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(54) Titre : METHODE ET APPAREIL DE SYNTHÈSE RAPIDE DE PEPTIDES OU D'OLIGONUCLEOTIDES LIBRES OU
LIES AU SUBSTRAT, MATERIAU PLAT SYNTHETISE DE CETTE FACON ET UTILISATION DE CE MATERIEL
(54) Title: METHOD AND APPARATUS FOR THE RAPID SYNTHESIS OF SUBSTRATE-BOUND OR FREE PEPTIDES
OR OLIGONUCLEOTIDES, FLAT MATERIAL SYNTHESIZED IN THIS WAY AND THE USE OF SUCH MATERIAL

(57) **Abrégé/Abstract:**

The invention relates to a process for the rapid synthesis of support-bound or free peptides, a device for carrying out the process, a flat material with peptides bound to it which are prepared according to the process, and to a use for the flat material.



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Abstract

The invention relates to a process for the rapid synthesis of support-bound or free peptides, a device for carrying out the process, a flat material with peptides bound to it which are prepared according to the process, and to a use for the flat material.

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METHOD AND APPARATUS FOR THE RAPID SYNTHESIS OF SUBSTRATE-BOUND
OR FREE PEPTIDES OR OLIGONUCLEOTIDES, FLAT MATERIAL SYNTHESIZED
IN THIS WAY AND THE USE OF SUCH MATERIAL

- 5 Proteins play a decisive role in practically all
biological processes such as enzymatic catalysis,
transport and storage, coordinated movement, mechanical
supporting function, immune protection, conduction of
nerve stimuli and control of growth and differentiation.
- 10 Proteins are made up of one or more linear polypeptide
chains. Their multifarious functions are achieved through
three-dimensional folding and the association of these
chains. The folding and 3D structure is determined by the
primary amino acid sequence of the chains. The multi-
- 15 plicity of protein structures in nature is produced by
combining twenty amino acid components. Short partial
sequences (oligopeptides) can to a certain extent imitate
the local structure and function of a sequence in the
total protein formation. Oligopeptides in turn can be
- 20 prepared by chemical syntheses. Synthetic oligopeptides
are thus an important aid in the analysis of the struc-
ture and function of proteins.

A.

A L K P M S T T C K W P P G H F E S M T

B

A L K P M S T T R Q

T

S S

WS

W E

E F

P F

P W F

P E K

W K

P P G

H S I

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Because of the size and complexity of proteins, very many peptides are used in systematic studies.

5 A) Localisation of a functional region within a relatively long protein chain using overlapping peptides (cf. M. Z. Atassi, Eur. J. Biol. 145, 1-20 (1984); H. M. Geysen, R. H. Meloen and S. J. Barteling, Proc. Natl. Acad. Sci. USA 81, 3998 (1984)).

10 B) Deciphering the amino acid residues which are essential for function by means of substitution analysis (cf. R. A. Houghton, Proc. Natl. Acad. Sci. USA 82, 5131 (1985); H. M. Geysen, R. H. Meloen, and S. J. Barteling, Proc. Natl. Acad. Sci. USA 81, 3998 (1984)).

15 Biological test reactions such as enzymatic reaction and antibody binding are very sensitive, so that even small quantities (ng to μ g) of peptide substrates are sufficient. For the rapid and simple implementation of such tests it is advantageous if the peptide substrate is immobilized on a solid support material and can be readily removed from the reaction solution. The reaction
20 with the peptide can then be measured quantitatively either in the remaining reaction solution or by subsequent analysis of the support-bound material.

The extent to which such systematic investigations can be carried out depends essentially on the speed
25 of the peptide synthesis and on the technical and the chemical characteristics of the support. Rapid peptide syntheses can be carried out according to the principle developed by Merrifield of step-wise synthesis on a solid phase (R. B. Merrifield, J. Amer. Chem. Soc. 85, 2149
30 (1963)).

A process will be described below by which very many (several hundred) immobilized oligopeptides can advantageously be prepared per working day in quantities adequate for test purposes and on a suitable support
35 material or flat material.

For this purpose, the present invention provides

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a process for the rapid synthesis of support-bound or free peptides, starting from one or more functionalized supports and synthesizing the required peptides by a step by step coupling of an amino acid, dipeptide or oligopeptide

5 corresponding to the given amino acid sequences according to a suitable synthesis procedure, characterized in that:

a flat material is used as the support,

at each synthesis step, for coupling of amino acids or peptides, small aliquots of a solution of the
10 required amino acid or the required dipeptide or oligopeptide, in the form of their activated derivatives, are transferred to the planar (i.e., flat) support in the shape of spots or lines at spot-shaped or linear reaction areas;

15 the coupling is carried out in the reaction area which develops by wetting; and

at least two peptides are synthesized simultaneously alongside each other at at least two spot-shaped or linear reaction areas which are not in contact
20 with each other.

The present invention also provides a process for the rapid synthesis of support-bound or free oligonucleotides, starting from one or more functionalized supports and synthesizing the required oligonucleotides by a
25 step by step coupling of a mononucleotide, dinucleotide or oligonucleotide corresponding to the given nucleotide sequences according to a suitable synthesis procedure, characterized in that:

a flat material is used as the support;

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at each synthesis step, for coupling of nucleotides, small aliquots of a solution of the required nucleotide or the required dinucleotide or oligonucleotide, in the form of their activated derivatives, are transferred
5 to the support in the shape of spots or lines at spot-shaped or linear reaction areas;

the coupling is carried out in the reaction area which develops by wetting; and

at least two oligonucleotides are synthesized
10 simultaneously alongside each other at at least two spot-shaped or linear reaction areas which are not in contact with each other.

The present invention also provides a device for carrying out the process according to the invention. The
15 device comprises an automatic pipetting device with a fitted planar (i.e., flat) support, wherein the automatic pipetting device delivers aliquots of 10nl to 10 μ l to spot-shaped or linear reaction areas on the planar support, and wherein the planar support is adapted to be installed on a washing or
20 filtering device. The device may also comprise a plurality of small sealable receptacles for solutions of the amino acid, dipeptide, oligopeptide, nucleotide, dinucleotide or oligonucleotide to be coupled; means for air-drying the support; and means for sucking solutions through the planar
25 support.

According to an embodiment, the synthesized peptides and/or oligonucleotides are separated from the planar support using a cleaving solution.

According to a special embodiment, several to very
30 many different peptides are synthesized simultaneously alongside each other on the flat support on spot-shaped or

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3b

linear reaction areas which are not in contact with each other.

According to a further special embodiment, the flat support employed is a porous or non-porous film, membrane, or sheet (e.g. a sheet of paper) of this nature, which possess a chemically reactive functionality which is suitable for coupling amino acids or peptides.

A mechanically stable flat material which is chemically inert towards the reagents and solvents employed is used as the support material. It can be porous or non-porous

for use in the biological test reactions. In this case, the peptides which have been prepared can be directly employed without isolation and subsequent immobilization, if the linkage between the peptide and the support material has been chosen so that it is stable against all the chemical reactions during the peptide synthesis.

If the support material is porous, its inner surface is also available for the synthesis. In this case, washing can also be performed by sucking solutions through the material. Non-porous support material has a very much smaller surface area. Washing can be performed by simple rinsing.

Suitable flat materials for peptide synthesis according to the claimed process are, for example, those composed of cellulose (which bear hydroxyl functionalities and can be employed directly), glass or plastics, such as polyethylene, polypropylene and Teflon*, onto whose surface suitable chemical functionalities have been introduced, for example by graft polymerization of acrylic acid or its derivatives. The functionalization of the surfaces can additionally take place, for example, by the introduction of aminopropyl groups or glycine or diglycine anchor groups.

The size of the particular reaction area depends on the volume applied to the support, the absorptive properties of the support material and the volatility of the solvent. Depending on the selected support, for example on the specific functionality of a paper support, the particular magnitude of the fluid volume will naturally also depend on the chosen scale of the peptide synthesis. The magnitude of the particular fluid volume also determines the minimum distance between two reaction areas and thus, indirectly, the number of reaction areas which can be accommodated on a given support; cf., for example, Table 2.

In each case according to the nature and chemical pretreatment of the support, the surface-specific loading with reactive functionalities may be varied over a wide

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range (e.g. 1 pmol to 1 $\mu\text{mol}/\text{cm}^2$).

Specific examples of papers which may be employed are Whatman* Chr1, Whatman* 540 and Whatman* 3MM. Whatman* 540 is preferable to Whatman* Chr1 because of its greater mechanical stability. The size of the sheet of paper, as well as the arrangement of the reaction areas or patches or spots, is arbitrary. Immunologists will prefer the format of a microtiter plate for 8x spots. In this case, a standard microtiter plate can be taken in which small holes have been bored in the centers of the wells and these standard microtiter plates can be used as templates for cutting out paper sheets (8.5 x 12.5 cm) and marking the positions of the spots with a pencil.

The peptides are then built up on the surface functionalities stepwise and corresponding to the predetermined sequences according to known methods for solid-phase peptide synthesis (E. Wunsch et al. in Houben-Weyl: Methoden der organischen Chemie (Methods of Organic Chemistry), 4th Edition, Volume 15 (E. Müller, Editor), Thieme, Stuttgart, 1974; G. Barany, R. B. Merrifield in The Peptides, Vol. 2 (E. Gross, U. Meienhofer, Eds; Academic Press, New York, 1970, p. 1). Preferably, the Fmoc/tBu method is adopted (C.-D. Chang, U. Meienhofer, Int. J. Peptide Protein Res. 11, 246 (1978); E. Atherton, H. Fox, D. Harkiss, C. J. Logan, R. C. Sheppard, B. J. Williams, J. Chem. Soc. Chem. Commun. 537 (1978). Besides the twenty essential amino acids, modified and non-natural amino acids may also be incorporated, as required.

The amino acid components (suitably protected and activated amino acid derivatives) which are necessary for the chemical synthesis of the peptides, are provided as concentrated solutions (0.1 to 0.3 M) in a suitable solvent, which preferably has a high boiling point and low evaporation rate (e.g. N-methyl-pyrrolidinone, b.p. 203°C), in small sealable receptacles.

The linking of amino acids to peptides takes place stepwise, beginning with the C-terminal end, and,
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where appropriate, in parallel for many peptides by the cyclical performance of the same two reaction steps on each occasion. The linking may be illustrated, for example, as follows:

- 5 1) Peptide bond formation: an aliquot (0.1 to 10 μ l)
of the activated amino acid solution is added to a
predetermined dot or line on the planar support. As a
result of wetting, a reaction area (patch) is formed
which is defined by the volume applied. If many different
10 peptides are to be synthesized in parallel on a corres-
pondingly large support surface, the sites for applica-
tion are located at distances which ensure that these
reaction areas cannot intersect. The reaction time is 5
to 30 minutes, for example. After this, the support is
15 washed several times, for example with dimethylformamide
(DMF), in a suitable sealable receptacle or on a filter-
ing device. The sites for application can be established
by marking the support (e.g. pencil markings on cellulose
paper). However, the free amino functionalities on the
20 reaction surface may also be rendered directly visible by
means of a color indicator (e.g. Bromphenol Blue;
V. Krchnak, J. Vagner, P. Safar, M. Lebl, Collect. Czech.
Chem. Commun. 53,2542 (1988) so that no other marking is
necessary.
- 25 2) Elimination of the N-terminal protective group:
for this purpose, the support is treated with sufficient
cleavage solution. When the preferred Fmoc/tBu method is
implemented, the Fmoc protective group is eliminated with
20 percent piperidine in DMF, for example. After 2 to 10
30 minutes, for example, washing is carried out several
times with DMF. Where appropriate, treatment is then
carried out with a solution of the color indicator until
the reaction areas become visible. Then washing is
carried out several times with ethanol, for example, and
35 the material is air-dried (where appropriate using a
hairdryer at max. 40°C.

When all the peptide sequences have been completed, the N-terminal amino groups are preferably

blocked, for example by acetylation with acetic anhydride. For this purpose, the support can be treated with acetylation solution (e.g. 5 percent acetic anhydride and 5 percent diisopropylethylamine in DMF) and thereafter washed several times with DMF and ethanol.

After this, the protective groups on the amino acid side chains can be eliminated by a suitable acid treatment (depending on the chosen synthesis method). For this purpose, the support is treated with cleavage solution. According to a special embodiment using the Fmoc/tBu method (see the following list of amino acid derivatives employed), treatment is carried out, for example, for 10 minutes with 50 percent trifluoroacetic acid (TFA), 5 percent anisole and 1 percent thioanisole in dichloromethane. After that, the support is washed several times with dichloromethane, then DMF and then ethanol, and air-dried. The removal of the protective groups can also be achieved by treating with 50 percent TFA and 2 percent triisobutylsilane in DCM in the course of about 60 min., after which washing is carried out, for example with DCM, DMF and ethanol.

The peptides are now prepared for biological use as immobilized substrates. For this, the support, with the peptides covalently bound to it, can either be treated as a whole, e.g. with a test reaction solution, or else the individual reaction areas can be separated by cutting up the sheet and then treated individually.

The simplest method for constructing peptides on supports in the manner described is to link the corresponding C-terminal amino acid components directly to the reactive functionalities on the support surface via stable amide or ester bonds. According to more specific embodiments, a so-called bifunctional linker reagent (cf. E. Atherton et al. J. Chem. Soc. Perkin Trans. I 538-546 (1981)) can first of all be linked to the support functionalities. The peptide, which is in turn constructed on it, can then, depending on the chemical nature of the peptide-linker bond, be cleaved

off, e.g. under suitable reaction conditions with a C-terminal carboxyl or carboxamide group, and employed in soluble form for test purposes (labile linker!). However, the linker reagent may also be employed as a spacer, in order to increase the availability of the peptide for large reaction partners such as antibodies or enzymes (stable linker!).

When using paper supports, a two-step procedure may be employed.

10 First step (A): A uniform distribution of reactive amino functionalities is provided. For this purpose, the hydroxyl groups of the cellulose are usually esterified with an N-Fmoc-protected amino acid derivative and the amino groups are subsequently liberated by treatment with 20 percent piperidine solution in DMF.

15 Second step (B): A further N-Fmoc-amino acid is attached in a spot-shaped or linear manner to the amino functionalities which have been introduced. Subsequently, all the remaining amino functionalities are blocked by acetylation and the Fmoc is cleaved off.

20 Fmoc-glycine was also used for both derivatization steps within the scope of the present invention. However, not inconsiderable quantities of the Gly-Gly anchor were lost during removal of the Fmoc protective group and during extended storage, presumably as a result of diketopiperazine formation. Furthermore, the Gly-Gly end of the peptides appears to interfere with antibody binding, and some sera also appear to bind non-specifically to Ac-Gly-Gly. Fmoc- β -Ala is a better choice. β -Alanine is an unusual amino acid which is not present in natural proteins, while the β -Ala- β -Ala anchor is not subject to any diketopiperazine formation and the spacer is enlarged. Dried, flat materials which had been derivatized with patches of β -Ala- β -Ala and stained with BPB showed no loss of amino acid functionalities after several months of storage at -20°C . For more extended storage at temperatures up to 30°C , an Fmoc-protected anchor is even more stable. For clarification, the reader

is referred to Figure 6. This figure shows a typical arrangement of patches on a paper sheet (β -Ala- β -Ala anchor stained with BPB). In this case, the format conformed to that of a microtiter plate for 96 patches.

5 When compared to the state of the art (Geysen et al. Proc. Natl. Acad. Sci. USA 81, 3998 (1984); Frank and Krause, International Patent Publication No. WO91/06558 published on May 16, 1991), the process which has been presented has the following advantages:

10 - extremely simple operation; for the chemical couplings of the amino acid derivatives to peptides, only small volumes of the activated amino acid solutions need be dotted onto the planar support;

15 - even smaller reaction volumes are possible, e.g. 0.1 to 1 μ l, resulting in even more economical consumption of the expensive amino acid derivatives;

 - the supports such as cellulose papers or glass fiber papers are readily available or can be obtained commercially;

20 - the scale of synthesis of individual peptides can be selected within a wide range because of the variable surface-specific loading with reactive functionalities;

25 - the pipetting steps (in particular when the process is automated) can be carried out extremely quickly so that still more peptides can be prepared per unit time.

 The process described above can also be enlisted in a corresponding manner for the rapid synthesis of support-bound or free oligonucleotides. With regard to the chemistry and the performance of oligonucleotide synthesis, reference can be made, for example, to the European Patent 84 100 059.9.

30 The process described here may also be automated. In this case, automatic pipetting devices can be employed for aliquotting and spotting of the activated amino acid solutions or nucleotide solutions as well as addition of the other washing and reaction solutions. The planar support must then be installed on an appropriate washing or filtering device.

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Known devices do not make use of planar supports. Rather they are designed to deliver their droplets to reaction vessels, since according to the art, synthesis of peptides or oligonucleotides are not carried out on supports, but in solution. The claimed device is not provided with reaction vessels, but instead with planar supports.

The x/y-programmable TLC spotters or pipetting workstations which are obtainable commercially are suitable devices for the automated implementation of the process according to the invention. Volumes down to 10 nl can be added precisely at each desired location, so that several thousand peptides can be synthesized on a readily manageable flat material support. This is 2 orders of magnitude below the lithographic technique of Fodor et al. Science, 251, 767-773 (1991). Even in the smallest quantities of fluid (about 10 nl), each peptide is formed in nmol quantities, which are sufficient for isolation and exhaustive characterization.

20 **Implementation Example 1:**

Analysis of an immunogenic segment (CMV26) of the 36/40K protein of cytomegalovirus. **Figure 1:** primary amino acid sequence of the cloned, immunogenic segment CMV26. Overlapping decapeptide sequences, in each case displaced by one amino acid, were derived from this. **Figures 2 A - C:** List of all the decapeptides to be synthesized with the allocated numbering and instructions for the simultaneous parallel implementation of the amino acid couplings.

Planar support: A 5.5 x 10.5 cm large sheet of cellulose paper (Chrl or 540, Whatman, England) was cut out. 50 spots at a distance of 1 cm were then marked on it with

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10a

pencil. The sheet was then placed in a cylindrical glass vessel which could be sealed with a ground stopper and treated at room temperature for 3 hours with 1 ml of a solution of

- 5 0.2 M Fmoc-glycine
- 0.3 M Hydroxybenzotriazole (HOBt)
- 0.24 M Diisopropylcarbodiimide (DIC)
- 0.2 M N-methylimidazole (MeIm)

in DMF,

- 10 and subsequently washed three times with 10 ml of DMF on each occasion, then treated for 10 minutes with 20 percent piperidine in DMF (elimination of the Fmoc group), thereafter washed three times with 10 ml of DMF

on each occasion and twice with 10 ml of ethanol (abs) on each occasion, and dried with a hairdryer. The quantity of glycine (free amino groups) esterified to the hydroxyl groups of the cellulose paper was determined quantitatively 0.2 as 0.3 $\mu\text{mol}/\text{cm}^2$.

5 The sheet was placed in a shallow glass or polyethylene dish. Aliquots (1 μl) of a solution of 0.3 M Fmoc-glycine, 0.45 M HOBt and 0.36 M DICD in N-methylpyrrolidinone (MPD) were then added to each of the pre-
10 marked spots on the paper and the dish was covered with a glass plate. After 20 minutes, the sheet was placed in the glass cylinder and washed three times with 10 ml of DMF on each occasion. Then all the free amino groups of the first glycine coupling which were still present on
15 the paper were blocked by acetylation. For this purpose, the sheet was treated for 10 minutes with 3 ml of 5 percent acetic anhydride and 5 percent diisopropylethylamine in DMF. Subsequently, it was washed three times with 10 ml of DMF on each occasion, then treated
20 with 20 percent piperidine in DMF and washed three times again with DMF. After that, 3 ml of 0.3 percent Bromphenol Blue (BPB) in DMF were added, as a result of which the reaction areas of the 2nd glycine coupling stain blue. The sheet was then washed twice with 10 ml of
25 ethanol on each occasion and dried.

The cellulose planar support which has been pretreated in this manner provides diglycine anchor groups with free primary amino functionalities at the marked reaction areas for the construction of peptides.
30 The 49 decapeptides listed in Figure 2A were numbered sequentially and synthesized simultaneously in parallel on the correspondingly numbered reaction areas as described above. The distribution of the activated amino acid solutions was carried out using the list shown in
35 Figure 2B and 2C.

Synthesis method: Fmoc/tBu method

Amino acid derivatives employed:

Fmoc Ala

Fmoc-Arg (Pmc)
 Fmoc-Asn (Tmob)
 Fmoc-Asp (OtBu)
 Fmoc-Cys (Butthio)
 5 Fmoc-Gln (Tmob)
 Fmoc-Glu (OtBu)
 Fmoc-Gly
 Fmoc-His (Boc)-Pfp
 Fmoc-Ile
 10 Fmoc-Leu
 Fmoc-Lys (Boc)
 Fmoc-Met
 Fmoc-Phe
 Fmoc-Pro
 15 Fmoc-Ser(tBu)
 Fmoc-Thr(tBu)
 Fmoc-Trp
 Fmoc-Tyr (tBu)
 Fmoc-Val

20 All the protective groups of the amino acid side chains (-), with the exception of (Butthio) on Cys, can be removed with 20 to 50 percent TFA in dichloromethane. The Butthio group can then subsequently be removed with, for example, dithiothreitol (DTT) or dithioerythritol
 25 (DTE).

The activation of the amino acid derivatives can be effected according to standard methods, e.g. by 1.5-fold excess of 1-hydroxybenzotriazole (HOBt) and 1.2-fold excess of diisopropylcarbodiimide (DIC) in N-methyl-
 30 pyrrolidinone for one hour. Other activation methods, for example using symmetrical anhydrides, pentafluorophenol esters or BOP, are also possible. In accordance with the consumption that was calculated in advance, 0.2 to 1 ml of the solutions was made up. Aliquots of 1 μ l were
 35 applied. After 5 to 20 minutes, the color of the reaction areas stained blue with BPB changed to yellow, indicating complete reaction of the free amino groups. Elimination of the Fmoc protective group was carried out using

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20 percent piperidine in DMF for 5 minutes. Terminal acetylation of the completed peptides was achieved by treatment with 5 percent acetic anhydride and 5 percent diisopropylethylamine in DMF for 10 minutes. The protective groups on the amino acid side chains were removed using 20 percent TFA and 1 percent triisobutylsilane in dichloromethane for 20 minutes, and the support was subsequently washed, using on each occasion 10 ml portions, three times with dichloromethane, three times with DMF and three times with ethanol, and dried.

Using these peptides, the sequence-specific binding of anti-CMV26 antibodies in a rabbit serum, for example, was demonstrated by means of a color test.

Figure 3: Schematic representation of the color test. The surface-bound peptides (P) are incubated with the rabbit anti-CMV26 serum, excess serum is washed off and incubation is then carried out with a goat anti-rabbit antibody, which is covalently linked to the enzyme β -galactosidase. Only if the anti-CMV26 antibodies can bind the peptide is the enzyme bound via the second antibody. This enzyme can then be detected by its activity in cleaving 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, resulting, in the presence of a mild oxidising agent such as potassium hexacyanoferrate(III), in the formation of a blue indigo dye which is insoluble in water and alcohol.

Figure 4: Photograph of the flat cellulose support after the color test. The peptides on the reaction areas 11 to 16 and 30 to 33 are reacting positively.

The bound antibodies can be detached once more by treating the support with proper reagents (such as urea, sodium dodecyl sulfate and mercaptoethanol). The indigo dye can be washed off with DMF. After that the support with the immobilized peptides can be re-used. This can be repeated several times as long as no reaction conditions are used which detach or destroy the peptides. After more than ten antibody binding tests with the same support,

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some reaction areas were cut out and the peptides detached with 1 M NaOH. It was possible unambiguously to detect the CMV26-Gly-Gly peptides both in HPLC analysis and by FAB mass spectroscopy.

5 **Implementation Example 2:**

Analysis of the same immunogenic segment (CMV26) of the 36/40K protein of cytomegalovirus.

Planar support: A 5.5 x 10.5 cm large sheet of glass fiber paper (QM-A*, Whatman, England) was cut out; 50
10 spots were then marked on it with a pencil at a distance of 1 cm in each case. The sheet was boiled overnight under reflux in a conical flask (250 ml) with a ground stopper together with 50 ml of 2 percent aminopropyl triethoxysilane in abs. toluene. Subsequently the sheet
15 was washed three times with toluene and three times with acetone, on each occasion with portions of 10 ml, and dried at 110°C for 1 hour. The aminopropyl groups (free amino groups) introduced in this manner were quantitatively estimated to be 30 to 50 nmol/cm².

20 All further chemical operations were carried out exactly as described in Implementation Example 1 for cellulose paper after the first glycine coupling. The result of the antibody binding test was identical.

The Implementation Examples 1 and 2 illustrate an
25 important aspect of the use of the flat materials, with their bound peptides, which are prepared by the claimed process. They can be employed for diagnostic purposes in human and veterinary medicine for the very specific detection of bacterial and viral infections. Such infec-
30 tions lead, by immune reactions within the affected organism, to the formation of antibodies against viral or bacterial proteins. Using virus-specific or bacterium-specific peptides, such antibodies can be detected in the serum and the nature and subclass of the causative
35 organism can thereby be determined. The same process can in turn be employed in order to identify the specific peptides, by (as shown in the implementation examples) preparing and testing series of peptides bound to flat

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material.

Implementation Example 3:

Four oligopeptides related to CMV26 were synthesized by spot-shaped synthesis on paper supports with Boc-Lys-Pro derivatization and cleaved from the supports using diketopiperazine formation. Details may be obtained from Figure 5 A-B and Table 1. These show:

5

Figure 5 A: HPLC traces of the crude peptides;

Figure 5 B: FAB mass spectrum of peptide 15 (based on positive ions);

10

Table 1: Tabulated data relating to the synthesized peptides.

Peptide Sequence No.	Molecular mass ^{a)} (theor.) [M+H] ⁺	Amino acid analysis ^{b)}					Amount				
		D	S	G	A	L	P	K	[nmol]		
14	AC-G-G-S-L-S-S-DKP	755.4	756.4	-	2.88	1.98	-	1.00	x	x	66
15	AC-G-S-L-S-S-L-DKP	811.4	812.4	-	2.90	0.97	-	2.00	x	x	52
16	AC-S-L-S-S-L-A-DKP	825.4	826.4	-	2.90	-	0.99	2.00	x	x	54
17	AC-L-S-S-L-A-N-DKP	852.4	853.4	0.89	1.98	-	1.00	2.00	x	x	57
R	AC-DKP	268.2	267.2	-	-	-	-	-	x	x	ca. 75

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- (a) Ac-DKP was determined in the FAB mass spectrum only as (M-H)⁻ based on negative ions.
- (b) The DKP moiety was not completely degraded under standard hydrolysis conditions; x signifies that Pro and Lys were present in equal quantities, although not equimolar in relation to the amino acid residues in the peptide chain.

Implementation Example 4:

Volumes down to 0.1 μ l were dotted on by hand using an ordinary micropipette which was fitted with a polypropylene tip. Some typical data, which were obtained using two different paper qualities, are assembled in Table 2 below.

Table 2: Relation between dot volume, patch size and functionality.

A: Whatman* 540			B: Pre-treated Whatman* 3 MM		
β -ala anchor			β -ala anchor		
Volume	Diameter	Anchor	Volume	Diameter	Anchor
[μ l]	[mm]	[μ mol]	[μ l]	[mm]	[μ mol]
0.1	3	0.02	1	4	0.10
0.5	5	0.07	5	7	0.45
1.0	7	0.13	10	10	0.85
2.0	9	0.25	20	14	1.70
5.0	12	0.60	50	22	4.40

Values are $\pm$ 5 percent

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CLAIMS:

1. A process for a rapid synthesis of a plurality of peptides, each of which has an amino acid sequence and is free or bound to a support, which process comprises:

5 providing the support that is a flat porous film, membrane or sheet and has a functionality suitable for coupling with an amino acid or peptide;

coupling step by step an amino acid, dipeptide or oligopeptide corresponding to the amino acid sequence onto
10 the support, thereby synthesizing the peptides bound to the support; and

when the peptides freed from the support are required, separating the synthesized peptides from the support by using a suitable cleaving solution, wherein:

15 at each coupling step, a small aliquot of a solution of the amino acid, dipeptide or oligopeptide, in the form of an activated derivative, is transferred to the support in the shape of a spot or line at a spot-shaped or linear reaction area;

20 the coupling is carried out in the reaction area which develops by wetting; and

the peptides are synthesized simultaneously alongside each other at at least two spot-shaped or linear reaction areas which are not in contact with each other.

25 2. The process according to claim 1, wherein the synthesized peptides are kept bound to the support.

3. The process according to claim 1 or 2, wherein the coupling is conducted by an Fmoc/tBu method.

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4. The process according to any one of claims 1 to 3, wherein the support is composed of cellulose, glass or an organic polymer and has the functionality on a surface thereof.

5 5. The process according to any one of claims 1 to 4, wherein the functionalized support has an aminopropyl group or a glycine or diglycine anchor group as the functionality.

6. The process according to any one of claims 1 to 5, wherein the coupling of the amino acid, dipeptide or
10 oligopeptide takes place starting with a C-terminus of each peptide to be synthesized.

7. The process according to any one of claims 1 to 6, wherein the functionalized support has a surface-specific loading with a reactive functionality of from 1 pmol
15 to 1 μmol per cm^2 .

8. A process for a rapid synthesis of a plurality of oligonucleotides, each of which has an oligonucleotide sequence and which is free or bound to a support, which process comprises:

20 providing the support that is a flat porous film, membrane or paper and has a functionality suitable for coupling with a nucleotide;

coupling step by step a mononucleotide, dinucleotide or oligonucleotide corresponding to the given
25 nucleotide sequence according to a suitable synthesis procedure, thereby synthesizing the oligonucleotides bound to the support; and

when the oligonucleotides freed from the support are required, separating the oligonucleotides so synthesized
30 from the support, wherein:

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at each coupling step, a small aliquot of a solution of the required mononucleotide, dinucleotide or oligonucleotide, in the form of an activated derivative, is transferred to the support in the shape of a spot or line at
5 a spot-shaped or linear reaction area,

the coupling is carried out in the reaction area which develops by wetting, and

the oligonucleotides are synthesized simultaneously alongside each other at at least two spot-
10 shaped or linear reaction areas which are not in contact with each other.

9. The process according to claim 8, wherein the support is composed of cellulose, glass or an organic polymer and has the functionality on a surface thereof.

15 10. The process according to claim 8 or 9, wherein the functionalized support has an aminopropyl group or a glycine or diglycine anchor group as the functionality.

11. The process according to any one of claims 8 to 10, wherein the functionalized support has a surface-
20 specific loading reactive functionality of from 1 pmol to 1 μmol per cm^2 .

12. The process according to any one of claims 8 to 11, wherein the coupling takes place starting with a 3'-terminus of each oligonucleotide to be synthesized.

25 13. The process according to any one of claims 8 to 12, wherein the synthesized oligonucleotides are kept bound to the support.

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14. The process according to any one of claims 8 to 12, wherein the synthesized oligonucleotides are freed from the support.

15. A device for carrying out the process as defined
5 in any one of claims 1 to 7, which comprises:

an automatic pipetting device;

a support in the form of a flat porous film,
membrane or sheet which has a plurality of spot-shaped or
linear reaction areas on a flat surface thereof, the
10 reaction areas having a functionality suitable for coupling
with the amino acid, dipeptide or oligopeptide to be coupled
according to the process;

a washing or filtering device on which the support
is adapted to be installed;

15 a plurality of small sealable receptacles each for
containing therein a solution of the amino acid, dipeptide
or oligopeptide;

means for air-drying the support; and

means for sucking a washing liquid through the
20 support,

wherein the automatic pipetting device delivers
aliquots of 10 nl to 10 µl to the spot-shaped or linear
reaction areas on the support.

16. A device for carrying out the process as defined
25 in any one of claims 8 to 14, which comprises:

an automatic pipetting device;

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a support in the form of a flat porous film, membrane or sheet which has a plurality of spot-shaped or linear reaction areas on a flat surface thereof, the reaction areas having a functionality suitable for coupling
5 with the nucleotide, dinucleotide or oligonucleotide to be coupled according to the process;

a washing or filtering device on which the support is adapted to be installed;

a plurality of small sealable receptacles each for
10 containing therein a solution of the nucleotide, dinucleotide or oligonucleotide;

means for air-drying the support; and

means for sucking a washing liquid through the support,

15 wherein the automatic pipetting device delivers aliquots of 10 nl to 10 μ l to the spot-shaped or linear reaction areas on the support.

17. The device according to claim 15 or 16, wherein the support is a sheet of paper made of cellulose or glass
20 fiber and having a surface thereof functionalized with a free primary amino group.

18. The device according to claim 17, wherein the surface of the support has an aminopropyl group or an anchor selected from the group consisting of a Gly-Gly anchor and a
25 β -Ala- β -Ala anchor.

19. The process according to any one of claims 1 to 8, wherein the synthesized peptide is specific to a virus or bacteria and causes an immuno reaction with an antibody against the virus or bacteria.

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20. A flat porous film, membrane or sheet with peptides bound to a surface thereof, prepared according to the process of any one of claims 1 to 7.

21. A flat porous film, membrane or sheet with
5 oligonucleotides bound to a surface thereof, prepared according to the process of any one of claims 8 to 13.

22. Use of the flat porous film, membrane or sheet according to claim 20 or 21 for a diagnostic purpose in human or veterinary medicine for detection of a bacterial or
10 viral infection.

23. Use of the flat porous film, membrane or sheet according to claim 21 for a hybridization purpose.

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Figure 1

CMV26 (36/40K protein)

ARGSIEGRGKSRGGGGGSSLANAGGLHDDGPGLDNDLMNEPMGLGGLGGGGGGKKHLDPSTCSQAC

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Fig. 2 A

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A:CMV26PEP

		Len	M.W.	Protection/ Modification
1- 1A	IEGRGKSRGG	10	1015.6	
2- 2A	EGRGKSRGGG	10	959.6	
3- 3A	GRGKSRGGGG	10	887.6	
4- 4A	RGKSRGGGGG	10	887.6	
5- 5A	GKSRGGGGGG	10	788.5	
6- 6A	KSRGGGGGGG	10	788.5	
7- 7A	SRGGGGGGGS	10	747.4	
8- 8A	RGGGGGGGSL	10	773.5	
9- 9A	GGGGGGGSLS	10	704.4	
10- 10A	GGGGGSLSS	10	734.4	
11- 11A	GGGGSLSSL	10	790.5	

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FIG. 2A

12- 12A	GGGSLSSIA	10	804.5	
13- 13A	GGSLSSLAN	10	861.5	
14- 14A	GGSLSSLANA	10	875.5	
15- 15A	GSLSSLANAG	10	875.5	
16- 16A	SLSSLANAGG	10	875.5	
17- 17A	ISSLANAGGL	10	901.6	
18- 18A	SSLANAGGLH	10	925.6	
19- 19A	SLANAGGLHD	10	953.6	
20- 20A	LANAGGLHDD	10	981.5	
21- 21A	ANAGGLHDDG	10	925.5	
22- 22A	NAGGLHDDGP	10	951.5	

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Restrictions as to Use

FIG. 2A

23- 23A	AGGLHDDGPG	10	894.5	
24- 24A	GGIHHDDGPGL	10	936.5	
25- 25A	GLHDDGPGLD	10	994.5	
26- 26A	LHDDGPGLDN	10	1051.6	
27- 27A	HDDGPGLDND	10	1053.5	
28- 28A	DDGPGLDNDL	10	1029.5	
29- 29A	DGPGLDNDLM	10	1045.5	
30- 30A	GPGLDNDLMN	10	1044.5	
31- 31A	PGLDNDLMNE	10	1116.6	
32- 32A	GLDNDLMNEP	10	1116.6	
33- 33A	LNDNDLMNEPM	10	1190.6	

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FIG. 2A

34- 34A	DNDLMNEPMG	10	1134.5	
35- 35A	NDLMNEPMGL	10	1132.6	
36- 36A	DLMNEPMGLG	10	1075.6	
37- 37A	LMNEPMGLGG	10	1017.6	
38- 38A	MNEPMGLGGL	10	1017.6	
39- 39A	NEPMGLGGLG	10	943.5	
40- 40A	EPMGLGGLGG	10	886.5	
41- 41A	PMGLGGLGGG	10	814.5	
42- 42A	MGLGGLGGGG	10	774.5	
43- 43A	GLGGLGGGGG	10	700.4	
44- 44A	LGGLGGGGGG	10	700.4	

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45- 45A	GGGCGGGGGG	10	644.4	
46- 46A	GLGGCGGGGG	10	644.4	
47- 47A	LGCGGGGGGK	10	715.5	
48- 48A	GGCGGGGGKK	10	730.5	
49- 49A	GGCGGGGGKH	10	810.5	

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FIG. 2B/2C

Cycle 0

G: 1 2 3 4 5 6 15 16 21 23 34 36 37 39 40 41 42 43 44
 45 46 (21/0)
 L: 8 11 17 24 28 35 38 (7/0)
 D: 19 20 25 27 (4/0)
 N: 13 26 30 (3/0)
 S: 7 9 10 (3/0)
 A: 12 14 (2/0)
 M: 29 33 (2/0)
 H: 18 49 (2/0)
 P: 22 32 (2/0)
 K: 47 48 (2/0)
 E: 31 (1/0)

Cycle 1

G: 1 2 3 4 5 6 7 16 17 22 24 35 37 38 40 41 42 43 44
 45 46 47 (22/0)
 L: 9 12 18 25 29 36 39 (7/0)
 D: 20 21 26 28 (4/0)
 N: 14 27 31 (3/0)
 S: 8 10 11 (3/0)
 M: 30 34 (2/0)
 K: 48 49 (2/0)
 P: 23 33 (2/0)
 A: 13 15 (2/0)
 E: 32 (1/0)
 H: 19 (1/0)

Cycle 2

G: 2 3 4 5 6 7 8 17 18 23 25 36 38 39 41 42 43 44 45
 46 47 48 (22/0)
 L: 10 13 19 26 30 37 40 (7/0)
 D: 21 22 27 29 (4/0)
 N: 15 28 32 (3/0)
 S: 9 11 12 (3/0)
 A: 14 16 (2/0)
 P: 24 34 (2/0)
 M: 31 35 (2/0)
 K: 49 (1/0)
 E: 33 (1/0)
 R: 1 (1/0)
 H: 20 (1/0)

Cycle 3

G: 3 4 5 6 7 8 9 18 19 24 26 37 39 40 42 43 44 45 46
 47 48 49 (22/0)
 L: 11 14 20 27 31 38 41 (7/0)
 D: 22 23 28 30 (4/0)
 S: 1 10 12 13 (4/0)
 N: 16 29 33 (3/0)
 A: 15 17 (2/0)
 P: 25 35 (2/0)
 M: 32 36 (2/0)
 H: 21 (1/0)
 R: 2 (1/0)
 E: 34 (1/0)

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FIG. 2B/2C

Cycle 4

G: 4 5 6 7 8 9 10 19 20 25 27 38 40 41 43 44 45 46 47
 48 49 (21/0)
 L: 12 15 21 28 32 39 42 (7/0)
 D: 23 24 29 31 (4/0)
 S: 2 11 13 14 (4/0)
 N: 17 30 34 (3/0)
 A: 16 18 (2/0)
 P: 26 36 (2/0)
 M: 33 37 (2/0)
 K: 1 (1/0)
 E: 35 (1/0)
 R: 3 (1/0)
 H: 22 (1/0)

Cycle 5

G: 1 5 6 7 8 9 10 11 20 21 26 28 39 41 42 44 45 46 47
 48 49 (21/0)
 L: 13 16 22 29 33 40 43 (7/0)
 D: 24 25 30 32 (4/0)
 S: 3 12 14 15 (4/0)
 N: 18 31 35 (3/0)
 A: 17 19 (2/0)
 P: 27 37 (2/0)
 M: 34 38 (2/0)
 K: 2 (1/0)
 E: 36 (1/0)
 R: 4 (1/0)
 H: 23 (1/0)

Cycle 6

G: 2 6 7 8 9 10 11 12 21 22 27 29 40 42 43 45 46 47 48
 49 (20/0)
 L: 14 17 23 30 34 41 44 (7/0)
 D: 25 26 31 33 (4/0)
 S: 4 13 15 16 (4/0)
 N: 19 32 36 (3/0)
 A: 18 20 (2/0)
 P: 28 38 (2/0)
 R: 1 5 (2/0)
 M: 35 39 (2/0)
 E: 37 (1/0)
 H: 24 (1/0)
 K: 3 (1/0)

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FIG. 2B/2C

Cycle 7

G: 1 3 7 8 9 10 11 12 13 22 23 28 30 41 43 44 46 47 48
 49 (20/0)
 L: 15 18 24 31 35 42 45 (7/0)
 D: 26 27 32 34 (4/0)
 S: 5 14 16 17 (4/0)
 N: 20 33 37 (3/0)
 A: 19 21 (2/0)
 P: 29 39 (2/0)
 R: 2 6 (2/0)
 M: 36 40 (2/0)
 E: 38 (1/0)
 H: 25 (1/0)
 K: 4 (1/0)

Cycle 8

G: 2 4 8 9 10 11 12 13 14 23 24 29 31 42 44 45 47 48 49
 (19/0)
 L: 16 19 25 32 36 43 46 (7/0)
 D: 27 28 33 35 (4/0)
 S: 6 15 17 18 (4/0)
 N: 21 34 38 (3/0)
 M: 37 41 (2/0)
 A: 20 22 (2/0)
 P: 30 40 (2/0)
 R: 3 7 (2/0)
 E: 1 39 (2/0)
 H: 26 (1/0)
 K: 5 (1/0)

(18/1)

Cycle 9

G: 3 5 9 10 11 12 13 14 15 24 25 30 32 43 45 46 48 49
 L: 17 20 26 33 37 44 47 (7/7)
 D: 28 29 34 36 (4/4)
 S: 7 16 18 19 (4/4)
 N: 22 35 39 (3/3)
 M: 38 42 (2/2)
 A: 21 23 (2/2)
 P: 31 41 (2/2)
 R: 4 8 (2/2)
 E: 2 40 (2/2)
 H: 27 (1/1)
 I: 1 (1/1)
 K: 6 (1/1)

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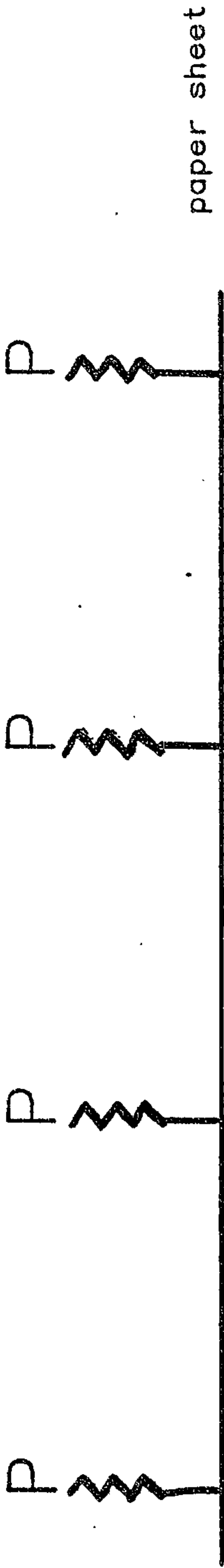
Figure 3

5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

β -galactosidase
5-bromo-4-chloro-indigo
blue color

Goat anti Rabbit

Rabbit anti CMV26



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Fig. 4

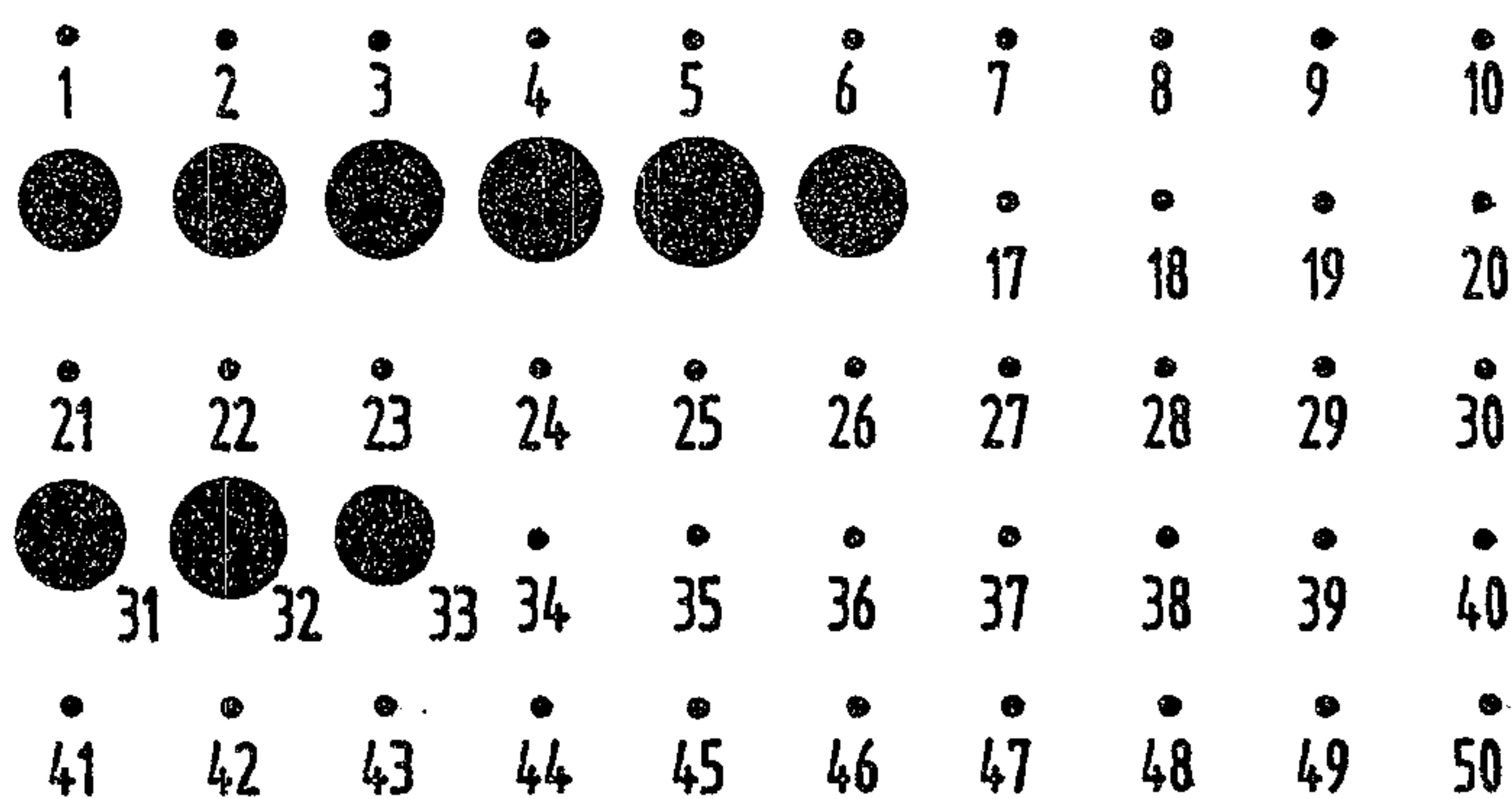
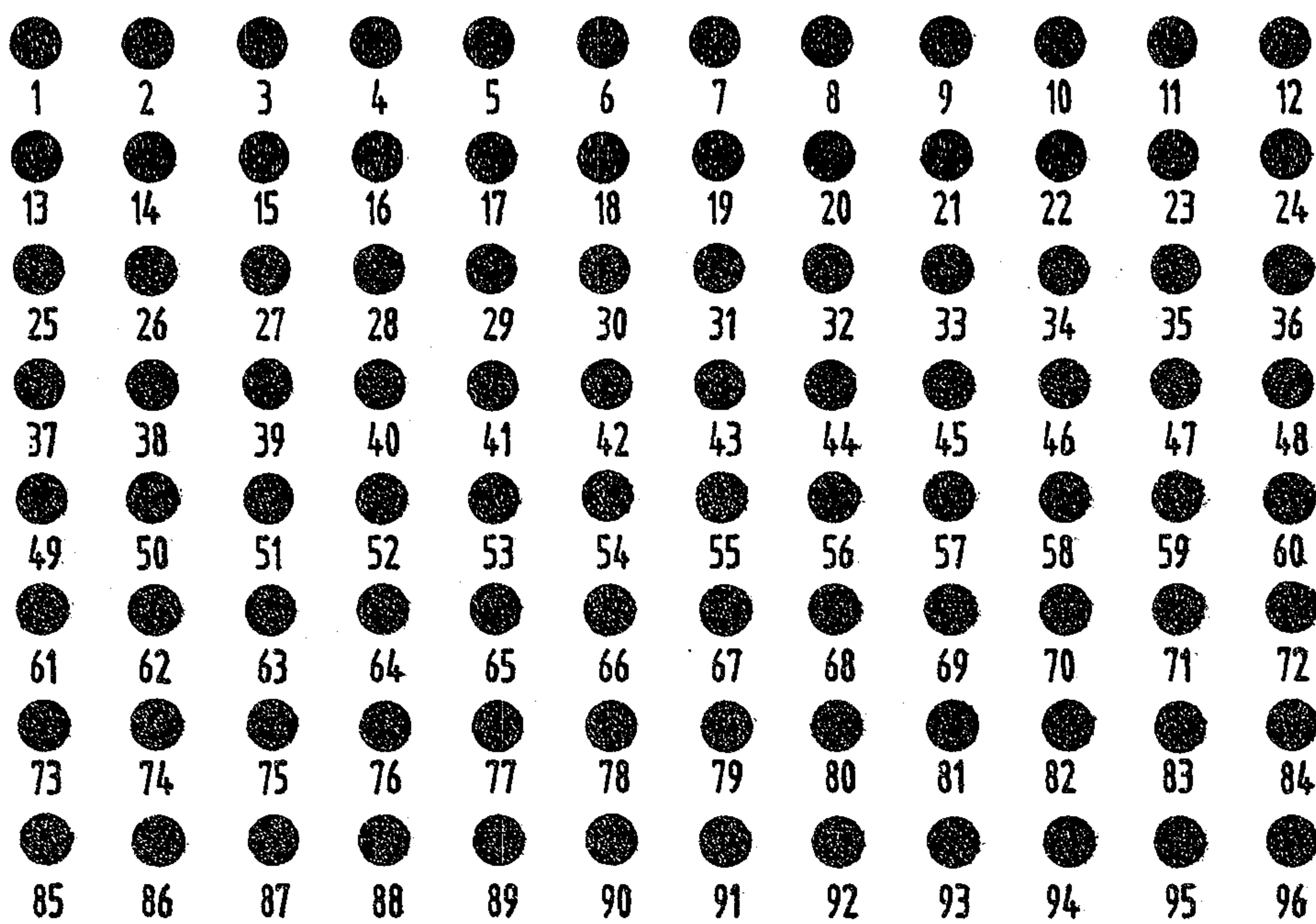


Fig.6



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Fig. 5A

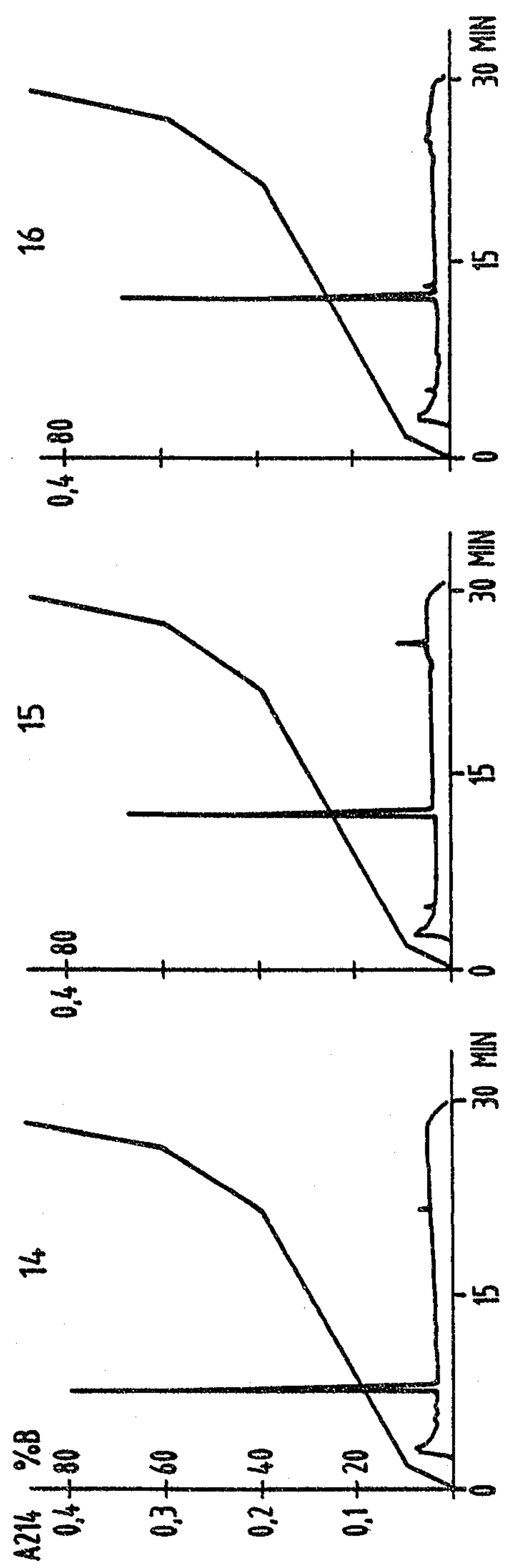
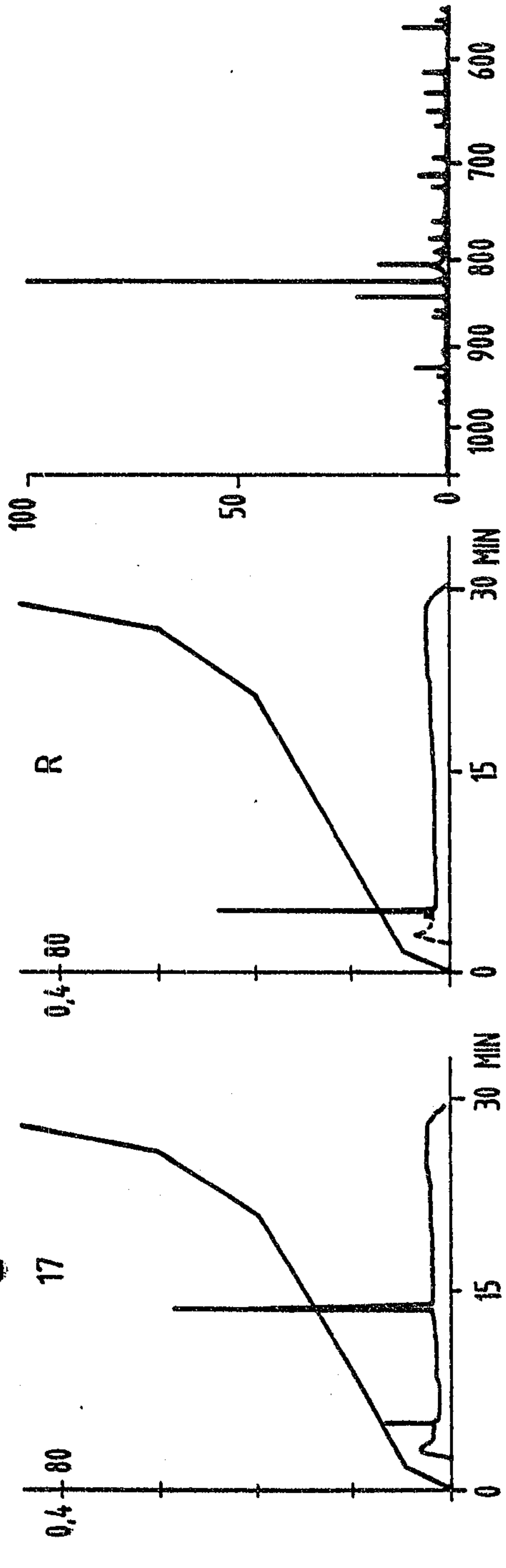


Fig. 5B



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