*Journal of Steroid Biochemistry*, Vol. 10, pp. 535 to 540
Pergamon Press Ltd 1979. Printed in Great Britain

# PURIFICATION AND CHARACTERIZATION OF HUMAN SEX HORMONE BINDING GLOBULIN

M. JAWED IQBAL and MICHAEL W. JOHNSON
Department of Biochemical Endocrinology, Chelsea Hospital for Women and Institute of Obstetrics and Gynaecology, Dovehouse Street, London S.W.3 6LT, England

(*Received* 31 *July* 1978)

## SUMMARY

Sex hormone binding globulin (SHBG) was isolated from post-partum plasma in a three stage procedure involving affinity chromatography on androstanediol-3-hemisuccinate aminohexyl Sepharose 4B followed by affinity chromatography using Cibacron Blue F3G-A-Sepharose 4B and finally gel filtration on Sephadex G-150. The purified protein was homogeneous in polyacrylamide gel electrophoresis and had retained its binding activity for 5α-dihydrotestosterone (5α-DHT) with 1.08 binding site per mol. The procedure yielded 5 mg of protein with an overall purification of about 3700. The association constant for 5α-DHT at 4°C was $0.6 \times 10^9$ L/M; the molecular weight based on sodium dodecyl sulphate polyacrylamide gel electrophoresis was 80,000 and the latter technique also demonstrated four components of almost identical mobilities. Amino acid and carbohydrate compositions were determined for the purified protein.

## INTRODUCTION

There have been several successful and partially successful attempts at purification of human [1–7] and bovine [8] Sex Hormone Binding Globulin, (SHBG). Noteworthy among them are those by Bohn[3], Rosner and Smith[4] and Mickelson *et al.*[5]. With the exception of Suzuki *et al.*[8], the approaches that have resulted in substantial purification of this protein have employed multiple steps. Affinity chromatography has been used in conjunction with ammonium sulphate precipitation, repeated gel filtration and isoelectric focusing [4], preparative gel electrophoresis [5] or multiple conventional separation procedures alone have been employed [3]. As has been demonstrated previously [7, 8], ammonium sulphate precipitation and repeated gel filtration, ion-exchange chromatography and isoelectric focusing steps are not required if a good affinity gel is available. We have reported previously a new approach to purification of this binding protein [7]. The use of this technique, which involves electrophoretic desorption of the immobilised protein from the affinity column, on a preparative scale is still under investigation. In this report we describe the isolation of SHBG in a fully active form in just three purification steps.

## MATERIALS AND METHODS

Radioactive steroids were purchased from the Radiochemical Centre, Amersham, U.K. All steroids were purchased from Steraloids Ltd., U.K. Sepharose 4B, Sephadex LH-20 and Sephadex G-150 were purchased from Pharmacia, G.B. Ltd. N,N-Dicyclohexyl carbodiimide, human serum albumin and bovine serum albumin were purchased from Sigma Chemical Co. Ltd, U.K. Myoglobin, cytochrome C, ovalbumin, carboxypeptidase and L-fucose dehydrogenase were purchased from Miles Laboratories Ltd., U.K. α-lactalbumin was a gift from the Imperial Cancer Research Fund, U.K. All electrophoresis reagents were obtained from Bio-Rad Laboratories, U.K. Tris buffer was used as before [7].

*Preparation of 5α-androstane-3β,17β-diol 3-hemisuccinate (androstanediol succinate).* 20 g of epiandrosterone were dissolved in a minimum volume of pyridine with 200 μCi of dehydroepiandrosterone, DHA. To this were added 24 g of succinic anhydride. The solution was heated for 2 h and allowed to cool to room temperature, it was then poured into ice-cold water, pH 3. The precipitate formed was washed with ice-cold acidified water, then with ice-cold acidified 20% methanol, pH 3, and finally with ice-cold ether. The precipitate was dried *in vacuo* (yield 24 g) and 20 g of this were dissolved in 800 ml of absolute ethanol. The solution was then treated at room temperature with 20 g of sodium borohydride for 15 min, acidified and poured into ice-cold water at pH 3. The resulting precipitate was collected, washed as above and recrystallised from ethanol. 19.2 g of androstanediol-3-hemisuccinate were obtained, specific activity = 10,600 d.p.m./mg. (m.p. = 215°C).

*Coupling of androstanediol-3-hemisuccinate to aminohexyl-Sepharose 4B.* Aminohexyl-Sepharose 4B was prepared according to the procedure of Cautrecasas[9]. 14 g of androstanediol-3-hemisuccinate were dissolved in 500 ml of 80% dimethyl formamide and the washed aminohexyl-Sepharose 4B added to this followed by the addition of 7 g of N,N-dicyclohexyl

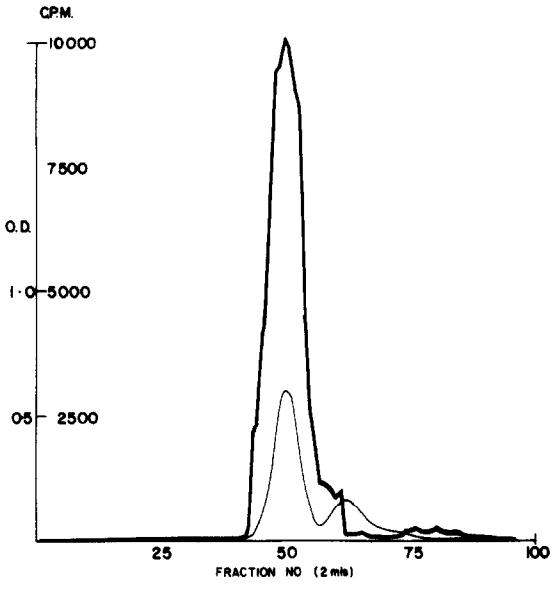


Fig. 1. Sephadex G-150 chromatography. The bold line represents the [$^3$H]-DHT bound and the thin line represents the optical density.

carbodiimide. The mixture was stirred for 4 h and washed with 10 l. of 80% dimethyl formamide, followed by 50% dimethyl formamide and then by 15 l. of distilled water. No radioactivity was detectable in the washes. The gel was finally washed with 1 l. of Tris buffer. The resultant affinity gel contained 37 $\mu$M steroid per ml of gel.

Cibacron Blue F3G-A-Sepharose 4B was prepared by the method of Heyns and De Moor [10]. The gel was capable of binding 15 mg human serum albumin per ml.

*Assay of the capacity of androstanediol-Sepharose for SHBG.* To 0.25 g of the affinity gel were added varying amounts of a 1:5 dilution of post-partum plasma, 2, 4, 8, 16 and 32 ml, and incubated for 45 min at 4°C. The supernatant was drained through pasteur pipettes fitted with fibre plugs and 0.4 ml of the drained supernatants incubated with 20,000 c.p.m. [$^3$H]-DHT for 45 min at 4°C. The two-tier column assay was then performed on the incubates as previously described [11].

*Assay for SHBG activity.* The two-tier column method [11] was employed for the determination of SHBG binding capacity, BC, through most of the purification steps. For the very dilute protein concentrations obtained after affinity chromatography the same method was still employed with the modification that the samples to be incubated were made up in 1:5 heat denatured plasma. Binding activity was determined after the gel filtration step in the fraction collected by adding 100 $\mu$l of the sample of de-albuminised, heat denatured plasma [11] and using the charcoal separation method [12].

*Isolation of SHBG*

*Affinity chromatography on androstanediol-Sepharose.* To 3.8 l of post-partum plasma 250 ml androstanediol-Sepharose were added and the mixture was allowed to stir for 16 h at 4°C. The supernatant was removed by suction on a coarse sintered glass funnel and washed with Tris buffer (0.05 M Tris, 0.005 M $CaCl_2$, pH 7.4) containing 1 M NaCl until the U.V. absorbance at 280 nm was nil. The gel was then washed with 1 l. Tris without NaCl. The affinity gel was then transferred to a beaker and 300 ml of 0.1 mM DHT (4000 c.p.m./$\mu$g) in Tris buffer were added to it. The suspension was stirred for 4 h at room temperature and then eluted on a coarse sintered glass funnel. This step was repeated twice with 300 ml of DHT–Tris buffer. The affinity eluate was concentrated to 30 ml on the Amicon ultrafiltration cell, model 402, using PM 10 ultrafiltration membranes with a cut off molecular weight of 10,000. The protein concentration of the eluate was determined by the Lowry technique [13]. Scatchard analysis was performed on 1:20 dilution of this 30 ml fraction using the two-tier column method [11]. 100 $\mu$l of the 1:20 diluted protein solution were added to 400 $\mu$l of a 1:5 diluted heat denatured plasma and the assay was performed on this in the usual manner.

*Affinity chromatography on Cibacron Blue F3G-A-Sepharose.* The eluate was transferred to a beaker and 10 g of Cibacron Blue-Sepharose were added to it. The mixture was stirred for 45 min in an ice bath. The solution was then drained on a coarse sintered glass funnel and washed with 4 × the column volume with ice-cold Tris buffer. The eluate was again transferred to the ultrafiltration cell (which was kept in an ice bath) and dialysed with ice-cold Tris buffer. No radioactivity could be determined in the ultrafiltrate. The volume in the ultrafiltration cell was reduced to 10 ml and the protein concentration determined by the Lowry technique.

*Sephadex G-150 filtration.* To a 2.5 × 60 cm column of Sephadex G-150 packed in Tris buffer 10 ml of the affinity eluate were applied and eluted with Tris buffer. 2 ml fractions were collected; U.V. absorbance at 280 nm and DHT binding activity were determined for all fractions as described above. The fractions showing binding activity were pooled, concentrated again to 10 ml and subjected to Scatchard analysis as above; the total protein concentration of the purified material was determined by the Lowry method.

*Characterization of SHBG*

*Polyacrylamide gel electrophoresis, PAGE, to determine the purity of the material isolated.* The protein solution was concentrated to give 1 mg protein per ml using the Amicon minicon concentrator cells (A 25). Analytical PAGE was performed using the method of Smith[14]; the total gel concentration employed was 18% with 0.66% cross linkage.

*Determination of molecular weight.* The molecular weight of purified SHBG was determined using the sodium dodecyl sulphate-polyacrylamide gel electrophoresis, SDS-PAGE, technique of Weber and

Table 1. Comparison of binding data for original plasma and purified material

| | Protein Conc. (Mg/ml) | Binding capacity (nmol/ml) | Total binding capacity (nmol) | Specific activity (nmol/mg) | Purification factor | % Recovery |
|---|---|---|---|---|---|---|
| Original plasma | 99.0 | 0.359 | 1364.2 | 0.00363 | | |
| Androstanediol-Sepharose 4B eluate | 1.80 | 11.954 | 358.6 | 6.64110 | 1830 | 26.29 |
| Cibacron Blue F3G-A-Sepharose 4B eluate | 1.20 | | | | | |
| Sephadex G-150 eluate | *0.509 | 6.837 | 68.4 | 13.4320 | 3700 | 5.01 |

Binding capacity measurements were not carried out for the Cibacron Blue F3G-A-Sepharose 4B eluate.
* This mass was calculated from the carbohydrate and amino-acid analyses and represents the sum total of carbohydrate and polypeptide concentrations.

Osborn[15]. The total gel concentration employed was 10%. Human serum albumin, bovine serum albumin, myoglobin, ovalbumin, α-lactalbumin, cytochrome C, carboxypeptidase and L-fucosedehydrogenase were used as standards. The experiment was performed once after pre-incubation of the protein solutions with 1% SDS and 1% β-mercaptoethanol for 2 h at 45°C and once after pre-incubation with 1% SDS in the absence of β-mercaptoethanol.

*Amino acid and carbohydrate analysis.* The sample of the purified material, 1 mg/ml, was hydrolysed in 6 N HCl at 110°C for 24 h and the amino acid composition was determined using a Rank Hilger amino acid analyser. Cysteine which is determined as cysteic acid and methionine which is determined as methionine sulphoxide were not determined as these required a separate run and therefore a further supply of the material. The carbohydrate composition was determined by the method of Clamp *et al.*[16]. The mass of the material was again calculated from these analyses.

## RESULTS

Analytical PAGE carried out to assess the purity of the isolated material showed one sharp band of protein with an $R_F$ value of 0.28 (Fig. 2). SDS-PAGE of the purified material sample that had been incubated with 1% SDS in the absence of β-mercaptoethanol showed one sharp band which approximated to a molecular weight value of 86,000. When the sample was incubated with 1% SDS and 1% β-mercaptoethanol, four components of almost identical electrophoretic mobilities were observed, each component approximating to a molecular weight of 20,000 ± 500 (this is a mean of four different runs). A molecular weight of 80,000 was therefore estimated by adding up the values for the four components.

The purified SHBG was found to contain 34.4% carbohydrate. The compositions of individual sugars and amino acids are listed in Table 2. No lysine or arginine could be detected and the ammonia peak which is normally observed when amino acids are analysed was missing, however, preceding the region where ammonia is normally detected a long sharp peak of unidentifiable material was observed.

## DISCUSSION

Suzuki *et al.*[8] and Iqbal *et al.*[7] have shown previously that the use of a high affinity gel makes the more conventional techniques unnecessary. We were able to manufacture an affinity gel for SHBG, androstanediol-3-hemisuccinate-aminohexyl-Sepharose 4B which was capable of immobilising SHBG in 25.6 ml of post-partum plasma per ml of gel and was still unsaturated. We were able to couple 37 μmol steroid per ml of gel whereas Mickelson *et al.*[5] were able to couple only 2 μmol/ml of gel using the 17 position of 5α-DHT. It is not clear why these authors have used the 17 position for linkage when they report themselves that the incorporation of the succinate group at the 17 position reduces the binding affinity relative to DHT by a factor of about 600. What is more important, the unavailability of the 17β group on the steroid might have caused their gel to be less specific as well.

The results of previous attempts at the purification and characterization of human SHBG [3–5] are conflicting, and this report will add further to the controversy although it is broadly in agreement with one of these earlier studies [4].

The molecular weight for human SHBG reported by Bohn[3], for example, is 65,000. Bohn on the basis of his experiments with analytical sodium dodecylsulphate polyacrylamide gel electrophoresis, also postulates that the SHBG molecule is probably composed of four sub-units which possess a molecular weight of 16,000 each. A similar finding is reported by Suzuki *et al.*[8] for bovine SHBG which breaks up into three sub-units on treatment with guanidine chloride. Rosner and Smith[4] report a molecular weight for human SHBG of 94,000 on the basis of a modified SDS-PAGE technique of Weber and Osborn[15], but find no evidence for subunit or polymeric structure. The most recent report is by Mickelson *et al.*[5] who calculated a molecular weight of 36335 for this protein by equilibrium sedimentation

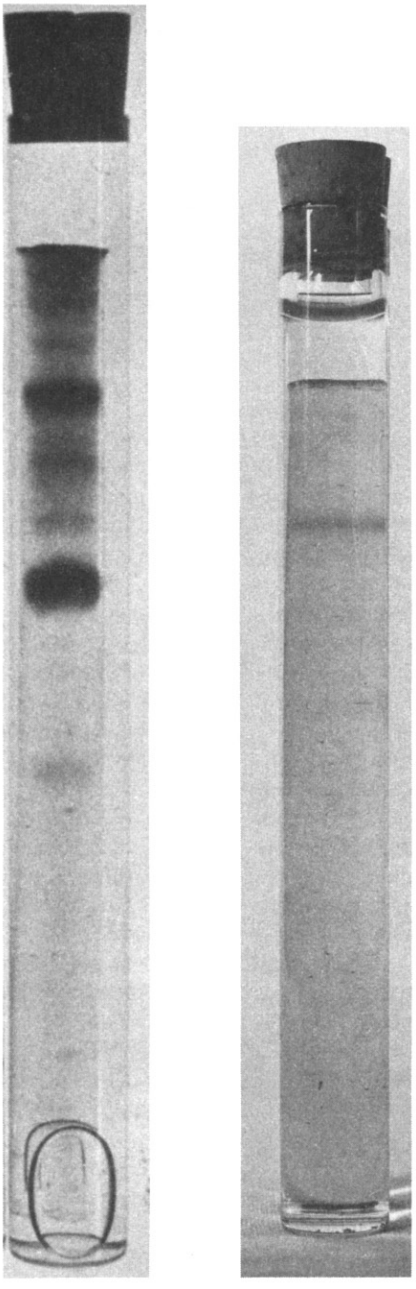

Fig. 2. Analytical polyacrylamide gel electrophoresis of original plasma (left) and purified material (right). Total gel concentration was 18% with 0.66% cross linkage.

in the presence of 0.1 M mercaptoethanol and again found no evidence for it being a tetramer.

Using the SDS-PAGE technique of Weber and Osborn[15], without reduction of disulphide bonds we observed a single sharp band which approximated to a molecular weight of 86,000. This molecular weight is likely to be an overestimate due to decreased SDS binding; therefore, the molecular weight used in calculations was 80,000 which was derived by adding up the molecular weights of the four components observed when the sample was run after preincubation with $\beta$-mercaptoethanol. The four components observed were very close together and their individual electrophoretic mobilities approximated to a molecular weight of 20,000 $\pm$ 500. In the light of these findings and those of Bohn[3] for human and Suzuki *et al.*[8] for bovine SHBG, it is tempting to suggest that human SHBG is a tetramer with monomers differing only slightly in their molecular weights.

SDS-PAGE is perhaps not the most reliable technique for the determination of molecular weights of

Table 2. Amino-acid and carbohydrate compositions

| Amino acid | g/100 g polypeptide | mol/mol SHBG |
|---|---|---|
| Asp | 12.76 | 76.8 |
| Thr | 5.48 | 36.8 |
| Ser | 6.86 | 52.3 |
| Glu | 12.61 | 68.6 |
| Gly | 6.28 | 67.0 |
| Ala | 8.08 | 72.6 |
| Val | 10.48 | 71.7 |
| Ile | 5.74 | 35.1 |
| Leu | 11.62 | 71.0 |
| Tyr | 5.22 | 23.1 |
| Phe | 5.10 | 24.7 |
| His | 3.07 | 15.8 |
| Pro | 6.70 | 46.6 |
| Carbohydrate | *g/100 g carbohydrate* | |
| Fucose | 8.70 | |
| Mannose | 14.36 | |
| Galactose | 16.75 | |
| N-acetyl glucosamine | 27.33 | |
| Sialic acid | 32.83 | |
| Total carbohydrate content 34.4% | | |

For aminoacid analysis the sample was hydrolysed in 6 N HCL at 110°C for 24 h.

The molecular weight used for the calculation of the molar ratios of the aminoacids was 80,000.

glycoproteins as pointed out by Mickelson *et al.*[5] who obtain three different molecular weight values for human SHBG using three different techniques. Gel filtration again is not a reliable technique for glycoproteins as these are relatively more hydrated and give falsely elevated molecular weight values. We used SDS-PAGE because it was one of the two techniques available to us (the other being gel filtration) and also because it served as a good means of comparison between our purified material and that of Bohn[3] and Rosner and Smith[4] who also employed similar techniques. Accepting the discrepancies in the molecular weight determination using the SDS-PAGE, our value of 80,000 is in good agreement with the value of 94,000 reported by Rosner and Smith[4].

Obviously the molecular weight values given by ourselves, Bohn[3] and Rosner and Smith[4] are subject to error and it would appear that equilibrium sedimentation had best be applied for the determination of molecular weight of glycoproteins as the technique likely to give the most accurate results [5]. Mercier *et al.*[2] did indeed use equilibrium sedimentation for determining the molecular weight of SHBG, their data as shown by Rosner and Smith[4] approximates to a molecular weight value of around 100,000. This would seem to agree more with our figure than with that reported by Mickelson *et al.*[5].

Using SDS-PAGE Mickelson *et al.*[5] calculate a molecular weight of 52,000 for SHBG; the authors suggest that this low molecular weight value is probably due to the low carbohydrate content (18%) of their purified material. It seems unlikely that the higher molecular weight obtained by ourselves and Rosner and Smith[4] is due to an impurity as Mickelson *et al.*[5] seem to imply since none was detected by electrophoretic techniques. Besides, the figures for number of binding sites per mole DHT given by ourselves and Rosner and Smith seem to suggest a highly purified material in both cases.

Discrepancies in the results of individual amino acid content and sugar analysis pose another problem. Cysteine and methionine were not measured and tryptophan is destroyed under the conditions of hydrolysis; the absence of lysine and arginine is remarkable. Remarkable also is the peak of unidentified material which precedes the expected but unobservable peak of ammonia; it is suggested [17] that this peak may be due to sialic acid and that arginine, lysine and ammonia may be "bound up" with this peak and therefore avoid detection. To what extent this phenomenon is common to glycoproteins is uncertain and the suggestion that the carbohydrate moeity interferes with the detection of certain amino acids must remain an hypothesis. In the data presented by Suzuki *et al.*[8] for amino acid analysis of bovine SHBG, cysteine is missing, it is not stated whether an attempt was made to measure it as cysteic acid.

In spite of the conflicting results, it is, nevertheless, encouraging that the isolated protein retains all of its binding activity and shows 1.08 binding sites per mol of DHT and that our results are in agreement with those of Rosner and Smith[4] on this and on the carbohydrate content, our value of 34.4% carbohydrate compares well with their 32.1%. We get very poor recovery, however, 5% as compared to about 35% reported previously [4, 5]. The heavy loss of material after the first purification step is perhaps due to high ratio of steroid per ml of Sepharose which may require an even higher concentration of the competing ligand in the elution buffer. Electrophoretic elution [7] may provide the answer to this if we are able to overcome the problem of denaturation caused by this technique.

*Acknowledgements*—We are grateful to Sister Mylrea of Queen Charlotte's Maternity Hospital for providing us with post-partum plasma and to Dr. J. Walker of the Institute of Cancer Research, London S.W.3 for amino acid analysis. We are also grateful to Dr. M. A. Bukhari of the Institute of Cancer Research, Sutton, Surrey for carbohydrate analysis. This work was funded by a grant from Wellcome Trust, U.K.

## REFERENCES

1. Burstein S. H.: The removal of testosterone binding globulin from plasma by affinity chromatography. *Steroids* **14** (1969) 263–267.
2. Mercier-Bodard C., Alfsen A. and Baulieu E. E.: Sex steroid binding plasma protein (SBP). *Acta endocr., Copenh. Suppl. No.* **147** (1970) 204–238.
3. Bohn H.: Isolierung, Charakterisierung und quantitative immunologische Bestimmung des Steroid-bindenden β-Globulins. *Blut* **XXIX** (1974) 1–15.

4. Rosner W. and Smith R.: Isolation and characterization of the testosterone-oestradiol-binding globulin from human plasma. Use of a novel affinity column. *Biochemistry* **14** (1975) 4813–4819.
5. Michelson K. E., Teller D. C. and Petra P. H.: Characterization of the sex steroid binding protein of human pregnancy serum. Improvements in the purification procedure. *Biochemistry* **17** (1978) 1409–1415.
6. Hampl R., Taborsky O., Musli J. and Starka L.: The use of an Enzacryl AA derivative for affinity chromatography of sex hormone binding globulin. *J. Chromatogr.* **97** (1974) 57–63.
7. Iqbal M. J., Ford P. and Johnson M. W.: Purification of sex hormone binding globulin using an affinity matrix in an electrophoretic cell system. *FEBS Lett.* **87** (1978) 235–238.
8. Suzuki Y., Itagaki E., Mori H. and Hosoya T.: Isolation of testosterone binding globulin from bovine serum by affinity chromatography and its molecular weight characterization. *J. Biochem.* **81** (1977) 1721–1731.
9. Cautrecasas P. and Anfinson C. B.: Affinity chromatography. *Methods Enzymol.* **XXII** (1971) 345–378.
10. Heyns W. and De Moor P.: A3 (17)$\beta$-Hydroxysteroid dehydrogenase in rat erythrocytes. Conversion of 5$\alpha$-Dihydrotestosterone into 5$\alpha$-Androstane-3$\beta$, 17$\beta$-diol and purification of the enzyme by affinity chromatography. *Biochem. biophys. Acta* **358** (1974) 1–13.
11. Iqbal M. J. and Johnson M. W.: Study of steroid-protein binding by a novel "two-tier" column employing Cibacron Blue F3G-A-Sepharose 4B. I—Sex hormone binding globulin. *J. steroid Biochem.* **8** (1977) 977–983.
12. Iqbal M. J. and Johnson M. W.: Determination of binding capacity of testosterone-oestradiol binding globulin, TeBG, with prior immunoprecipitation of albumin. *I.R.C.S. Med. Sci.* **4** (1976) 231.
13. Lowry O. H., Rosenbrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the Folin phenol reagent. *J. biol. Chem.*, **193,** (1951) 265–275.
14. Smith I. ed (1968) In *Chromatographic and Electrophoretic Techniques*. Vol. 2. Heinemann, London. pp. 365–399.
15. Weber K. and Osborn M.: Reliability of molecular weight determinations by dodecyl sulphate—polyacrylamide gel electrophoresis. *J. biol. Chem.* **16** (1969) 4406–4412.
16. Clamp J. R., Bhatti T. and Chambers R. E.: The determination of carbohydrate in biological materials by gas liquid chromatography. *Meth. biochem. Anal.* **19** (1971) 229–334.
17. Walker J.: Personal Communication (1978).