(CONVENTION. By one or more persons and/or a Company.) Form 4

626540

COMMONWEALTH OF AUSTRALIA

*Patents Act 1952-1969*

# CONVENTION APPLICATION FOR A PATENT

(1) Here insert (in full) Name or Names of Applicant or Applicants, followed by Address (es).

~~I~~ / We (1) BEHRINGWERKE AKTIENGESELLSCHAFT

of D-3550 Marburg, Federal Republic of Germany

(2) Here insert Title of Invention.

hereby apply for the grant of a Patent for an invention entitled: (2)

MAGNETIC PROTEIN CONJUGATES, A PROCESS FOR THE PREPARATION THEREOF, AND THE USE THEREOF

(3) Here insert number(s) of basic application(s)

which is described in the accompanying complete specification. This application is a Convention application and is based on the application numbered (3)

P38 07 904.6

(4) Here insert Name of basic Country or Countries, and basic date or dates

for a patent or similar protection made in (4) Federal Republic of Germany on 10th March 1988

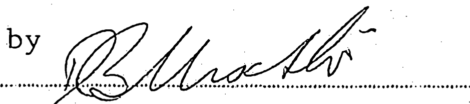
~~My~~ / Our address for service is Messrs. Edwd. Waters & Sons, Patent Attorneys, 50 Queen Street, Melbourne, Victoria, Australia.

DATED this 8th day of March 1989

(5) Signature (s) of Applicant (s) or Seal of Company and Signatures of its Officers as prescribed by its Articles of Association.

(5) BEHRINGWERKE AKTIENGESELLSCHAFT

by



D. B. Mischlewski

Registered Patent Attorney

M007236 09/03/89

To:

Form 8

COMMONWEALTH OF AUSTRALIA

Patents Act 1952

DECLARATION IN SUPPORT OF A CONVENTION APPLICATION UNDER PART XVI. FOR A PATENT.

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In support of the Convention application made under Part XVI. of the Patents Act 1952 by BEHRINGWERKE AKTIENGESELLSCHAFT of D-3550 Marburg, Federal Republic of Germany for a patent for an invention entitled:

MAGNETIC PROTEIN CONJUGATES, A PROCESS FOR THE PREPARATION THEREOF, AND THE USE THEREOF

We, Philipp Stein, 28 Höhenweg, D-3550 Marburg,
Heribert Bug, 7 Amselweg, D-3551 Niederweimar,
Federal Republic of Germany

do solemnly and sincerely declare as follows:

1. We are authorized by BEHRINGWERKE AKTIENGESELLSCHAFT the applicant for the patent to make this declaration on its behalf.

2. The basic application(s) as defined by Section 141 of the Act was (were) made at München in the Federal Republic of Germany under No. P 38 07 904.6

on March 10, 1988

by BEHRINGWERKE AKTIENGESELLSCHAFT

3. a) Peter Hermentin, 30 Barfüßertor, D-3550 Marburg
b) Reiner Dönges, 7 Bachstraße, D-3563 Dautphetal
c) Karlheinz Enßle, 4 Salegrund, D-3550 Marburg
d) Roland Kurrle, 18 Schenkendorfweg, D-3550 Marburg
e) Friedrich Robert Seiler, 10 Oberer Eichenweg, D-3550 Marburg
a) - e) Federal Republic of Germany

is/are the actual inventor(s) of the invention and the facts upon which BEHRINGWERKE AKTIENGESELLSCHAFT is entitled to make the application are as follows:

The said BEHRINGWERKE AKTIENGESELLSCHAFT is the assignee of the said Peter Hermentin, Reiner Dönges, Karlheinz Enßle, Roland Kurrle, Friedrich Robert Seiler

4. The basic application referred to in paragraph 2 of this Declaration was (were) the first application(s) made in a Convention country in respect of the invention the subject of the application.

DECLARED at Marburg, Federal Republic of Germany

this 16th day of January 1989

To the Commissioner of Patents

BEHRINGWERKE AKTIENGESELLSCHAFT

Prokurist
ppa. Stein

Prokurist
ppa. Bug

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(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 626540

(54) Title
**MAGNETIC PROTEIN CONJUGATES, A PROCESS FOR THE PREPARATION THEREOF, AND THE USE THEREOF**

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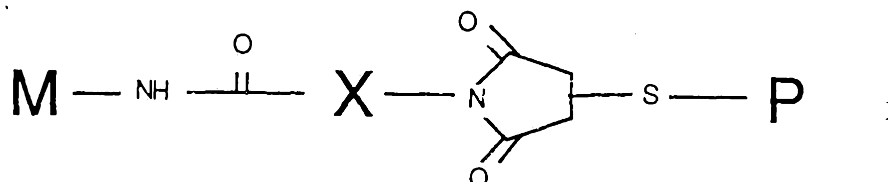
(57) Claim

1. A magnetic protein conjugate of the formula I




in which M is a dispersible magnetically reacting material or particle which carries amino groups, P is an immunoglobulin or immunoglobulin residue, preferably a monoclonal antibody or a Fab, Fab' or $F(ab')_2$ fragment which carries one or more mercapto groups, and X is an organic chemical structure which links the two ligands by chemical means.

21. The use of a magnetic protein conjugate as claimed in claim 1 for the specific removal of cells or soluble antigens, receptors, substrates, cofactors or carbohydrate determinants from aqueous salt solutions or body fluids, or the use within the framework of a diagnostic method or as a diagnostic aid.

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Form 10

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952-69

# COMPLETE SPECIFICATION

(ORIGINAL)

Class Int. Class

Application Number:
Lodged:

Complete Specification Lodged:
Accepted:
Published:

Priority:

Related Art:

Name of Applicant: BEHRINGWERKE AKTIENGESELLSCHAFT

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Complete Specification for the invention entitled:

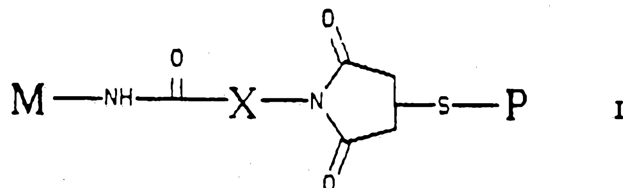
MAGNETIC PROTEIN CONJUGATES, A PROCESS FOR THE PREPARATION THEREOF, AND THE USE THEREOF

The following statement is a full description of this invention, including the best method of performing it known to : us

BEHRINGWERKE AKTIENGESELLSCHAFT    88/B 005 - Ma 679.
Dr. Ha/Sd.

Magnetic protein conjugates, a process for the preparation thereof, and the use thereof

The invention relates to magnetic protein conjugates of
5 the general formula I

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to which the following applies:

M is a dispersible magnetically reacting material or particle which carries amino groups, P is a protein which
15 carries one or more mercapto groups, and X is an organic chemical structure which links the two ligands by chemical means.

X is preferably an aliphatic, aromatic, alicyclic, ali-
20 cyclic-aliphatic or aromatic-aliphatic spacer which can optionally be substituted in a suitable manner in each case, preferably $-(CH_2)_n-$ with n = 1 - 8, preferably with n = 1 - 5 and particularly preferably with n = 2 or 3, or

25 X is preferably phenylene or substituted phenylene, it being possible for the two ligands to be in the ortho, meta or para positions, and it being possible for a substituent which is optionally present in the phenylene ring to be a methyl, hydroxyl, methoxy, acetoxy, nitro
30 or cyano group or a chlorine or bromine atom, or

X is preferably phenylene-$(CH_2)_n-$ with n = 1 - 5, preferably with n = 3, with the phenylene group being linked to the succinimidyl group, or
35

X is preferably -CHR-, where R represents an amino acid side chain, preferably the side chain of the amino acids

alanine, serine, threonine or methionine and particularly preferably the side chain of alanine, or

X is preferably methylenecyclohexyl, where the methylene group is linked to the succinimidyl group and, with respect to the carbonyl group linked to the cyclohexyl ring, is preferably linked in position 4 of the cyclohexyl ring.

The nature of X can also be such that it can be cleaved by chemical or enzymatic means.

P can be a protein in which the mercapto groups either are present in the natural way or are generated by reduction of disulfide linkages or are introduced by a chemical reaction.

P is, in particular, an immunoglobulin or immunoglobulin residue, preferably a monoclonal antibody or a Fab, Fab' or $F(ab')_2$ fragment, an antigen or a residue of an enzyme, hormone, lectin or growth factor.

P is preferably a monoclonal antibody of the IgG or IgM class, in particular a monoclonal antibody which is directed against an antigen which is present in dissolved form in aqueous salt solutions or body fluids, or a monoclonal antibody which is directed against an antigen which is expressed on cells, it being possible for the cells expressing the antigen to be, in particular, cells of the myeloid or lymphatic system, cells of the peripheral blood, in particular B lymphocytes, T lymphocytes or the precursor cells thereof, or tumor cells, in particular tumor cells of the bone marrow. Such cells can also be erythrocytes, bacteria, mycoplasmas or protozoa. However, viruses are also to be understood to be cells within the scope of the invention.

M is preferably a dispersible particle with a metal oxide core and with an enveloping coat carrying amino groups, it

being possible for the metal oxide core to include a group of paramagnetic substances, preferably a particle whose diameter is between about 0.1 μ and about 100 μ, but preferably between about 0.1 μ and about 1.5 μ.

5

The invention also relates to a process for the preparation of a magnetic protein conjugate of the general formula I and to the use of a conjugate of the formula I for the specific removal of cells or soluble antigens, receptors, substrates, cofactors or carbohydrate determinants from aqueous salt solutions or body fluids, and to the use within the framework of a diagnostic method or as a diagnostic aid, and, in particular, to the use for bone marrow depletion or for HLA typing.

15

Bone marrow transplantation is often the only therapeutic option for, inter alia, the treatment of certain types of leukemia and of panmyelopathy (panmyelophthisis).

20 In cases of leukemia and certain lymphoid neoplasias, the patients have hitherto been subjected to whole-body irradiation with an extremely high dose and/or to aggressive chemotherapy. With a treatment of this type the normal stem cells of the bone marrow, the precursors of
25 all blood cells, are completely destroyed. Hence bone marrow from a suitable donor is reinfused into the patient, and cells from this colonize the medullary cavities of the recipient and thus permit redevelopment of the hemopoietic and immune systems. This method is called allo-
30 geneic bone marrow transplantation.

The high risk of allogeneic bone marrow transplantation derives, inter alia, from the T lymphocytes of the donor, which are carried over into the patient in the reinfused
35 bone marrow and which recognize the cells of the recipient as foreign and thus attack and destroy them. This bone marrow intolerance, which is often life-threatening for the patient, is called the graft-versus-host reaction or graft-versus-host disease (GVHD). The risks of this

graft-versus-host disease can, on the one hand, be diminished by, if possible, reinfusing accurately typed bone marrow from particularly suitable donors, usually from among the relatives, into the patient. However, on the other hand, they can also be reduced by selectively eliminating undesired cell populations, as may be represented by, for example, T lymphocytes of the donor bone marrow, before reinfusion into the patient. This elimination of donor T cells can be carried out, for example, by selective lysis, in the presence of complement, of the cells which are to be removed, or by selective killing of the T cells with the aid of so-called immunotoxins, or by another method, for example by magnetic cell depletion of the bone marrow.

Bone marrow cell depletion of this type can be carried out in a relatively straightforward manner, in such a way that the bone marrow is incubated with a monoclonal mouse antibody which, for example, is directed specifically against the T cells of the bone marrow and, as a consequence, binds only to the T cells. T cells of this type, to which monoclonal mouse antibodies are attached, can now be removed in a second step by incubating them for example with rabbit anti-mouse immunoglobulin which is bound to magnetic particles, by which means the magnetic material is attached in a specific manner to the T lymphocytes so that they can be removed from the bone marrow with the aid of a magnet (in this context, see Vartdal et al., Transplantation (1987), 43, 366-371 and the literature cited therein).

It is also possible in an analogous manner to remove from the bone marrow other cell populations, for example tumor cells, which is of importance for what is called autologous bone marrow transplantation (in this context, see Kvalheim et al., Cancer Research (1987), 47, 846-851 and the literature cited therein). Moreover, it is also possible, as described by Kvalheim et al., ibid., to bind the monoclonal antibody which recognizes the tumor cells

directly onto the magnetic particles so that the above-mentioned second antibody (rabbit anti-mouse) is no longer necessary.

5 The method, described above, for bone marrow depletion with the aid of monoclonal or polyclonal antibodies which are bound to magnetic particles is still very new and requires further development and testing. A very wide variety of types of magnetic particles suitable for 10 this purpose are now commercially available, and the preparation thereof has been described more than once in the patent literature (see, for example, Chagnon et al., EP 0,125,995 A2 (priority US 493,991 of May 12, 1983), Advanced Magnetics, or Ughelstad et al., WO 8,303,920 of 15 Nov. 10, 1983, SINTEF). It is known of these magnetic particles that they are composed of a metal oxide core, in which paramagnetic substances may be included, and that the core is surrounded by an enveloping coat which may carry reactive groups such as, for example, amino-20 phenyl, amino, carboxyl, hydroxyl or sulfhydryl groups, which can be used for coupling proteins (Chagnon et al., EP 0,125,995 A2).

It is known, for example, that particles carrying car-25 boxyl groups can be induced to react with amino groups of proteins in the presence of a condensing agent (Chagnon et al., EP 0,125,995 A2).

Also known is the coupling of proteins to the magnetic 30 particles carrying amino groups, by use of glutaraldehyde, the coupling taking place via the amino groups in each case (Chagnon et al., EP 0,125,995 A2).

Furthermore, it is known that particles carrying hydroxyl 35 groups can be activated by reaction with p-toluenesulfonyl chloride, and that particles activated in this way can be induced to react with amino groups of proteins (Kvalheim et al., Cancer Research (1987), 47, 846-851).

It is common to all these coupling methods that the protein is, in each case, linked to the particles via its free amino groups. However, coupling of this type via amino groups may be a considerable disadvantage with mono-
5 clonal antibodies because, in this case, occasionally the specificity and reactivity of the antibodies is impaired. This is a consequence of the fact that the amino groups in an antibody are distributed more or less at random over the entire molecule, and thus are also located in
10 the antigen-binding site of the Fab fragment, which brings about a loss of specificity where there is coupling via these amino groups.

It is furthermore known that antibodies can be taken up
15 on magnetic particles even without chemical linkage, purely by adsorption, if the particles are enveloped in a styrene/divinylbenzene copolymer, because protein is known to bind non-specifically to polystyrene.

20 However, even with this method an impairment of the antibody specificity and reactivity must be expected. Another serious disadvantage of this method comprises, however, the possibility that antibodies bound by adsorption become detached again during bone marrow depletion and thus are
25 also administered to the patient on reinfusion of the depleted bone marrow, which might result in serious side reactions, especially where there has been a previous attempt at therapy with monoclonal antibodies. However, this problem is known, and the aim is to overcome it by
30 covalent linkage of the antibodies to the magnetic particles.

It is also known that magnetic particles with a polystyrene envelope have the serious disadvantage that they
35 tend to aggregate and, moreover, attach themselves non-specifically to cells.

Based on this prior art, the object of the present invention is to develop a method in which monoclonal

antibodies are coupled to magnetic particles a) covalently and b) not via their amino groups. Hence, in other words, the object of the present invention is to find a coupling method in which the antigen binding site of the antibody is not altered, or the coupling of the antibody takes place away from the antigen binding site.

This object according to the invention is achieved by the preparation of magnetic protein conjugates of the general formula I on page 1.

It has now been found that magnetic particles which carry as reactive groups free amino groups can be converted in a straightforward manner into magnetic particles which carry as reactive groups maleimido groups. Particles of this kind are new.

It has additionally been found that magnetic particles which carry maleimido groups can be conjugated without difficulty with proteins which have mercapto groups, it being possible for the mercapto groups in the protein to be either already present naturally or introduced by chemical means or generated by reduction of disulfide linkages which are present.

In particular, it has been found that magnetic particles which carry maleimido groups can be conjugated without difficulty with monoclonal antibodies if the interchain disulfide linkages of the antibodies are converted by selective reduction into free SH groups which can be induced to react with the maleimido groups of the magnetic particles to form a stable thioether linkage. This way of coupling monoclonal antibodies to magnetic particles is likewise new.

It has been found, surprisingly, that the specificity and reactivity of the antibodies coupled to magnetic particles via thioether linkages is completely retained because the coupling of the antibody via its hinge region does not

alter or impair its antigen-binding site. Herein lies a particular advantage of the invention compared with the coupling methods hitherto disclosed, in which the antibodies are, as described above, taken up on magnetic
5 particles either purely by adsorption or by reaction of their amino groups, which may impair both the specificity and the reactivity of the conjugated antibodies. Moreover, compared with coupling by adsorption, the present invention has the advantage that the antibodies are chemically
10 bonded to the magnetic particles and, as a consequence, do not become detached from the particles when magnetic antibody conjugates according to the invention are used, for example for bone marrow depletion.

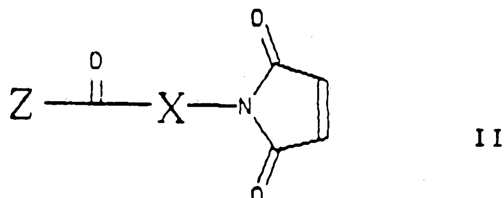
15 It has additionally been found that the magnetic antibody conjugates according to the invention prove to be particularly advantageous, for example for the depletion of bone marrow, because of their high specificity.

20 In addition, it has been found that the magnetic antibody conjugates according to the invention also prove advantageous, becauses of their high specificity, within the framework of a diagnostic method or as a diagnostic aid, especially, for example, for HLA typing.
25

The preparation of magnetic antibody conjugates according to the invention is described hereinafter by way of example for various monoclonal antibodies which are directed against cells of the bone marrow; the specified examples
30 do not, however, restrict the invention. In addition, the use of the magnetic antibody conjugates, which have been prepared by way of example, for the depletion of cells of the bone marrow and for HLA typing is likewise described by way of example without restricting the use
35 to the specified examples.

Process for the preparation of a magnetic protein conjugate of the general formula I

Magnetic particles M carrying amino groups are reacted
5 in a suitable solvent with a maleimido spacer of the general formula II

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which reacts with amino groups and in which Z is a suitable reactive leaving group, with the formation of an amide linkage, to give a compound of the general formula III

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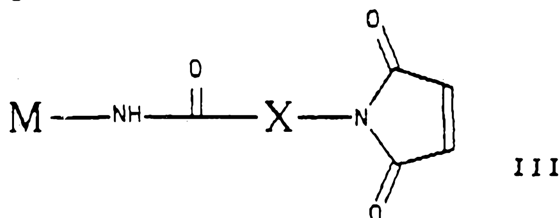


which is finally reacted, in a suitable aqueous salt-
20 containing solvent which does not denature proteins, such as, for example, physiological saline or a phosphate-buffered saline, with a protein P carrying mercapto groups to give a compound of the general formula I.

25 The nature of solvents suitable for the coupling of a compound of the formula II to magnetic particles must be such that the solvent which is used does not impair the physical and magnetic properties of the magnetic particles which are used for the coupling in each case, especi-
30 ally their size, dispersibility and surface characteristics. An example of a solvent found suitable for magnetic particles as described, for example, in EP 0,125,995 A2 or WO 8,303,920 is a mixture of water and dimethylformamide, where the substituent Z in the formula II is,
35 for example and preferably, succinimidyloxy. However, another possible example of a suitable solvent is anhydrous acetone, in which case the substituent Z in the formula II can, besides succinimidyloxy, also be a halogen such as, for example, chlorine or bromine, or a pseudo-

halide such as, for example, cyanide, or an acyl group such as, for example, acetyl or p-nitrobenzoyl, or another reactive leaving group such as, for example, a tosyl group, with the reaction being carried out in all the
5 cases where Z is not succinimidyloxy preferably in the presence of a suitable base such as, for example, sodium carbonate.

Method for cell depletion

10

A suspension of a cell mixture which is to be depleted, in a salt-containing, preferably physiological aqueous solution or in a body fluid, is incubated with a compound of the formula I at a suitable temperature between, for
15 example, 0°C and 40°C, preferably with shaking, likewise preferably under sterile conditions, for a suitable period, and then the magnetic particles are removed from the solution by a suitable magnet.

20 Examples of suitable temperatures are 0°C, room temperature or 37°C, but room temperature is preferred. The duration of the incubation depends in each case on the incubation temperature used and on the binding reactivity of the antibody and may be, for example, from a few
25 minutes up to, for example, 2 hours. Incubation is preferably carried out at, for example, room temperature for a period of, for example, 10 to 20 minutes.

Method for isolating soluble bioorganic molecules

30

This method essentially follows the method for cell depletion.

Examples:

35

The examples which follow serve to illustrate the invention further but do not restrict the invention.

Magnetic particles which have been induced to react in

the manner described with monoclonal antibodies are called "magnetobeads" hereinafter, with their specificity being indicated in each case by prefixing the particular antibody name.

5

Example 1:

Conjugation of magnetic particles according to EP 0,125,995 A2 using a gamma-maleimidobutyrate spacer

10

0.5 ml of a commercially available suspension of magnetic particles (BioMag M4100, Sebak[R]) was washed 3 x with phosphate-buffered saline pH 7.2 (PBS) and resuspended in 6 ml of PBS. To this suspension was added a freshly prepared solution of 20 mg N-(gamma-maleimidobutyryloxy)succinimide (GMBS, Calbiochem) in 4 ml of dry dimethylformamide, and the mixture was shaken at room temperature for 1 h. The particles were then spun down at 3000 x g, washed 3 x with 20 ml of PBS each time and resuspended in 6 ml of PBS.

15

20

Example 2:

General procedure for coupling monoclonal antibodies* to magnetic particles carrying maleimido groups from Example 1

25

*) Note: All the monoclonal antibodies used were lyophilized in a concentration of 1 mg/ml in a solution of 0.1 mol/l sodium citrate buffer pH 6.6 and 50 g/l sucrose.

30

1 mg of lyophilized monoclonal antibody was dissolved in 0.5 ml of water, 2 mg of dithiothreitol were added, and the mixture was incubated at room temperature for 30 min. The reduced antibody was isolated by means of gel filtration on Sephadex G25 in isotonic saline pH 7.2 in an elution volume of 4 ml, and was added to the suspension of magnetic particles prepared as in Example 1. The mixture (10 ml) was incubated at room temperature for

35

45 min, with shaking. The particles were then spun down at 3500 x g, washed 3 x with 20 ml of PBS each time, re-suspended in 10 ml of PBS pH 7.2, and stored at 4°C.

5 Where appropriate, 0.2% sodium azide or 0.2% sodium azide + 0.1% BSA (bovine serum albumin) + 0.2% Tween 20 was added to the suspension.

The monoclonal antibodies coupled as in Example 2 are
10 summarized in Table 1.

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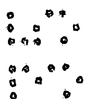

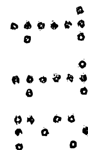

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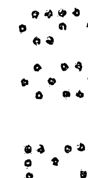

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Table 1: Magnetobeads prepared as in Example 2

| Monoclonal antibody | Class and isotype | Specificity |
|---|---|---|
| BMA 0110 | IgG2b | CD2 |
| BMA 0111 | IgG1 | CD2 |
| BMA 030 | IgG2a | CD3 |
| BMA 033 | IgG3 | CD3 |
| BMA 031 | IgG2b | TCR |
| BMA 041 | IgM | CD4 |
| BMA 0117 | IgG2a | CD7 |
| BMA 081 | IgG2a | CD8 |
| VIL-A1 | IgM | CD10 |
| SV393 | IgG1 | Beta-2-M |
| BMA 022 | IgG2a | HLA-DR |
| BMA 0210 | IgM | monocytes |
| 84-24/91-8 | IgG1 | TSH |
| BB 10 | IgG2b | CD10/CALLA |
| BMA 0160 | IgM | Glycophorin A |
| BMA 0112 | IgG1 | CD7 |
| 704/152/5 | IgG3 | Neuroblastoma |
| 575/931/2 | IgG1 | SCLC |
| A-h-TSH 5404 | IgG1 | TSH |
| 383/44 | IgG1 | IgE |
| 393/57 | IgG2b | IgE |
| 84-9/6 | IgG2a | IgE |
| 209/2 | IgG2a | CD2 |
| RAM | polyclonal | mouse gamma-globulin |

explanation of abbreviations see Table 2

Example 3:

Positive reaction of BMA 041 magnetobeads with T cells

5 40 µl of a suspension of BMA 041 magnetobeads in PBS prepared as in Example 2 were incubated with 120 µl of a T cell suspension (clone 5/87-7-A3; $CD4^+$, $CD8^-$, $CD2^+$, $CD3^+$, T-cell receptor-positive; $1 \times 10^4$ cells/µl in RPMI 1640) at room temperature for 10 min, with shaking, and, without delay, were
10 inspected under the microscope in a counting chamber and photographed. The cells had formed rosettes or aggregates with the magnetic particles.

Example 4:

15

Negative reaction of BMA 041 magnetobeads with B cells

40 µl of a suspension of BMA 041 magnetobeads in PBS prepared as in Example 2 were incubated with 120 µl of a B
20 cell suspension (B cell line GR, $1 \times 10^4$ cells/µl in RPMI 1640) as in Example 3 and were then inspected under the microscope and photographed. No formation of rosettes or aggregates was observed.

25 Example 5:

Positive reaction of BMA 081 magnetobeads with T cells

40 µl of a suspension of BMA 081 magnetobeads prepared
30 as in Example 2 were incubated as in Example 3 with T cells (clone 5/87-4-B6; $CD8^+$, $CD4^-$, $CD2^+$, $CD3^+$, T-cell receptor-positive) and then inspected under the microscope and photographed. The negative control was B cells analogous to Example 4 (B
35 cell line GR). The T-cell receptor-positive cells had formed rosettes or aggregates with the magnetic particles. No formation of rosettes or aggregates was observed with the negative control.

Example 6:

Positive reaction of BMA 031 magnetobeads with T cells

5 40 µl of a suspension of BMA 031 magnetobeads prepared as in Example 2 were incubated in analogy to Example 3 with T cells (clone 5/87-4-B6; $CD8^+$, $CD4^-$, $CD2^+$, $CD3^+$, T-cell receptor-positive) and then inspected under the microscope and photographed. The negative control was B cells
10 analogous to Example 4 (B cell line GR). The antigen-positive cells had again formed rosettes or aggregates with the magnetic particles; the antigen-negative cells were unassociated.

15 Example 7:

Depletion of cell populations from mononuclear cells

Mononuclear cells (MNC) were isolated from freshly dona-
20 ted human blood on a Ficoll gradient in a manner known per se (Boyum, Scand. J. Immunol. (1976), Suppl. 5, 9 - 15).

For the depletion, $3 \times 10^7$ MNC in 2 ml of PBS containing
25 1% BSA (w/v, Seromed) in plastic tubes (Falcon, No. 2051) were mixed with 1 ml of a suspension of 2 mg/ml magnetobeads in PBS and incubated at room temperature, shaking continuously, for 15 min. The magnetobeads and the cells bound thereto were then removed with the aid of a perma-
30 nent magnet. The cells remaining in suspension were pelleted at 400 x g and resuspended in a suitable medium, for example PBS or RPMI 1640. The depletion efficiency was determined by means of indirect immunofluorescence in a cytofluorograph (Ortho).

35

For this purpose, before and after depletion of a particular cell population $1 \times 10^6$ cells were labeled with 1 - 10 µg/ml first antibody (a monoclonal antibody of suitable specificity in each case) and then with 10 -

50 µg/ml second antibody (rabbit anti-mouse immunoglobulin, $F(ab)_2$ fragment, FITC-labelled, Behringwerke) in a manner known per se and evaluated in a cytofluorograph, and the depletion efficiencies were determined to be above 95% in each case.

The cells depleted as in Example 7 are listed in Table 2.

The results achieved for the depletion with BMA 041 (anti-CD4, Behringwerke AG) magnetobeads and BMA 081 (anti-CD8, Behringwerke AG) magnetobeads are depicted by way of example in Figure 1. This entailed the depletion efficiency being determined by labeling the unbound cell population in each case with BMA 031 (anti-T-cell receptor, Behringwerke AG), BMA 041 or BMA 081. After incubation with FITC-labeled second antibody (see above) the fluorescence intensity in each case was evaluated in a cytofluorograph with linear amplification.

Table 2: Depletion of cells by means of magnetobeads prepared as in Example 2

| Coupled monoclonal antibody | Antigen recognized (specificity) | Depleted cell population |
|---|---|---|
| BMA 0110 | CD2 | $CD2^+$-T-cells |
| BMA 0111 | CD2 | $CD2^+$-T-cells |
| BMA 030 | CD3 | $CD3^+$-T-cells |
| BMA 033 | CD3 | $CD3^+$-T-cells |
| BMA 031 | TCR | $TCR^+$-T-cells |
| BMA 041 | CD4 | $CD4^+$-T-cells |
| BMA 0117 | CD7 | $CD7^+$-T-cells |
| BMA 081 | CD8 | $CD8^+$-T-cells |
| VIL-A1 | CD10 | $CD10^+$-B-cells |
| SV 393 | Beta-2-M | HLA-class $I^+$-cells |
| BMA 022 | HLA-DR | HLA-class $II^+$-cells |
| BMA 0210 | monocytes | monocytes |
| BB 10 | CD10/CALLA | $CD10^+$- or $CALLA^+$-cells |
| BMA 0160 | Glycophorin A | erythrocytes |
| BMA 0112 | CD7 | $CD7^+$-cells |
| 704/152/5 | Neuroblastoma | Neuroblastoma-cells |
| 209/2 | CD2 | $CD2^+$-cells |

explanation of abbreviations:

CD : clusters of differentiation
TCR : T-cell receptor
Beta-2-M: $\beta_2$-microglobulin
HLA-DR : human leucocyte antigen, subclass DR
TSH : thyroid-stimulating hormone
CALLA : common acute lymphoblastic leukaemia antigen
SCLC : small cell lung cancer
IgE : immunoglobulin E
RAM : rabbit anti-mouse

Example 8:

Detection of the proliferation of T cells in culture after incubation with uncoupled magnetic particles (BioMag M4100, Sebak[R])

Mononuclear cells were isolated from human blood in analogy to Example 7 and incubated with a suspension of uncoupled magnetic particles (BioMag M4100, Sebak[R]) in analogy to Example 7. The magnetic particles were then removed with the aid of a permanent magnet. The remaining MNC were incubated in 96-well flat-base titer plates (Nunc) at a concentration in each case of $1 \times 10^5$ MNC per well in serum-free culture medium (Iscove, Behring modification) in the presence of pokeweed mitogen (PWM, Gibco; 1 : 3000 final dilution) or of 10 µg/ml phytohemagglutinin (PHA, Wellcome) in a $CO_2$ incubator at $37^oC$ for 64 hours. After a culture time of 48 hours $2.7 \times 10^3$ Bq of $^{14}C$-thymidine (Amersham) were added to each of the cultures. After a further culture time of 14 hours, the cells were filtered off with suction on glass fiber filter disks using a cell harvester (Innotec). The radioactivity on the filters was determined in a β counter (Packard) after addition of scintillation fluid.

Figure 2 shows the evaluation of an experiment of this type and demonstrates that the stimulation of MNC with PHA or PWM is not impaired by depletion with uncoupled magnetic particles (BioMag M4100, Sebak[R]).

Example 9:

Detection of the proliferation of T cells in culture in the presence of uncoupled magnetic particles (BioMag M4100, Sebak[R])

Various amounts of uncoupled magnetic particles (BioMag M4100, Sebak[R]) were added to MNC which were then distributed in 96-well plates and cultivated with PHA (10 µg/ml),

BMA 030 (Behringwerke, mitogen for T lymphocytes) (0.01 µg/ml) or Staphylococcus aureus capsid (SAC, Behringwerke, mitogen for B lymphocytes, final dilution 1 : 3000) in analogy to Example 8.

5

It is evident from Figure 3 that magnetic particles (BioMag M4100, Sebak^R) do not, at a concentration of 2 - 20 µg/ml, have an adverse effect on the proliferation of B and T cells.

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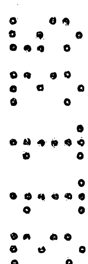
Key to the figures

Fig. 1: Depletion of T cell subpopulations with BMA 041 and BMA 081 magnetobeads



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PBL: original cell population

$CD4^-$: population after depletion with BMA 041 magnetobeads

$CD8^-$: population after depletion with BMA 081 magnetobeads

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Fig. 2: Proliferation of T cells in culture after incubation with uncoupled magnetic particles (BioMag M4100, Sebak^R) and stimulation with phytohemagglutinin (PHA) or pokeweed mitogen (PWM) compared with medium control

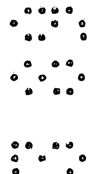

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Fig. 3: Proliferation of T cells in culture in the presence of uncoupled magnetic particles (BioMag M4100, Sebak^R) after stimulation with phytohemagglutinin (PHA), anti-CD3 monoclonal antibody BMA 030, or Staphylococcus aureus capsid (SAC) compared with medium control

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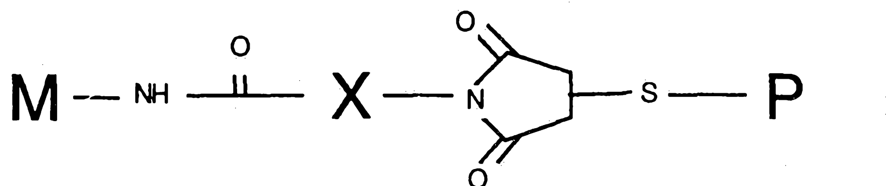
THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A magnetic protein conjugate of the formula I




in which M is a dispersible magnetically reacting material or particle which carries amino groups, P is an immunoglobulin or immunoglobulin residue, preferably a monoclonal antibody or a Fab, Fab' or $F(ab')_2$ fragment which carries one or more mercapto groups, and X is an organic chemical structure which links the two ligands by chemical means.

2. A magnetic protein conjugate as claimed in claim 1, wherein X is an aliphatic, aromatic, alicyclic, alicyclic-aliphatic or aromatic-aliphatic spacer which can optionally be substituted in a suitable manner in each case.

3. A magnetic protein conjugate as claimed in claim 2, wherein X is $-(CH_2)_n-$ with n = 1 - 8, preferably with n = 1 -5 and particularly preferably with n = 2 or 3.

4. A magnetic protein conjugate as claimed in claim 2, wherein X is phenylene, it being possible for the two ligands to be in the ortho, meta or para positions.

5. A magnetic protein conjugate as claimed in claim 2, wherein X is phenylene-$(CH_2)_n$- with n = 1 -5, preferably with n = 3, with the phenylene group being linked to the succinimidyl group.

6. A magnetic protein conjugate as claimed in claim 2, wherein X is -CHR-, where R represents an amino acid side chain, preferably the side chain of the amino acids alanine, serine, threonine or methionine, and particularly preferably the side chain of alanine.

7. A magnetic protein conjugate as claimed in claim 2, wherein X is methylenecyclohexyl, where the methylene group is linked to the succinimidyl group and, with respect to the carbonyl group linked to the cyclohexyl ring, is preferably linked in position 4 of the cyclohexyl ring.

8. A magnetic protein conjugate as claimed in claim 2, wherein X is as in claim 4 but the phenylene ring is substituted by a methyl, hydroxyl, methoxy, acetoxy, nitro or cyano group or by a chlorine or bromine atom.

9. A magnetic protein conjugate as claimed in claim 1, wherein the mercapto groups of the protein P either are present in the natural way or are generated by reduction of disulfide linkages or are introduced by a chemical reaction.

~~10. A magnetic protein conjugate as claimed in claim 1, wherein P can be an immunoglobulin or immunoglobulin residue, preferably a monoclonal antibody or a Fab, Fab' or F(ab')$_2$ fragment, an antigen or a residue of an enzyme, hormone, lectin or growth factor.~~

10. A magnetic protein conjugate as claimed in claim 1 , wherein P represents a monoclonal antibody of the IgG or IgM class.

11. A magnetic protein conjugate as claimed in claim 1 , wherein P represents a monoclonal antibody which is directed against an antigen which is present in dissolved form in aqueous salt solutions or body fluids.

12. A magnetic protein conjugate as claimed in claim 1 , wherein P represents a monoclonal antibody which is directed against an antigen which is expressed on cells, especially on cells of the myeloid or lymphatic system or of the peripheral blood, especially on B

lymphocytes, T lymphocytes or the precursor cells thereof, or on tumor cells, especially on tumor cells of the bone marrow.

13. A magnetic protein conjugate as claimed in claim 12, wherein P represents a monoclonal antibody which is directed against an antigen which is expressed on bacteria, mycoplasmas or protozoa or else on viruses.

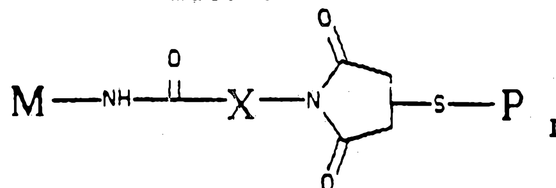
14. A magnetic protein conjugate as claimed in claim 1, wherein P represents an antigen.

15. A magnetic protein conjugate as claimed in claim 1, wherein M is a dispersible particle with a metal oxide core and an enveloping coat carrying amino groups, it being possible for the metal oxide core to include a group of paramagnetic substances.

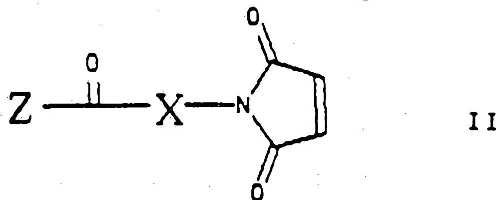
16. A magnetic protein conjugate as claimed in claim 15, wherein the diameter of the particles is between about 0.1 μ and about 100 μ, but preferably between about 0.1 μ and about 1.5 μ.

17. A magnetic protein conjugate as claimed in claim 1, wherein X can be cleaved by chemical or enzymatic means.

18. A process for the preparation of a magnetic protein conjugate of the formula I




which comprises magnetic particles M carrying amino groups being reacted with a maleimido spacer of the formula II which reacts with amino groups




II

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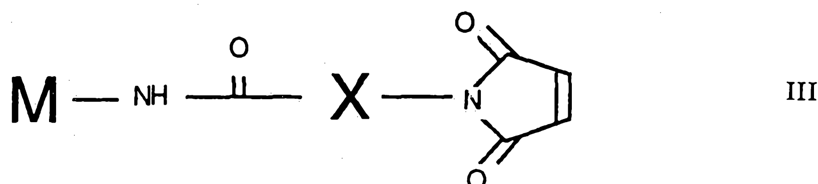
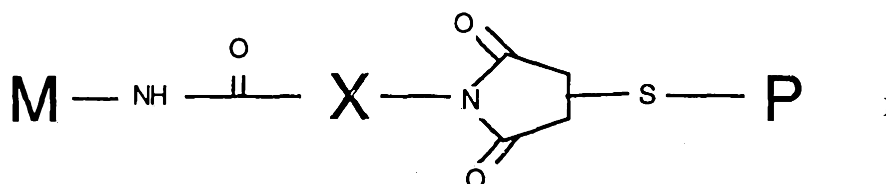
where Z is a reactive leaving group and X is an organic chemical linking structure, with the formation of an amide linkage, to give a compound of the formula III




III

which is finally reacted with a protein P carrying mercapto groups to give a compound of the formula I.

19. A method for removing a dissolved antigen, antibody, receptor, substrate, cofactor or carbohydrate determinant from aqueous salt solutions or body fluids, which comprises incubating the solution with a suitable magnetic protein conjugate of the formula I as claimed in claim 1

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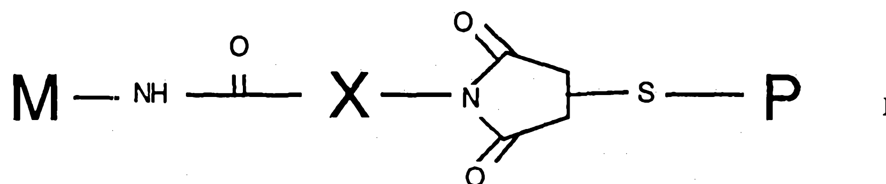
and, after specific adsorption of the component which is to be removed, removing the magnetic protein conjugate by magnetic means and, where appropriate, eluting the specifically adsorbed component again from the magnetic protein conjugate.

20. A method for removing cells from aqueous salt solutions or body fluids, which comprises incubating the suspension with a suitable magnetic protein conjugate of the formula I as claimed in claim 1

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and, after specific adsorption of the cells which are to be removed, removing the magnetic protein conjugate by magnetic means and, where appropriate, detaching the specifically adsorbed cells or particles again from the magnetic protein conjugate.

21. The use of a magnetic protein conjugate as claimed in claim 1 for the specific removal of cells or soluble antigens, receptors, substrates, cofactors or carbohydrate determinants from aqueous salt solutions or body fluids, or the use within the framework of a diagnostic method or as a diagnostic aid.

22. The use of a magnetic protein conjugate as claimed in claim 21 for removing cells as defined in claim 12 or 13, preferably for bone marrow depletion, or for HLA typing.

DATED this 8th day of March 1989.

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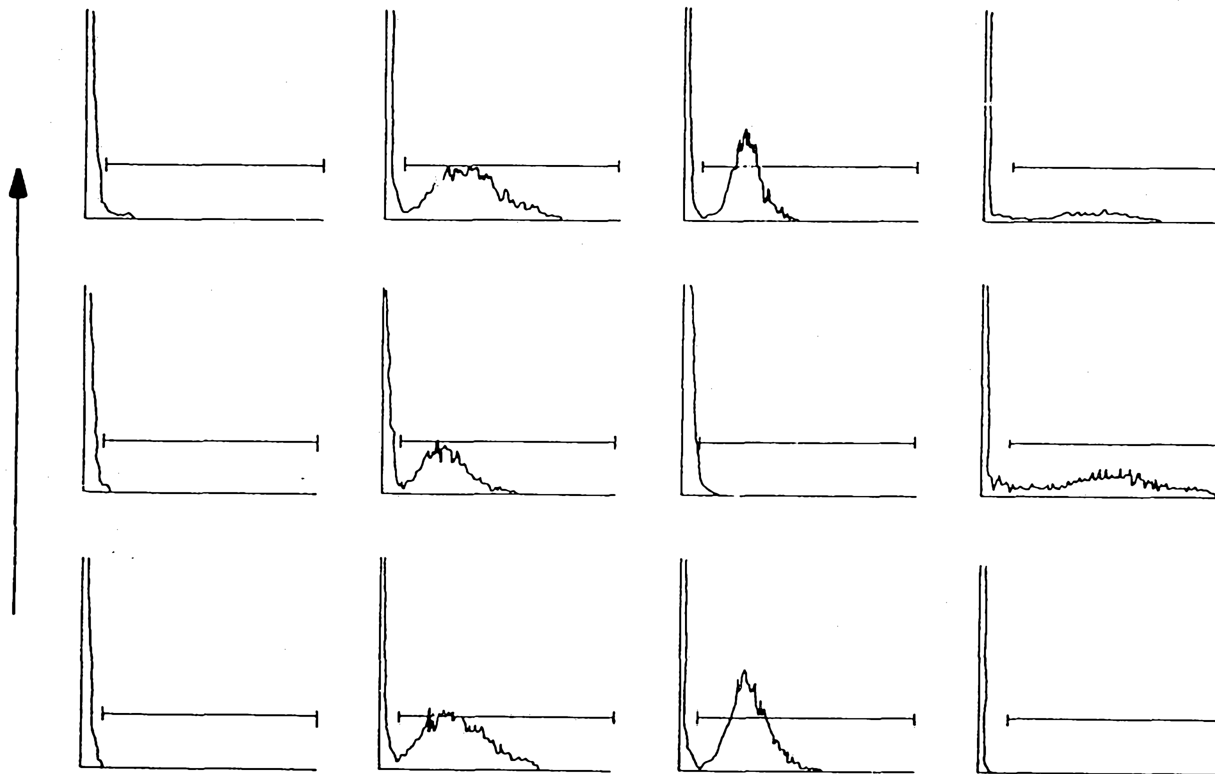
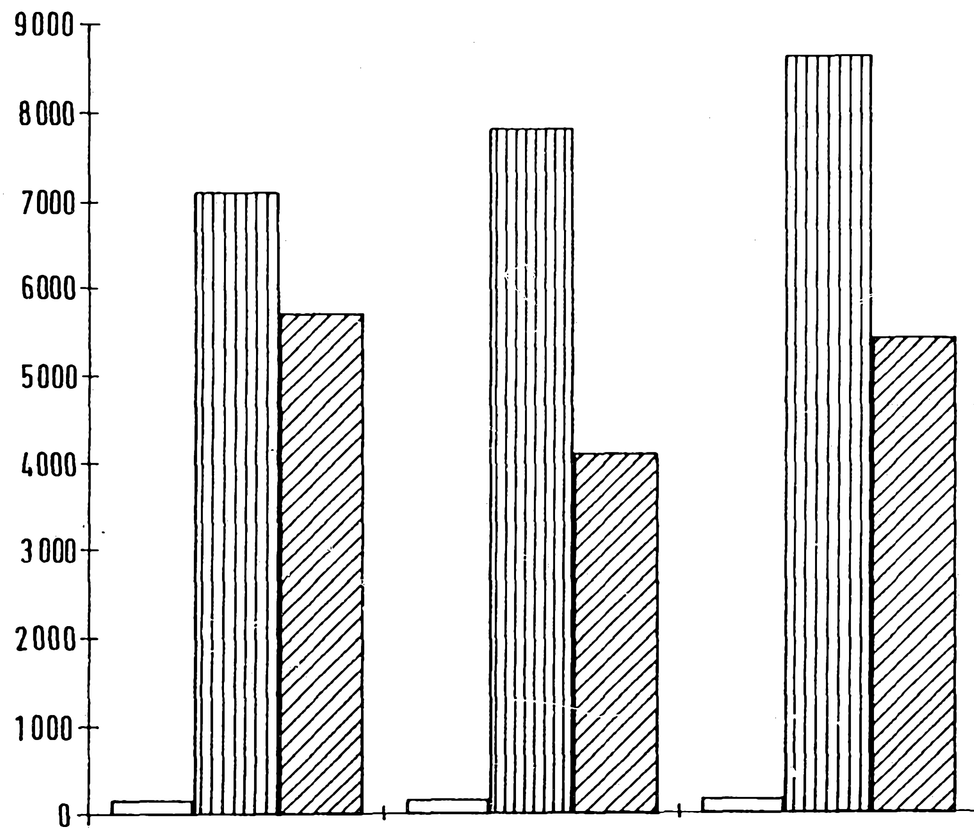


Fig.1



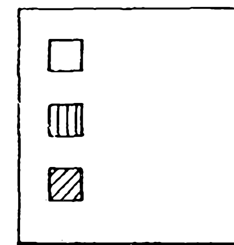



Fig. 2

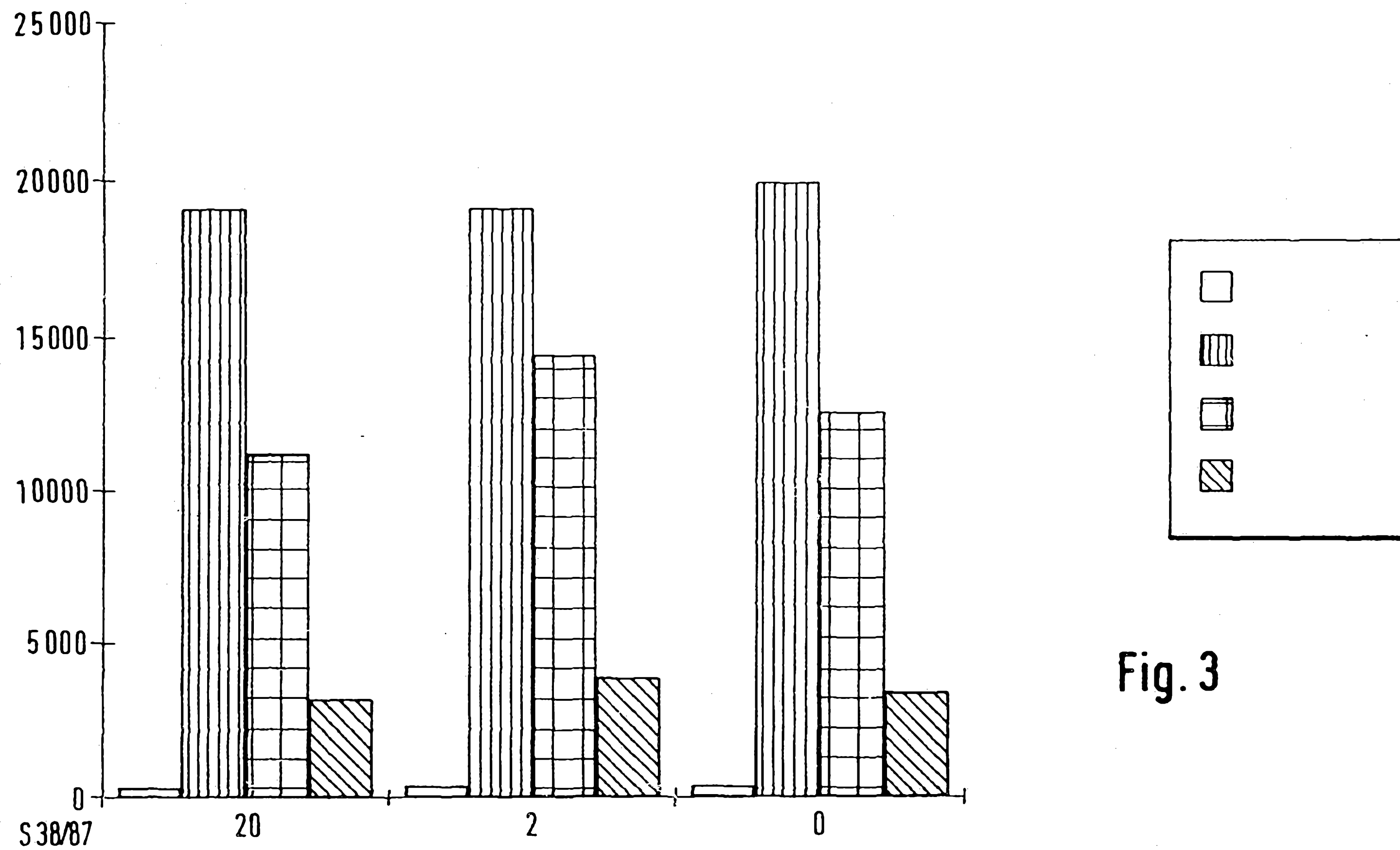



Fig. 3