, serum amyloid A protein (SAA) [15–18]. SAA (about 104 amino acid residues) is an apolipoprotein of high-density lipoproteins and has the same N-terminal amino acid sequence as protein AA (76 amino acid residues). Normally present in trace amounts, the concentration of SAA increases markedly in chronic inflammatory diseases, although only a few patients develop amyloidosis. It has been speculated whether one or several isoforms of SAA may be amyloidogeneous. Structural analyses of both the AA and SAA isoforms may therefore be useful in the elucidation of amyloidogenesis.

REFERENCES

1 G. C. Glenner, W. Terry, M. Harada, C. Isersky and D. Page, *Science*, 172 (1971) 1150.
2 E. C. Franklin, C. J. Rosenthal and M. Pras, in J. Hamburger, J. Crosnier and M. H. Maxwell (Editors), *Advances in Nephrology*, Vol. 5, Year Book Medical Publishers, Chicago, 1975, p. 89.
3 M. Pras, B. Frangione and E. C. Franklin, in G. C. Glenner, P. P. E. Costa and A. F. De Freitas (Editors), *Amyloid and Amyloidosis*, Excerpta Medica, Amsterdam, 1980, p. 249.
4 M. Pras, E. C. Franklin, F. Pulli and B. Frangione, *J. Exp. Med.*, 154 (1981) 989.
5 B. Kaplan and M. Pras, *Clin. Chim. Acta*, 163 (1987) 199.
6 B. Kaplan and M. Pras, *Biomed. Chromatogr.*, 5 (1991) 86.
7 M. Pras, M. Schubert, D. Zucker-Franklin, A. Rimon and E. C. Franklin, *J. Clin. Invest.*, 47 (1968) 924.
8 U. K. Laemmli, *Nature*, 227 (1970) 680.
9 B. Kaplan and M. Pras, *J. Chromatogr.*, 423 (1987) 376.
10 B. Kaplan and M. Pras, *Biomed. Chromatogr.*, 4 (1990) 89.
11 K. D. Ganchev, *God. Vissh. Khim-Tekhnol. Inst. Sofia*, 20 (1972) 295.
12 A. A. Ansari and R. G. Mage, *J. Chromatogr.*, 140 (1977) 98.
13 D. Mahuran, *J. Chromatogr.*, 172 (1979) 394.
14 P. Westermark, *Biochim. Biophys. Acta*, 701 (1982) 19.
15 D. C. Parmelee, K. Titani, L. H. Ericsson, N. Eriksen, E. Benditt and K. A. Walsh, *Biochemistry*, 21 (1982) 3298.
16 N. Eriksen and E. P. Benditt, *Methods Enzymol.*, 128 (1986) 311.
17 F. E. Dwulet, D. K. Walkace and M. D. Benson, *Biochemistry*, 27 (1988) 1677.
18 A. F. Strachan, F. C. De Beer, D. R. van der Westhuyzen and G. A. Coetzee, *Biochem. J.*, 250 (1988) 203.