Monatshefte für Chemie 114, 453—463 (1983)

Monatshefte für Chemie
Chemical Monthly

Cholesteryl Sulfate in Meconium: Inhibitory Action on Rabbit Anti-M and Anti-N Antisera

Diethelm Gauster and **Hans Tuppy***

Institut für Biochemie, Universität Wien, A-1090 Wien, Austria

(Received 13 September 1982. Accepted 29 September 1982)

A lipid present in meconium and capable of inhibiting the agglutination of human M and N erythrocytes by rabbit anti-M and anti-N antibodies has been isolated and identified as cholesteryl sulfate. The reaction of human anti-A and anti-B antisera with the respective human red blood cells is not impaired by cholesteryl sulfate. Cholesteryl hemisuccinate has been found to be an hemagglutination inhibitor qualitatively similar to but less active than cholesteryl sulfate.

(Keywords: Cholesteryl sulfate; Blood group M and N; Meconium)

Cholesterylsulfat in Mekonium:
Hemmende Wirkung auf Kaninchen anti-M und anti-N Antiseren

Eine lipophile Substanz, welche die Agglutination humaner Erythrocyten durch Kaninchen anti-M und anti-N Antiseren inhibiert, konnte aus Mekonium isoliert und als Cholesterylsulfat identifiziert werden. Die Reaktion humaner anti-A und anti-B Antiseren mit den jeweiligen A und B Erythrozyten wurde durch Cholesterylsulfat nicht beeinflußt. In bezug auf die Agglutinationshemmung verhält sich der Monocholesterylester der Bernsteinsäure ähnlich wie der der Schwefelsäure, ist jedoch erst in höherer Konzentration wirksam.

Introduction

Meconium is known to contain glycoproteins having blood group M and N activity[1,2]. In addition, a chloroform-methanol soluble material has been extracted from meconium by *R. H. Côté*[2] and reported to inhibit the agglutination of M and N erythrocytes by anti-M and anti-N antibodies. Although assumed to be of glycolipid nature[2], this or these hemagglutination inhibitor(s) have so far not been identified chemically nor has their serological specificity been determined. The present study was undertaken to isolate and characterize the inhibitory material.

0026-9247/83/0114/0453/$ 02.20

Experimental

Extraction of Lipids from Meconium

Meconium (300 g wet weight) was lyophilized. After removal of acetone-soluble compounds, the residue was extracted with 1 l each of chloroform-methanol mixtures 2:1, 1:1, and 1:2, and finally with 1 l methanol. The combined extracts were evaporated to dryness in vacuo. The dry material was dissolved in 100 ml hot methanol, the solution cooled down to room temperature, the resulting precipitate rejected, and the solution evaporated again; the same procedure was repeated using 70 ml propanol-1. The residue (800 mg) obtained by evaporation of the propanolic solution contained the biological activity. It was washed with acetone and diethylether (1 l each) and stored in a dessicator over phosphorus pentoxide.

DEAE Cellulose Column Chromatography

The procedure used was that described by *Rouser* et al.[3]. A column 2.4 cm in diameter filled with DEAE cellulose to a height of 22 cm was employed repeatedly for the fractionation of 200 mg aliquots of meconium lipid. Elution was carried out by successively passing through the column 750 ml each of the following mixtures (*v/v*): 1. chloroform/methanol 9:1; 2. chloroform/methanol 7:3; 3. absolute methanol; 4. chloroform/acetic acid 5:1 followed by 500 ml absolute methanol; 5. chloroform/methanol 4:1 plus 10 ml concentrated ammonia per litre of the mixture; 6. chloroform/methanol 2:1 plus 40 ml concentrated ammonia per litre of the mixture; 7. acetic acid containing 0.1 mol/l ammonium acetate.

Silica Gel Column Chromatography

A column (1.4 × 20 cm) filled with silica gel (Merck, Kieselgel H 60, particle size 10–40 μm, previously freed from particles too fine or too large by sedimentation in chloroform/methanol 1:1) was used for the fractionation of 25 mg aliquots of the lipid which had emerged from the DEAE cellulose column with chloroform/methanol 4:1 + NH_3 (fraction 5). For elution a linear solvent gradient was prepared by adding 120 ml of solution II (chloroform/methanol/25% ammonia 60:30:4 *v/v/v*) to 80 ml of solution I (chloroform/methanol/25% ammonia 90:10:0.5 *v/v/v*). Fractions were collected on a LKB Ultrorac 7000 equipped with Uvicord II (UV-filter at 253 nm).

Preparative Thin Layer Chromatography

Polyamide foils (20 × 20 cm) covered with SiO_2 containing a fluorescence indicator (254 nm; Schleicher & Schüll F 1500 LS 254) were used after washing with propanol-1 and methanol. Per foil, 3–4 mg of sample were spotted on a line of 15 cm, with reference substances (lecithine, lysolecithine, and oleic acid) on either side. The chromatograms were developed twice with a mixture of dioxane/dimethylformamide/triethylamine 40:10:1 (*v/v/v*), dried in a vacuum dessicator and inspected under UV light (fluorescence quenching). The side edges of the foils were sprayed with ammonium molybdate[8]. Three bands whose migration was similar to that of oleic acid were scraped off and eluted with 90% methanol. Colloidal SiO_2 was removed by partition of the dried eluates between butanol-1 and water; the butanol phase, which contained the active material, was brought to dryness.

Preparative Thin Layer Electrophoresis

The foils used for electrophoretic separations (150 V cm^{-1}) were the same as for thin layer chromatography (see above). The buffer was a mixture of propanol-1/propanol-2/water 1:1:1 (*v/v/v*) + 1% acetic acid and 0.6% pyridine. Picric acid served as a marker. Detection and elution of the bands ($R_{\text{picric acid}}$ 0.5-0.6) were performed as in thin layer chromatography.

Analytical Thin Layer Chromatography

Polyamide foils and the following solvent mixtures were used for ascending chromatography: A: chloroform/methanol/25% ammonia/water 60:30:4:4 (*v/v/v/v*) lower phase; B: chloroform/methanol/acetic acid/water 60:30:4:4 (*v/v/v/v*); C: propanol-1/water/25% ammonia 6:2:1 (*v/v/v*); D: chloroform/methanol 9:1 (*v/v*); E: *n*-hexane/diethylether/acetic acid 100:30:1 (*v/v/v*); F: dioxane/dimethylformamide/triethylamine 40:10:1 (*v/v/v*).

Hemagglutination

25 µl of the antisera were serially diluted ($1:2^n$) on Salk plates with isotonic saline. To each dilution an equal amount of a 2% suspension of erythrocytes of the appropriate blood group was added. After 30 min at room temperature the plates were examined visually for agglutination. The highest dilution of the antiserum at which agglutination was detectible, was considered to contain 1 hemagglutination unit.

Hemagglutination Inhibition

25 µl of a solution containing the inhibiting material were serially diluted on Salk plates. Then an equal volume of antiserum (diluted so as to contain four hemagglutination units) was added. After incubation at room temperature for 30 min, 25 µl of a 2% erythrocyte suspension were added. 30 min later the Salk plates were inspected for hemagglutination.

Analytical and Synthetic Methods

Standard procedures were used to carry out N-acetylation[4], peracetylation[5], total acid hydrolysis[6], partial acid hydrolysis[6], mild basic methanolysis[7], acid methanolysis[24], acetolysis[9], determination of vicinal hydroxyl groups[25], and isotachophoretic determinations of sulfate according to *E. Kendler* and *J. F. K. Huber*[23]. The pyridinium salt of cholesteryl sulfate was prepared according to *McKenna* and *Norymbersky*[10]. Cholesteryl acetate[5], cholesteryl hemisuccinate[11], and cyclohexyl sulfate[12] were synthesized as described previously.

Adsorption of Cholesteryl Sulfate on Erythrocytes

200 µl of packed red blood cells (1 µl containing 1.15×10^7 cells[22]) added to a solution of 4 mg cholesteryl sulfate (Na salt) in 4 ml isotonic saline containing 15% propanol-2 were incubated for 15 min at 37 °C and subsequently for 2 h at room temperature. The cells were centrifuged and washed twice with 2 ml each of isopropanolic saline. In order to assess the amount of non-adsorbed cholesteryl sulfate the supernatants were collected and the slightly turbid solution was extracted three times with butanol. Upon evaporation of the butanol phases the residue was solvolyzed with 500 µl glacial acetic acid[9]. The

reaction mixture was diluted with 2 ml water and extracted three times with *n*-hexane. In the water phase sulfate was determined gravimetrically as $BaSO_4$ and compared with two blanks (omission of red blood cells and cholesteryl sulfate, respectively).

Rabbit Antisera

Anti-M (lots no. 19380; 19381; 19376; 19327) and anti-N (lots no. 21378; 21377; 21375) were obtained from Molter (Heidelberg, FRG); they failed to agglutinate neuraminidase-treated human red blood cells and thus proved to be sialic acid dependent. Anti-M (12303) from Biotest (Frankfurt FRG), anti-M from Behring (Marburg FRG) as well as anti-N from Behring (lots no. 4215A, resp. 6119C) and anti-N (3291/79) from Serco (Bonn FRG) were not examined for sialic acid dependency.

Human Antisera

Anti-A antisera were obtained from Immuno (Vienna, Austria, lot no. 2565), from Ortho (Don Mills, Ontario, U.S.A., lot no. AC 3601A) and from a patient immunised during gravity by fetal blood group A erythrocytes; the anti-B antiserum used was purchased from Immuno (lot no. 08372). A human anti-N antiserum, a generous gift of Dr. *M. N. Metaxas* (Zurich), which contained some anti-B and anti-T activity too, was absorbed on desialized B MM erythrocytes before use, and proved to be essentially inactive against desialized human NN erythrocytes.

Solvents and Reagents

Unless otherwise described, they were p.a. grade and purchased from Merck (Darmstadt, FRG).

Results

Isolation

The lipids extracted from meconium (800 mg) were fractionated on a DEAE cellulose column. Hemagglutination inhibiting activity was eluted by chloroform-methanol mixtures containing ammonia (fractions 5 and 6). The highest specific activity was found in fraction 5. Fraction 6, which contained considerable amounts of the active material, was rechromatographed on DEAE cellulose, the active fraction being combined with fraction 5. The combined fractions (80 mg) were subjected to silica gel column chromatography. The most active material (9 mg) inhibited agglutination of blood group N and M erythrocytes by anti-N and anti-M antisera at concentrations of 0.5 mg/ml and 1 mg/ml, respectively. When tested by qualitative thin layer chromatography, a group of three narrow bands having R_f values of about 0.6 in solvent systems A and B could be detected.

Using preparative thin layer chromatography on silica gel, the best resolution was achieved with a mixture of dioxane, dimethylformamide and triethylamine (solvent system F). The second of three closely

adjacent bands was strongest and contained most of the active material (4 mg). Thin layer electrophoresis allowed a further purification of the active compound (2.5 mg) from minor amounts of two substances both migrating ahead. This component inhibited agglutination of N and M erythrocytes by the appropriate antisera at concentrations of 0.25 mg/ml and 0.5 mg/ml respectively. It did not interfere with the agglutination of A and B erythrocytes by anti-A and anti-B antisera.

Identification

For identification the purified substance was subjected to various chemical treatments. Neither periodate oxidation, N-acetylation or peracetylation nor mild basic methanolysis or mild acid hydrolysis resulted in a detectable change of its chromatographic behaviour and its serological activity. Total hydrolysis, however, destroyed its activity. After partition of the reaction mixture between *n*-hexane and water, the hexane phase was shown to contain a single neutral lipid, which was identified as cholesterol by thin layer chromatography (systems D and E) and by mass spectroscopy. In the water phase which was submitted to thin layer chromatography (solvent system C) there was no substance that could be traced by UV absorption, fluorescence quenching, or colour reactions using ammonium molybdate, orcinol, resorcinol or periodate—benzidine[8]; a fast moving ion, however, was detected by isotachophoretic analysis and proved to be sulfate. Acid methanolysis differed from total acid hydrolysis in yielding, besides cholesterol, an anion which comigrated in isotachophoresis with synthetic methylsulfate. Acetolysis gave a neutral lipid, which could be extracted with hexane and was shown to be cholesteryl acetate by thin layer chromatography (system E), mass spectrometry, and by its melting point (114–116 °C), which did not change when mixed with synthetic cholesteryl acetate. Sulfate was identified in the water phase.

The identity of the active substance from meconium with cholesteryl sulfate was further proved by IR spectroscopy (Fig. 1), NMR spectra (Fig. 2) and mass spectroscopy.

Cholesteryl sulfate obtained by esterification of cholesterol had the same serological activities as the lipid isolated from meconium. Thus, for a further study of its biological properties, the synthetic cholesteryl sulfate was used.

Interaction of Cholesteryl Sulfate with Antisera and Red Blood Cells

Cholesteryl sulfate totally inhibited the agglutination of human N and M red blood cells by rabbit anti-N (Molter lot no. 21375) and anti-M

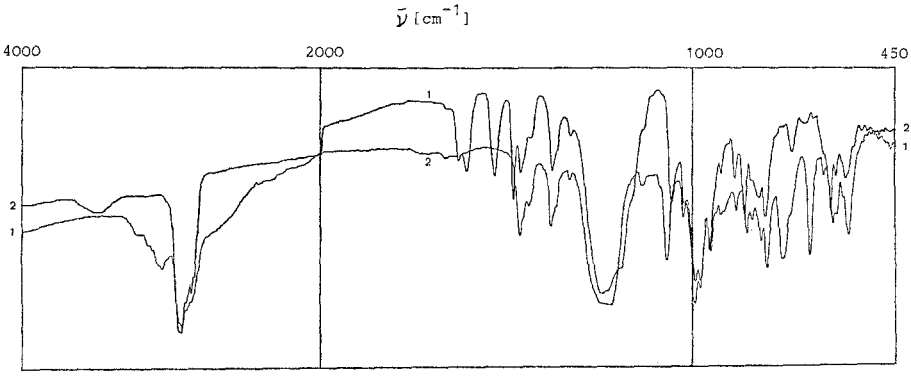

Fig. 1. IR spectra of cholesteryl sulfate (pyridinium salt) isolated from meconium (curve 1) and synthetic cholesteryl sulfate (Na salt) (curve 2). In addition to the absorption bands of cholesterol and pyridine the spectra show the strong absorption bands of the SO_4 group at 990 cm^{-1} and 800 cm^{-1} as well as at 1230 cm^{-1} the antisymmetric SO_4 oscillation[12,13]

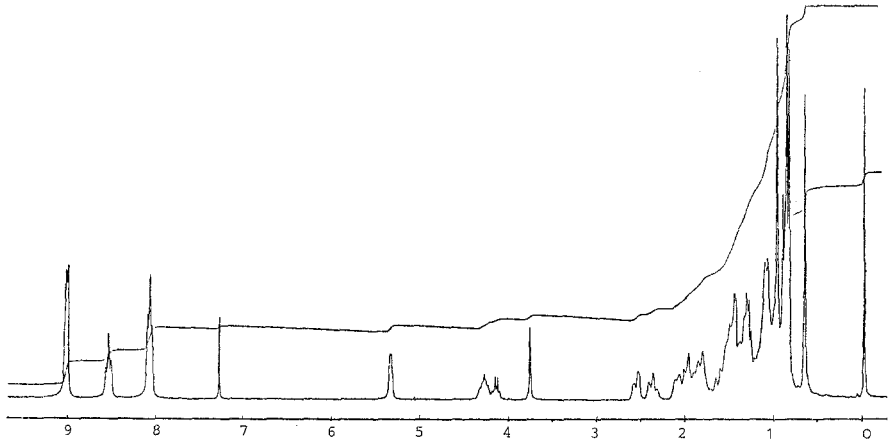

Fig. 2. 250 MHz-^{1}H NMR spectrum of cholesteryl sulfate (pyridinium salt) isolated from meconium. The signals at $\delta = 0.68$ ppm and 0.98 ppm are caused, respectively, by the two angular methyl groups C-18 and C-19. The three methyl groups of the side chain give rise to the doublets at 0.87 ppm (C-26 and C-27) and 0.91 ppm (C-27). Further characteristic signals are found at 5.35 ppm (1 proton in position 6), at 4.28 ppm (1 in position 3), and at 2.6/2.4 (2 protons at C-4, showing vicinal and geminal coupling). Pyridinium-proton signals are found at $\delta > 8$ ppm. The presence of the 5 methyl groups and the ratio of the signals of the protons in the positions 6, 3, 4, 18 (= 1:1:2:3) indicate that the cholesteryl moiety is intact. Synthetic cholesteryl sulfate pyridinium salt gives the same spectrum without the weak signals at 4.15 and 3.75 ppm which are obviously due to trace impurities

(Molter lot no. 19380) antisera at concentrations of 0.125 μmol/ml and 0.25 μmol/ml, respectively. These rabbit antisera contained anti-N and anti-M antibodies of the IgG class exclusively. Cholesteryl sulfate blocked several rabbit anti-N and anti-M antisera as well, though not in every case at the same concentration. In general, less cholesteryl sulfate was required to inhibit anti-N as compared with anti-M antisera (Table 1).

The agglutination of human A and B erythrocytes by anti-A (Immuno lot no. 2565, Ortho lot no. AC 3601 A) and anti-B (Immuno lot no. 08372) was not impaired by cholesteryl sulfate. As verified by chromatography on Sephadex G-200, the respective isoagglutinins were of the IgM type. From the serum of a patient who underwent immunisation against A erythrocytes in the course of pregnancy, IgG antibodies in addition to IgM were isolated by gel filtration, and cholesteryl sulfate was shown to be incapable of inhibiting the anti-A activity of IgG as well as of IgM at all concentrations tested (up to 2 μmol/ml).

Although the hemagglutination caused by human anti-A and anti-B active immunoglobulins was not affected, cholesteryl sulfate nevertheless interacted with these proteins. After addition of human anti-A and anti-B antisera the capacity of cholesteryl sulfate to inhibit the agglutination of M and N erythrocytes by the appropriate rabbit antisera was reduced. Several other proteins or protein fractions such as bovine serum albumin and human and rabbit IgG had a similar effect (Table 2). The uptake of cholesteryl sulfate by serum albumin and by the human IgG fraction could also be shown by conductivity measurements (unpublished results).

Cholesteryl sulfate is furthermore strongly absorbed by erythrocytes. Under conditions, however, where as much as 3.6 mg of cholesteryl sulfate had been taken up by 2.3×10^9 red blood cells, their agglutinability by antisera (anti-M, anti-N, anti-A and anti-B) was not changed.

Effect of Substances Structurally Related to Cholesteryl Sulfate

Cholesteryl hemisuccinate proved to be half as active as cholesteryl sulfate in inhibiting the hemagglutinating action of rabbit anti-M and anti-N antisera, whereas comparable concentrations of cyclohexyl sulfate had no detectable effect. Cyclohexyl sulfate at elevated concentrations ($\geqq 2$ μmol/ml) and sodium dodecyl sulfate ($\geqq 0.15$ μmol/ml) could not be tested on account of their hemolytic action.

Table 1. *The amount of cholesteryl sulfate (CS) required to inhibit the agglutination of human red blood cells by different antisera, diluted to four hemagglutination units (HU)*

anti-M antisera origin lot no.	HU of the undiluted sera	CS required μmol/ml	anti-N antisera origin lot. no.	HU of the undiluted sera	CS required μmol/ml
		Rabbit abtisera			
Molter			Molter		
19376	16	0.5	21375	24	0.125
19380	64	0.25-0.5	21377	16	0.25
19381	32	0.5	21378	16	0.25
Biotest			Serco		
12303	4	1	3291/79	8	1
Behring			Behring		
4215 A	96	0.125-0.25	6119 C	4	2
		Human antisera			
			Metaxas		
			143	16	1-2

Table 2. *Concentration of cholesteryl sulfate (μmol/ml) required to inhibit the agglutination of human N erythrocytes by rabbit anti-N antiserum (Molter 21378) in the presence of additional protein*

Protein added	Concentration of cholesteryl sulfate (μmol/ml)
Blank (pure saline)	0.312
Bovine serum albumin	0.625–1.25
Human IgG	1.25–2.5
Rabbit IgG	2.5

25 μl of a serially diluted cholesteryl sulfate solution was preincubated with 25 μl saline (blank) or 25 μl of a solution containing 0.625 μmol protein per ml isotonic saline for 30 min at room temperature before adding the rabbit antiserum as described under Experimental (hemagglutination inhibition).

Discussion

Cholesteryl sulfate is an ubiquitous entity in mammalian tissues and body fluids[13–16]. Small intestinal mucosa has been among the human tissues in which cholesteryl sulfate was identified[20]. In the metabolism of cholesterol esterification with sulfate is an important initial step[17–19]. Cholesteryl sulfate is taken up by cell membranes and has been found to stabilize red blood cells against hypotonic hemolysis[19]. In addition, various proteins, e.g. serum albumin, are capable of binding cholesteryl sulfate rather strongly[21].

As reported in this paper, cholesteryl sulfate turned out to account for most of the inhibitory action which lipid extracts of meconium exert on the agglutination of human M and N erythrocytes by the respective rabbit antibodies.

Since cholesteryl sulfate interacts with biological membranes as well as with soluble proteins, the question arises whether M and N erythrocyte membranes by incorporating cholesteryl sulfate are rendered inagglutinable by the antisera or, rather, immunoglobulins in the presence of cholesteryl sulfate are prevented from agglutinating the erythrocytes. The experimental data indicate that the accessibility to antibodies of M and N agglutinogens on the red cell membrane is not affected by cholesteryl sulfate, whereas anti-M and anti-N antisera preincubated with cholesteryl sulfate fail to agglutinate erythrocytes. The interference of cholesteryl sulfate with the agglutination of both M and N erythrocytes indicates that it lacks narrow serological specifity, although on the other hand, it does not impair the agglutination of human A and B erythrocytes by anti-A and anti-B antibodies and thus fails to be an universal hemagglutination inhibitor.

A human anti-N antiserum was blocked like several rabbit anti-N antisera by cholesteryl sulfate though at somewhat higher concentrations. Therefore, the interaction does not depend on some species-specific feature of rabbit antibodies. Furthermore, the fact that cholesteryl sulfate interferes with rabbit anti-N of the IgG type—but not with human anti-A of the IgG type—shows that its inhibitory action is not directed against G-type immunoglobulins in general. Thus, it appears that cholesteryl sulfate indeed reacts with the antigen-binding sites of anti-M and anti-N antibody molecules.

There is, however, no obvious resemblance between cholesteryl sulfate and the antigen determinant sites of M and N blood group substances, apart from the fact that sulfate groups, like the sialic acid moieties, are anionic sites, and that the cholesteryl moiety, like the N-terminal leucyl residue of glycophorin A of blood group N, might fit into a hydrophobic binding site.

Sulfate esters other than cholesteryl sulfate were tested for their potential inhibitory action on the agglutinating activity of anti-M and anti-N antibodies. Neither cyclohexyl sulfate nor dextrane sulfate had any observable effect. Sodium dodecyl sulfate could not be assessed in the hemagglutination inhibition assay system due to its strong hemolytic activity. It is evident, anyhow, that the esterified sulfate group by itself does not account for the inhibitory activity, since the sulfate group in cholesteryl sulfate can be replaced by the hemisuccinate group without marked loss of inhibitory capacity.

Acknowledgements

We thank Prof. *J. Spona* (2nd Gynaecological Clinic of the University of Vienna) for helping to provide meconium, and Prof. *W. Mayr* (Institute of Blood Group Serology) for erythrocytes and serum samples. We are particularly grateful to Dr. *H. Schenkel-Brunner* for helpful discussions, and to Dr. *E. Kendler* and Dr. *A. Heresch* for the performance and interpretation of isotachophoretic and mass spectroscopic determinations. The kind assistance by Dr. *E. Haslinger* and Dr. *W. Mikenda* with respect to registration and interpretation of NMR and IR spectra is sincerely appreciated.

References

[1] *Springer G. F., Nagai Y., Tegtmeyer H.*, Biochemistry **5**, 3254 (1966).
[2] *Côté R. H.*, Blood and Tissue Antigens (*Aminoff D.*, ed.), p. 249. Academic Press. 1970.
[3] *Rouser G., Kritchevsky G., Yamamoto A., Simon G., Galli C., Bauman A. J.*, Methods in Enzymology **14**, 272 (1969).
[4] *Lisowska E., Wasniowska K.*, Eur. J. Biochem. **88**, 247 (1978).

[5] *Kościelak J., Piasek A., Górniak H., Gardas A., Gregor A.*, Eur. J. Biochem. **37**, 214 (1973).
[6] *Takasaki S., Kobata A.*, Methods in Enzymology **50**, 50 (1978).
[7] *Vance D. E., Sweeley C. C.*, J. Lipid Res. **8**, 621 (1967).
[8] *Skipski V. P., Barclay M.*, Methods in Enzymology **14**, 530 (1969).
[9] *Bergelson L. D., Vaver V. A., Prokazova N. V., Ushakov A. N., Rozynov B. V., Stefanov K., Ilukhina L. I., Simonova T. N.*, Biochim. Biophys. Acta **260**, 571 (1972).
[10] *McKenna J., Norymberski J. K.*, J. Chem. Soc. **1957**, 3889.
[11] *Klein B., Kleinman N. B., Foreman J. A.*, Clin. Chem. **20**, 482 (1974).
[12] *Lloyd A. G., Tudball N., Dodgson K. S.*, Biochim. Biophys. Acta **52**, 413 (1961).
[13] *Moser H. W., Moser A. B., Orr J. C.*, Biochim. Biophys. Acta **116**, 146 (1966).
[14] *Bleau G., Chapdelaine A., Roberts K. D.*, Can. J. Biochem. **50**, 277 (1972).
[15] *Hochberg R. B., Ladany S., Lieberman S.*, Endocrin. **94**, 207 (1974).
[16] *Iwamori M., Moser H. W., Kishimoto Y.*, Biochim. Biophys. Acta **441**, 268 (1976).
[17] *Banerjee R. K., Roy A. B.*, Biochim. Biophys. Acta **137**, 211 (1967).
[18] *Young D. G., Hall P. F.*, Biochemistry **8**, 2987 (1969).
[19] *Lalumière G., Longpré J., Trudel J., Chapdelaine A., Roberts K. D.*, Biochim. Biophys. Acta **394**, 120 (1975).
[20] *Falk K. E., Karlsson K.-A., Leffler H., Samuelsson B. E.*, FEBS Letters **101**, 273 (1979).
[21] *Lalumière G., Chapdelaine A., Bleau G., Roberts K. D.*, Steroids **27**, 247 (1968).
[22] *Wintrobe M.*, Clin. Hematology. Philadelphia: Lea & Febiger. 1961.
[23] *Kendler E., Huber J. F. K.*, Mikrochim. Acta **1980**, **II**, 271.
[24] *Gray G. M.*, Methods in Enzymology **14**, 678 (1969).
[25] *Dittmer J. C., Wells M. A.*, Methods in Enzymology **14**, 482 (1969).