PREPARATION OF ^{131}I-ASIALO-α_1-ACID GLYCOPROTEIN

by

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Summary

α_1-Acid glycoprotein(orosomucoid) was prepared from a byproduct of the ethanol plasma fractionation by means of ion-exchange procedures. Immunoelectrophoresis suggested a high degree of purity; the purified protein contained 13.5% sialic acid and 17.8% hexose.
The α_1-acid glycoprotein was modified by removal of sialic acid with neurominidase(E.C. 3.2.1.18) followed by iodination with ^{131}I.
The purpose of the preparation, its potential use as a pharmacon for liver-function studies in nuclear medicine, is the subject of further study.

Introduction

In earlier studies[1] it has been shown that a number of asialo-glycoproteins on i.v. injection disappeare from the circulation in a short time and go to the liver. In case of α_1-acid-glycoprotein the disappearence seemed to be the fastest[2].
The material, prepared as will be described in this paper, is intended for study of its metabolism in the rat and its possible application in nuclear medicine in liver function tests. These studies will be described elsewhere.

Materials

Neuraminidase, from Vibrio cholerae(500U/ml), and N-acetylneuraminic acid were obtained from Calbiochem(Los Angeles, USA), cat.no.480717 and 110137, resp.; human α_1-acid glycoprotein, as standard for the immunologic assay, mono specific rabbit antihuman α_1-acid glycoprotein antiserum and M-Partigen radial immunodiffusion plates for α_1-acid

glycoproteins were obtained from Behring Werke AG (Marburg/Lahn, German Federal Republic) cat.no. TFC 05, RCW05 and TBX 03, resp.; rabbit antihuman antiserum was obtained from the Central Laboratory of the Blood Transfusion Service of the Netherlands Red Cross (Amsterdam, The Netherlands); carrier free $K^{131}I$, 25mCi/mL, free of reducing agents was obtained from Philips Duphar (Petten, The Netherlands), cat.no. DRN 5320; Sephadex G-75, DEAE Sephadex A-50 and QAE Sephadex A-50 were obtained from Pharmacia (Uppsala, Sweden); Monodur 31 from Vereinigte Seidenwebereien (Krefeld, German Federal Republic); Membrane filters no. 1121, 0.2µ, from Schleicher and Schüll (Dassel, German Federal Republic) and membrane filter 0.22µ from Millipore Benelux (Brussels, Belgium); Special Agar Noble from Difco Laboratories (Detroit, U.S.A.)
Recrystalized bovine serum albumin, obtained from Fluka AC (Buchs, Switzerland), cat.no. 05470, was dried over P_2O_5 before use as standard in the Folin reaction.
All other chemicals were of reagent grade.

Methods

Determination of protein

α_1-Acid glycoprotein concentrations were determined by the radial immunodiffusion technique of Mancini et al[3], as modified by the manufacturers of the plates, using the above mentioned α_1-acid glycoprotein from Behring Werke AG as standard; by A^{1cm}_{280nm} measured with a Zeiss PMQII spectrophotometer, using $A^{0,1\%,\ 1cm}_{280nm} = 0.89$ [4]; and by the Folin reaction [5], using recrystallized bovine serum albumin as standard.

Sialic acid analysis

Sialic acid was determined by the method of Warren [6].

Hexose analysis

Total hexose content was determined with the phenol reaction as described by Ashwell.[7]

Immunological technique

Immunoelectrophoresis(I.E.) was carried out in 1,3% Special Agar Noble, 0.05 M veronal buffer, pH 8.6, according to Peetoom.[8]
Macro-Ouchterlony (double agargel diffusion) plates were prepared according to Brummelhuis[9] in 0.9% agarose. The holes had a diameter of 0.7 cm, and the distance from core to core was 1.1 cm.
Antisera against the α_1-acid glycoprotein preparation were produced in rabbits. Intramuscular injections, totalling 1 ml 0.05% protein and 1 ml of Freund's complete adjuvant, were given divided over several parts of the body. Four weeks later this procedure was repeated with Freund's incomplete adjuvant. Antisera were collected three weeks after the second injection.

Preparation

Purification

The starting material consisted of the supernatant of fraction V, as obtained by fractionation of human plasma according to a Cohn VI procedure, as modified by Brummelhuis et al.[10] It constitutes a diluted plasma containing 40% ethanol, from which only a few, mainly acid, proteins have not been removed (ionic strength Γ = 0.2, pH 4.8-5.0, protein content less than 0.02%).
Fifty liters of this supernatant were diluted four times with H_2O, and the pH was adjusted to 4.5 with acetic acid.
At 4°C, 250 g of dry DEAE-Sephadex A 50 were added. The suspension was stirred

for 90 minutes and allowed to settle for 30 minutes. The clear supernatant was then pumped off and the slurry was transferred to a 30 l filterholder (Seitz) and collected on a nylon cloth with 30µ pores (Monodur 31).

Air pressure (0.2 atm) was applied to remove most of the fluid. The DEAE-Sephadex was then washed with 0.1 M acetate buffer, pH 4.5, (0.1 M acetic acid, pH adjusted with NaOH) in four portions of 6 l each. Elution was carried out with 5 l of 1 M acetate buffer pH 5.7 and fractions of 0.5 l were collected.

Eight fractions with $A_{280nm}^{1cm} > 0.7$ were pooled, dialyzed overnight against 200 l water at 4°C and lyophilized.

The powder was suspended in 120 ml water, dialyzed against water at room temperature until a conductivity of $0.014\Omega^{-1}cm^{-1}$, the value of 0.9% saline solution, was reached, and sterilized by filtration through a membrane filter (Schleicher and Schüll).

The crude concentrate of proteins with low I.E.P. obtained in this way contained appr. 5% protein, as was shown by its $A_{280\ nm}^{1\ cm}$.

The greater part was identified as α_1-acid glycoprotein by means of immunoelectrophoresis and Macro-Ouchterlony technique using a specific antiserum (Behring Werke AG).

An estimation based upon radial immunodiffusion indicated that roughly 85% of the protein in the preparation was α_1-acid glycoprotein.

In order to reach a higher degree of purity, chromatography was performed with a QAE-Sephadex A-50 column(30cm x 2cm) equilibrated with 0.02 M acetate buffer pH 4.5. Three milliliter of the crude α_1-acid glycoprotein concentrate was diluted ten times with water and applied to the column. Extensive washing was then carried out with successive 1 l portions of 0.02 M acetate buffer pH 4.5, 0.03 M acetate buffer pH 4.25, 0.05 M acetate buffer pH 4.00 and 0.5 M acetate buffer pH 3.75, while the $A_{280\ nm}^{1\ cm}$ of the eluate was continuously monitored.

Upon subsequent elution with 2 M acetate buffer pH 3.2, fractions with $A_{280\ nm}^{1\ cm} > 0.3$ were pooled, dialyzed against water, lyophilized, resuspended in water, dialyzed against water until a conductivity of $0.014\Omega^{-1}cm^{-1}$ was attained and sterilized by filtration through a membrane filter (Millipore).

Analysis

The 10 ml of 0.5% α_1-acid glycoprotein preparation, obtained as described above, were judged to be highly pure because of the single precipitation band in immunoelectrophoresis (Fig. 1). In order to further investigate its purity, antisera were produced in four rabbits against this purified preparation.

Macro-Ouchterlony analysis (Fig. 2) and immunoelectrophoresis (Fig. 3), employing these sera, revealed a very high grade of specificity since the serum from only one animal gave a faint indication of the presence of a minute amount of contaminant antigen in the α_1-acid glycoprotein preparation. On elution from a Sephadex G-75 column (35 cm x 2 cm) equilibrated with saline solution only one U.V. absorbing peak, without shoulders, was observed.

Protein concentration in the purified preparation was measured by radial immunodiffusion; by determination of $A_{280\ nm}^{1\ cm}$; and by the Folin reaction, resulting in 5.28 mg/ml, 6.20 mg/ml and 3.85 mg/ml, resp.. The last value represents purely the protein moiety. Correction for the carbohydrate content [11] yields 6.41 mg α_1-acid glycoprotein per ml. Determination of sialic acid and total hexose contents yielded 13.5% and 17.8% resp., both based on a protein content of 5.28 mg/ml. Heimburger et al.[11] reported for these values 12.1% and 14.7%, resp.

Removal of sialic acid

α_1-Acid glycoprotein was modified by the method of Morell et al.[12]
A solution of the protein and neuraminidase was incubated under conditions as described in the legend of Fig. 4.
At various times aliquots were analyzed for free sialic acid (Fig. 4).
The preparation from which the 10 hour aliqout was taken was used for iodination.

Iodination with ^{131}I

Asialo-α_1-acid glycoprotein was iodinated by the chloramine-T procedure[13], modified as follows: 0.55 ml of the mixture described above (which had been incubated for 10 hours with neuraminidase) was mixed with 0.25 ml 0.5 M sodium phosphate pH 7.7. Then 0.2 ml $K^{131}I$ was added, immediately followed by 1.0 ml 0.2% chloramine-T.
The solution was stirred for one minute after which 1.0 ml 0.75% $NaHSO_3$ and 2.0 ml 2% KI were added. The final product was passed through a Sephadex G-75 column (35 cm x 2 cm), equilibrated and eluted with saline solution, to seperate the iodinated protein from the excess $^{131}I^-$.
From the results of the ^{131}I-determination in the fractions of the eluate it was computed that approximately 90% of the radioactivity was protein bound, eluted well separated from the free $^{131}I^-$.

Discussion

The method of purification described above has the advantage that the starting material is rather cheap and readily available and that it yields a material of a high degree of purity as shown in Figs. 1, 2 and 3.
Furthermore, this procedure is relativily simple and lends itself

to scaling-up, as anticipated in case the material will be shown to be of clinical use. However, it is realized that in such a case not only problems of sterility and pyrogenity must be faced but also that such preparations must be free of neuraminidase, the presence of which could lead to antigen-antibody reaction after repeated parenteral administration. Work in progress aims at solving these problems by using a carrier-bound enzyme.

Whether the discrepancy between the results of the radial immunodiffusion and of the $A_{280}^{1\ cm}{}_{nm}$ measurements indicates an unidentified component, that escaped detection by the other immunotechniques, can not be answered at the present time.

A preparation as presented is, however, suitable for animal experiments. In these experiments, but also in the analytical procedures, the presence of neuraminidase does not interfere, since it represents less than 0.1% of the total protein content.

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Figure 1. Immunoelectrophoresis of the purified α_1-acid glycoprotein preparation (wells 1 and 3) against rabbit anti human serum (trough A) and commercially obtained specific rabbit anti-human α_1-acid glycoprotein (trough B);for reference the electrophoresis pattern of ten times diluted standard human plasma (well 2) is given.

Figure 2. Macro-Ouchterlony analysis of the purified α_1-acid glycoprotein preparation (central well) against antisera produced against it in two out of four rabbits. Wells A contain undiluted serum, wells B and C serum diluted 2 and 4 fold, resp.
The faint precipitation line near well A of rabbit 2 may hint at the presence of a slight amount of contaminating substance in the α_1-acid glycoprotein preparation. The three other rabbit sera did not show this phenomenon.

Figure 3. Immunoelectrophoresis of human standard serum (wells) against the antiserum (trough) produced against the purified α_1-acid glycoprotein preparation in rabbit 2 (see Fig.2).

Figure 4. Removal of sialic acid as function of time. A mixture of 1 ml of α_1-acid glycoprotein (= 5.28 mg), 0.07 ml 0.5 M acetate buffer, pH 5.65, containing 0.15 M NaCl, 0.02 ml 0.2 M $CaCl_2$. 0 aq. and 0.20 ml neuraminidase (= 100 U) was incubated at 37°C for up to 10 hours.
Plotted are the percentages of sialic acid liberated in the various aliquots as function of time.

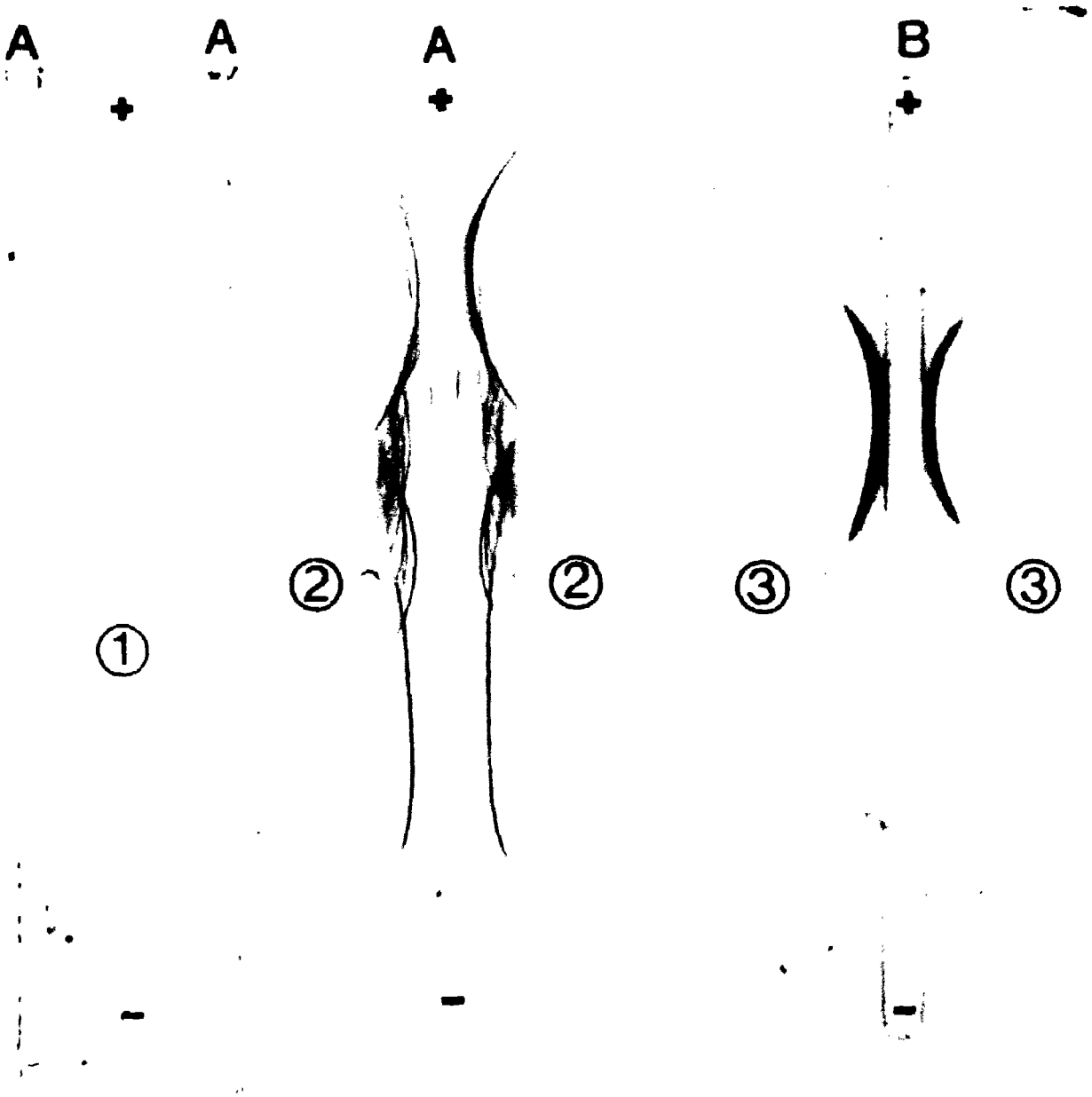

Fig. 1

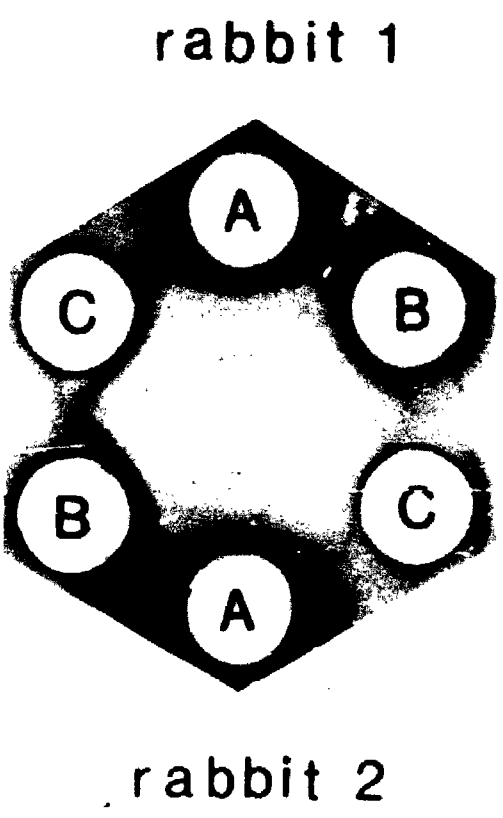

Fig. 2

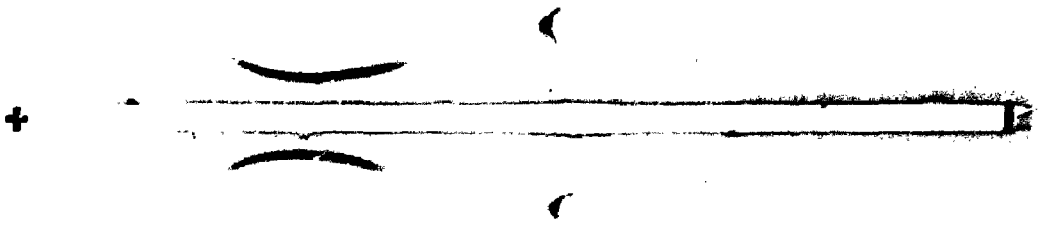

Fig. 3

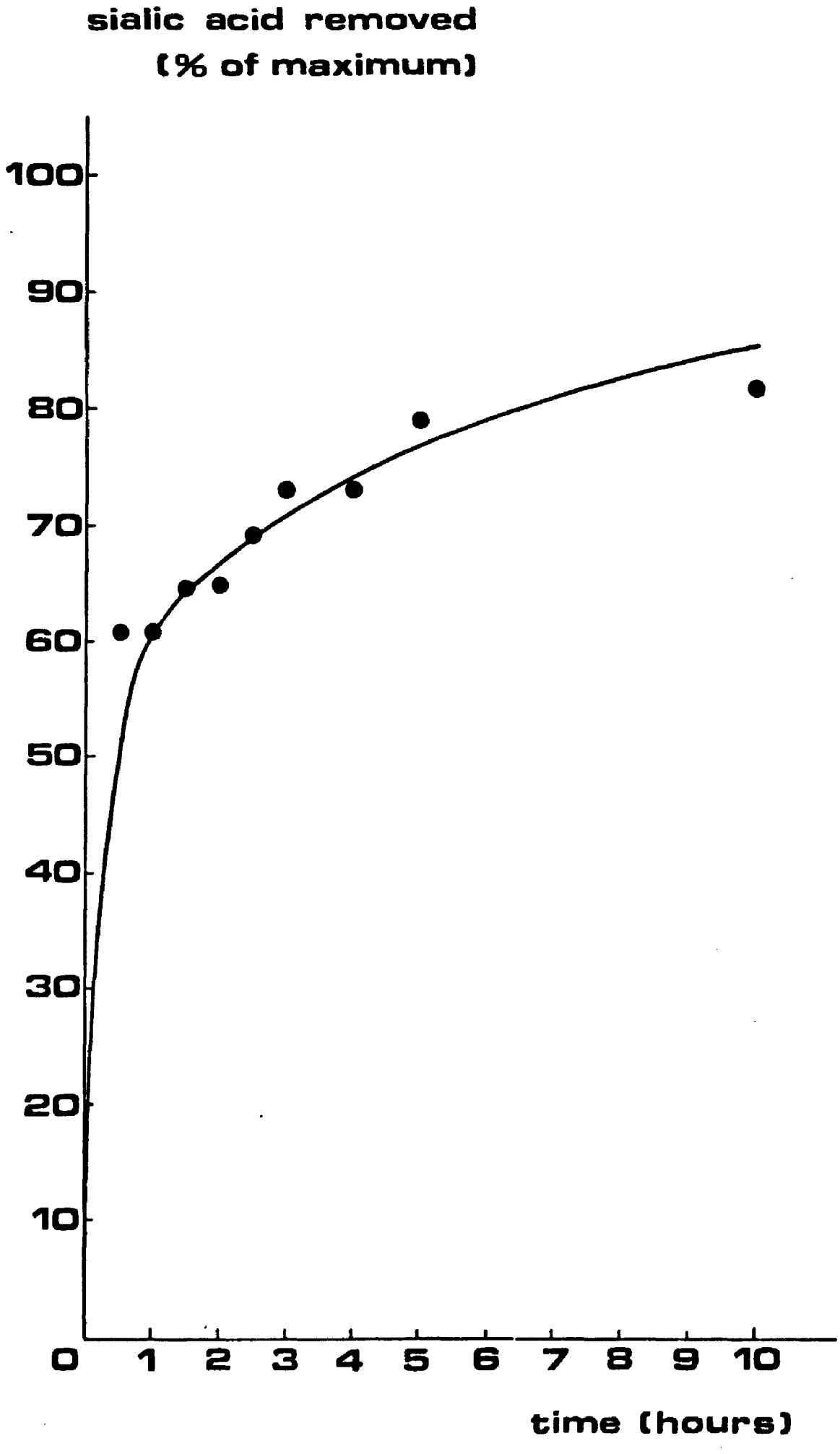
sialic acid removed
(% of maximum)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
time (hours)

Fig. 4