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(71) Applicant *(for all designated States except US):* AUTOMATED MICROBIOLOGY SYSTEMS LIMITED [GB/GB]; Grundy Building, Somerset Road, Teddington, Middlesex TW11 8TD (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only) :* BLOCH, Philip, Lyle [US/US]; 6807 Rosefield Drive, La Mesa, CA 92041 (US).

(74) Agent: AJELLO, Michael, J.; 38a Bramhall Lane South, Bramhall, Stockport, Cheshire SK7 1AH (GB).

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(54) Title: ELECTROPHORESIS GEL AND METHOD OF PRODUCTION

(57) Abstract

A method of producing and packaging a two-part or multiple-part polyacrylamide gel with a common buffer for electrophoresis, comprising the steps of casting within an upright narrow container, a stacking gel part followed, after polymerisation, with a resolving gel part. When the gel system is complete a part of the container is removed leaving the assembly on a backing sheet. The exposed upper surface of the gel is then covered by a protective film, and a plurality of gels fabricated in this manner may be stacked in superimposed relationship and packaged for storage. The gel system is fabricated using a single buffer for the stacking and resolving gel parts which may thus be contiguous when packaged. The totally pre-cast gel has a long shelf life and its fabrication produces minimum distorsion.

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ELECTROPHORESIS GEL AND METHOD OF PRODUCTION

THIS INVENTION concerns a method for constructing a gel matrix for the separation of molecules, particularly though not exclusively a method of applying gel electrophoresis theory to the construction of a polyacrylamide gel employed for the electrophoretic separation of polypeptides from polypeptide mixtures.

A polyacrylamide gel is constructed in two contiguous parts such that one part (stacking gel) is used for sample introduction and concentration, and the other part (resolving gel) is used for separation of individual polypeptides from a mixture. During the construction of the gel, it is impregnated with a gel buffer. The electrophoretic procedure is performed by connecting the gel to electrodes with wicks soaked in electrode buffer and placing the dissolved polypeptide mixture onto the surface of the stacking gel using a sample applicator. An electric field is then created with a regulated direct-current power supply. Under the influence of the electric potential a moving boundary of buffer ions is created, and the polypeptides move into the stacking gel, being concentrated into a very sharp zone within the moving boundary until they reach the resolving gel. As the polypeptides migrate

through the resolving gel their rates of travel are differentially affected by the frictional forces exerted by the resolving gel matrix so that the largest polypeptides move the least and the smallest polypeptides move the most. The extremely thin starting zone and the differential frictional forces combined with the high anticonvective and non-diffusional properties of the polyacrylamide gel, provide resolution of a cellular extract of about 1,000 proteins in up to 200 visible components.

An object of the present invention is to provide a new application of gel electrophoresis theory that allows the same buffer to be formulated for use within both the stacking and resolving portions of the gel such that once constructed the gel can be stored for long periods of time without deterioration of the quality of the resolution obtained. The invention also provides a special method of fabrication such that minimum distortion is obtained in the bands of resolved components.

Since its introduction in 1959, discontinuous polyacrylamide gel electrophoresis (PAGE) has continued to provide high quality results throughout the spectrum of biological research due to the very high resolution obtained. The combination of properties of PAGE that lead to very high resolution are: (i) very thin starting zones, of the order of ten microns, and (ii) non-convective and

anti-diffusional property of the gel. The power of PAGE to concentrate samples into very thin starting zones allows the application of dilute samples in large volumes (thick loading zone) without affecting the ultimate resolution of the system. Resolution of two components, for example, is achieved when the components migrate until one has travelled further than the other by at least one width of the zone created at the starting point. Since diffusion acts to broaden sample bands and thereby interfere with resolution, the non-diffusional property of the gel is important in achieving the high resolution of discontinuous PAGE.

Concentration of samples into thin starting zones (known as the stacking phenomenon) relies on adjusting the pH and chemical composition of the stacking gel and the electrode buffer such that Kohlrausch conditions are obtained. The required ionic constituents are a trailing ion (supplied by the electrode buffer), and a leading ion (supplied by the stacking and resolving gel). At the beginning of the run both leading and trailing ions migrate towards the anode. The leading ions have a higher electrophoretic mobility than the trailing ions, but do not outrace the latter. A steady state is set up such that the leading and trailing ions travel at the same speed. The trailing ions exist in a low conductance environment and the leading ions exist in a high conductance environment. The potential gradient is inversely related to the

conductance and the rate of travel of the ions is equal to the product of the electrophoretic mobility and the potential gradient. Thus the steady state condition is set up as follows:

$$R_t = R_l$$

$$m_t V_t = m_l V_l$$

where R = velocity, cm/sec,

m = electrophoretic mobility, cm^2/volt-sec,

and V = potential gradient,volts/cm.

subscripts: t = trailing, l = leading

Although the trailing ion has a lower mobility, it is travelling in an environment of a higher potential than the leading ion, and therefore a narrow zone or boundary is set up between them such that it moves at a constant velocity. Since polypeptides are less mobile than the leading ion they must travel behind the leading ions. At the same time the polypeptides are more mobile than the trailing ions and must travel in front of the them.

Although the mobility of polypeptides varies considerably it is possible to choose a small ion such as chloride that has a mobility greater than that of the fastest protein and another ion such as glycine for the

trailing component. The mobility of the glycine can be regulated by adjusting pH and thereby altering the net charge of the glycine. Since mobility is directly proportional to net charge, a pH can be imposed that will regulate the mobility to be less than that of the protein with the lowest mobility. The glycine and chloride therefore form a moving boundary which is very sharply defined and the proteins concentrate within the extremely narrow zone between them.

The polypeptides in this very narrow zone move through the stacking gel until the resolving gel is reached. The interface of these two gels is considered to be the starting zone. If the resolving gel is buffered at pH 8.9 the mobility of the glycine is high and so it quickly moves ahead of the concentrated polypeptides which therefore migrate into the resolving gel in a uniform voltage gradient. Thus the polypeptides are separated according to their differences in net rate of travel which is determined by a combination of their individual mobilities and their individual molecular sizes. The discontinuous gel system was first introduced in 1962 and involves a two-step process of making a stacking gel and a resolving gel each with its own buffer. Initially a basic pH system was presented and then soon expanded to an acidic and a neutral system. Strategies for setting up a discontinuous gel system at any pH range and buffer have

also been presented.

Other discontinuous systems were available, which were capable of producing narrow starting zones. One system relied upon the fact that polypeptide mobilities in the sample buffer loading zone are greater than in the polyacrylamide gel. Thus polypeptide would be concentrated in a narrow zone at the interface. The starting zones achieved were broader than those of the discontinuous system and the resolution not as great. A simplified discontinuous system which also relied on the sample buffer - gel interface for creating a narrow starting zone gave somewhat better results, by using gel buffers of reduced conductance. Another system that had limited concentrating powers relied on preparing the sample in gel buffer diluted 5-fold. In this way the sample was in a zone of lower conductance and migrated quickly to the gel - buffer interface where it was concentrated in a narrow zone. Since this technique was found to have limitations, it was modified by using a stacking gel and a polyacrylamide gradient. The buffer conductance was lowered in the stacking gel by a 5-fold dilution of the buffer used in the rest of the gel.

In 1979 there was introduced a modification of discontinuous PAGE, which has been used widely ever since. The modification was merely that of treating the proteins with the detergent sodium dodecyl sulfate (SDS) and the

sulfhydryl reagent-beta mercaptoethanol. This treatment dissociates the proteins into polypeptides and allows the SDS to bind to each polypeptide, denaturing them into uniformly shaped molecules with the same net charge density and hence the same electrophoretic mobility. The higher resolution of SDS-discontinuous polyacrylamide gel electrophoresis (SDS-PAGE) is probably due to the increased solubility of the modified polypeptides and their decreased interactions with each other and the gel matrix. Thus migration distance depends only on the size differences of the polypeptides.

In overview, because of the high resolution obtainable with discontinuous buffer systems, the SDS-discontinuous system is usually the system of choice for high resolution fractionation of protein mixtures under dissociating conditions. It appears that other systems for creating narrow starting zones are not in wide use because they do not work as well as the above system, or require modifying sample volumes and/or buffers.

According to the present invention there is provided a method of producing and packaging a two-part or multiple-part gel system comprising a stacking gel portion and a resolving gel portion, characterised in that the gel portions are impregnated with the same gel buffer, in that they are in direct contact and, preferably in that the

two-part or multiple-part gel is packaged between a pair of protective sheets.

In one example the buffer used for the two gels is 0.375 M Tris-HCl, pH 8.3 with 0.1% SDS. The electrode buffer is 0.025 M Tris, 0.192 M glycine, 0.1% SDS. Single gel buffer systems composed of Tris-HCl from pH 8.1 to pH 8.9 are capable of fractionating polypeptides however resolution, especially of the lower molecular weight proteins, is considerably sharpened at pH values between 8.1 and 8.3. pH 8.1 produced the greatest sharpness of resolved protein bands, however the top portion of the lane can be broadened in the horizontal direction. The speed of the electrophoresis changes with pH, being fastest at pH 8.9 and slowest at pH 8.1. pH 8.3 may therefore, be chosen as the optimum value for uniformity of the lane width, sharpness of the polypeptide bands, and speed of electrophoresis.

The gel system presented here is considered to work as follows. The leading ion is chloride, and the trailing ion is glycinate. The moving boundary is formed and travels at a rate determined by the pH established in the gel buffer. The polypeptides concentrate in the moving boundary and form a thin starting zone at the stacking - resolving gel interface. They enter the resolving gel and are subjected to differential frictional forces which act to separate the polypeptides on the basis of size. The

fundamental differences between this gel system and the standard discontinuous SDS-gel system are as follows.

In the standard system the stacking gel contains a Tris-HCl buffer at pH 6.8 and at a concentration of 0.125 M. This combination of pH and concentration produces a low conductance environment for the glycine and the sample. These with the chloride ion found in the stacking gel create the narrow starting zone.

Since the stacking gel contains a buffer which differs from that of the resolving gel, the stacking gel cannot be maintained in contact with the resolving gel without the two buffers becoming mixed by diffusion. Therefore, the stacking gel is poured just before use in order to maintain its effectiveness in creating a narrow starting zone.

In contrast, the new gel system does not rely on the lower ion concentration and lower pH in the stacking gel to provide the narrow starting zone. It only relies on the pH being set at 8.3 to form a moving boundary which creates the narrow starting zone. At pH 8.9 the glycine has a higher effective mobility, the conductance is higher and the starting zone broader. At pH 8.1 the glycine has a lower effective mobility, the conductance is lower and the starting zone sharper. In the gel system presented here, a

pH of 8.1 produces a higher potential gradient than a pHof 8.9. This is evidenced by the efficiency of the moving boundary in sweeping the polypeptide out of the sample buffer and into the stacking gel.

Furthermore, in the standard system, the polypeptides and the moving boundary enter the resolving gel which is at a higher pH (8.9) than the stacking gel (6.8). The effective mobility of the glycine increases at pH 8.9 and establishes a new steady state moving boundary. The velocity of this boundary is increased because of the higher pH and moves ahead of the polypeptides which are now being sieved while under the constant force of a uniform potential gradient. In contrast, the new gel system maintains the same pH in the resolving gel as in the stacking gel. Therefore the moving boundary is maintained and continues with the same sharpness and velocity. The polypeptides are subjected to frictional forces in the resolving gel which "drag" the ploypeptides out of the boundary interface. The smaller the polypeptide, the longer the distance needed to effectively retard it. Therefore the smaller the polypeptide, the longer it stays within the moving boundary. This maintains each polypeptide in a narrow zone for as long as possible, with the result that the effect of diffusion to cause band broadening acts over the least amount of time in the case of the smallest polypeptides. This is particularly

important in obtaining sharp bands for lower molecular weight polypeptides since they diffuse at a much faster rate than the larger molecules. In fact, because the particular purpose was to achieve band sharpness, a small amount of protein was allowed to remain in the moving boundary at the selected pH of 8.3.

The new rationale described above is in fact consistent with existing gel electrophoresis theory yet it represents a completely new application of that theory. This application can be generally applied to other matrices and electrophoretic separation of molecules such as agarose gel and starch gel and thin-layer electrophoresis. The gel system presented here represents only one of many different types of sieving gel systems that can be modified according to this new rationale. This new application has been used to construct a denaturing SDS-polyacrylamide gel which is run horizontally and is surface loaded. It is also considered to give superior results with a vertical gel which is top-edge loaded. It has also been used in native non-dissociating conditions to regulate the stacking and unstacking of polypeptides. It can also be used in the case of gradient gels (either continuous or stepwise) where the gel portion in which stacking occurs is an integral part of the gradient or is added separately.

For the polyacrylamide gel described here (the first practical application of the generalized strategy for using single buffer gels) there are three very clear advantages, as follows. The gel can be stored because the stacking and resolving portions of the gel contain the same buffer. The entire gel can be pre-electrophoresed to remove contaminants, such as persulfate, which can react with the proteins and adversely affect them. When compared to discontinuous gels, this polyacrylamide gel can be run at higher voltages without producing artifacts. It is felt that this is possible because the stacking gel does not have as low a conductance and that the potential gradient over the gel is more uniform.

The following further advantages are achieved.

(a) A matrix can be constructed which consists of a resolving and stacking region, both containing the same buffer. The buffer, at an appropriate pH when used with an appropriate electrode buffer at an appropriate pH (the electrode buffer might be the same as or different from the matrix buffer and might be at the same or different pH) is capable of concentrating the sample components in the stacking region. The stacking region may or may not exhibit any fractionation qualities of its own. The moving boundary may or may not be functional in the resolving zone of the matrix. The essential function of the buffers is to provide the leading and trailing components although in

some cases the sample can actually provide the trailing component. The buffers usually provide a criterion to satisfy the requirement for electroneutrality.

(b) The general approach of obtaining a narrow starting zone with a single buffer being used in the matrix can be applied to any electrophoretic separation such as thin layer electrophoresis, agarose electrophoresis and starch gel electrophoresis as well as polyacrylamide gel electrophoresis.

(c) The strategy for increasing resolution by keeping as many components as possible within the moving boundary, and for as long as possible.

(d) The general advantages conferred by using a single buffer for the electrophoretic matrix, namely longer shelf-life, the ability to place the stacking and resolving portions of the gel matrix in direct contact during fabrication and subsequent storage, without affecting the quality of results, the ability to pre-electrophorese the matrix, and the ability to use higher voltages.

Described below is another unique feature of an SDS-polyacrylamide gel made in accordance with the invention, which is responsible for lack of band distortion. The enhanced uniformity increases the effective

resolution and ease of analysis of the fractionation pattern.

A polyacrylamide gel is produced by placing a solution of monomers and catalysts in a mould. The catalysts bring about the polymerization of the monomers. The polymerization process is exothermic and autocatalytic. Following a latent period the polymerization process is initiated and occurs quickly. As polymerization proceeds the solution becomes heated. This heating produces convection within the polymerizing solution. This type of mixing has long been known to create mixing of density gradients of gradient gels resulting in distortion of fractionation patterns. A successful method for eliminating this mixing is to introduce a gradient of catalysts such that the gel begins polymerizing from the top surface down. By doing this a temperature inversion is formed and convective mixing does not occur.

Heretofore, it has not been realized that convective mixing per se in non-gradient gels such as the SDS-polyacrylamide described here can actually produce gels with discrete acrylamide concentration discontinuities. The mechanism by which this occurs is that polymerization and the accompanying heating occurs at different loci. The local heating causes convection of the yet unpolymerized gel solution. At the completion of polymerization, refractive index discontinuities are visible in the gel.

These discontinuities persist which appears to indicate that the per cent acrylamide in the gel differs or the polymer structure differs from place to place. The sieving effect of the gel is dependent on the percent acrylamide and the polymer structure, and therefore these discontinuities can degrade the quality of resolution of the gel. The severity of these gradients can be minimized by using the appropriate amount of catalysts; nevertheless the autocatalytic, exothermic nature of the polymerizing process dictates the formation of concentration discontinuities. To eliminate these discontinuities, a gradient of polymerizing agents is being used to initiate top to bottom polymerization of the gel. Any other method which results in top-to-bottom polymerization such as light induced polymerization initiated at the top or by eliminating oxygen at the top of the gel may be employed. Also the use of agents which increase the solution viscosity, for example glycerol or sucrose when used in conjunction with top-to-bottom polymerization are possible since these too will minimize the convective mixing as the gel polymerizes.

Fabrication of the gel structure is greatly simplified by having the stacking and resolving gels containing the same buffer. In one practical fabrication procedure, an upright narrow container whose lateral dimension in one horizontal direction is considerably less

than its height, is provided in the form of a protective backing sheet of acetate or mylar film as one side wall, together with a base and additional side walls which define a chamber in which the gels may be cast. Into this container is poured the constituents of one gel, for example the resolving gel. Once this has cured (polymerized) the other gel (having the same buffer) is introduced and this also is allowed to cure. Once the two-part gel is complete, the base and additional side walls of the container are removed leaving the gel assembly on the backing sheet. The fabrication is completed by placing a protective sheet in the form of a mylar film against the opposite exposed surface of the gel structure. This provides physical protection and acts as a moisture barrier.

The gels may be packaged singly or in multiples. For example, between 10 and 20 mylar-covered gels fabricated in this manner may be stacked in superimposed relationship and placed in a moisture-proof bag. Thus the gels packaged in the manner described may be stored or shipped. When required, the bag is opened and a covered gel is removed for use.

The protective mylar film should extend across the entire surface of the resolving gel and may extend further across part of the stacking gel. In one example, the film extends right across the stacking gel but contains a narrow

slot or a number of aligned apertures for the purpose of loading a sample upon which electrophoresis is to be carried out. Thus, the surface of the gel structure is prevented from coming into contact with moisture whilst in storage and particularly in use when condensation might otherwise occur. The barrier provided by the mylar film also minimises constituent losses from the surface during storage and use. The film used in packaging may or may not be the film used during use.

The depth of the gel structure will be in the region of 1mm however it is possible to construct gels on the order of 0.1mm to 10mm, whilst the thickness of the mylar film will depend on the particular application but in this example it can be several microns up to 400 microns.

Packaged gels manufactured as described, may be used readily by semi-skilled personnel since no specialised knowledge is required to bring together the stacking and resolving gels prior to electrophoresis.

CLAIMS

1. A method of producing and packaging a two-part or multiple-part gel system comprising a stacking gel portion and a resolving gel portion, characterised in that the gel portions are impregnated with the same gel buffer, and in that they are contiguous.

2. A method according to Claim 1, further characterised in that the gel structure is packaged between a pair of protective sheets.

3. A method according to Claim 1 or Claim 2, wherein the gel is polyacrylamide and the gel buffer is selected to have a pH value between 8.1 and 8.9.

4. A method according to any of Claims 1 to 3, wherein the gel buffer has a pH value of 8.3.

5. A method according to Claim 1, wherein the gel system comprises a leading ion of chloride and a trailing ion of glycinate.

6. A method according to any preceding claim, including the step of placing a solution of monomers and catalysts in a mould to bring about polymerisation, a gradient of catalysts being introduced such that the gel begins polymerising from the top surface down thereby

avoiding convective mixing.

7. A method according to any preceding claim, comprising the steps of providing an upright narrow container whose lateral dimension in one horizontal direction is considerably less than its height, and introducing into the container, initially, the constituents of one gel portion, and after this has cured, the constituents of the other gel portion, at least a part of the container being removed once the second gel portion has cured and being replaced by a protective sheet.

8. A method according to Claim 7, wherein said narrow container is provided in the form of a protective backing sheet of, for example, acetate or mylar film as one side wall, together with a base and additional side walls which define a narrow chamber in which the gels are cast, the base and additional side walls of the container being removed after formation of the gels, leaving the two-part or multiple-part gel assembly on the backing sheet, and subsequently placing a protective sheet of, for example, mylar film against the opposite exposed surface of the gel structure.

9. A method according to Claim 7 or Claim 8, in which the protective sheet extends right across the two-part or multiple-part gel systems but contains at least one aperture over the stacking gel portion for the purpose of

introduction of a sample upon which electrophoresis may be carried out.

10. A method according to any one of Claims 7 to 9, including the step of stacking a plurality of gels each between a backing sheet and a protective sheet in superimposed relationship, and placing the stack in a moisture-proof bag such that the gels may be removed one at a time from the stack for use.

11. A method according to Claim 8, wherein the gel system is fabricated to have a thickness in the region of 0.1mm to 10mm.

12. A method according to Claim 7, wherein said protective sheet is selected to be no greater than 400 microns in thickness.

13. A method according to any preceding claim, in which the gel buffer is 0.375 M Tris-HCl, pH 8.3 with 0.1% SDS.

14. A method according to any preceding claim, in which the gel system comprises a gradient gel (either continuous or stepwise) where the gel portion in which stacking occurs is an integral part of the gradient.

15. A method according to any of Claims 1 to 13, wherein the gel system is a gradient gel (either continuous or stepwise) where the gel portion in which stacking occurs is separately added to the gradient.

16. A method of producing and packaging a two-part or multiple-part gel system, substantially as hereinbefore described.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 87/00156

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: B 01 D 57/02; G 01 N 27/26

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	G 01 N; B 01 D

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US, A, 3384564 (L. ORNSTEIN et al.) 21 May 1968, see column 1, lines 11-19; column 2, line 65 - column 3, line 27; column 4, line 21 - column 6, line 51; figures 1-4	1-5,13
Y	US, A, 4481094 (A.F. DE CASTRO et al.) 6 November 1984, see abstract; column 3, lines 1-4; column 3, line 45 - column 4, line 54	1,5
Y	US, A, 4415428 (S. NOCHUMSON et al.) 15 November 1983, see abstract; column 8, example 5	3,4,13
Y	EP, A, 0113700 (DIRECTOR OF THE FINANCE DIVISION MINISTER'S SECRETARIAT SCIENCE AND TECHNOLOGY AGENCY) 18 July 1984, see abstract; page 1, lines 4-9; page 5, line 21 - page 6, line 32; figure 1	2
A		11,12
A	FR, A, 2179068 (BIOTEST-SERUM-INSTITUT) 16 November 1973, see page 1, lines 1-2;	2,10,11 ./.

* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26th May 1987	22 JUN 1987
International Searching Authority	**Signature of Authorized Officer**
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International Application No. PCT/GB 87/00156

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	page 2, lines 20-25; page 3, lines 27-29 --	
A	EP, A, 0159830 (FUJI) 30 October 1985, see abstract; page 4, lines 5-29; page 8, line 22 - page 9, line 30 --	10-12
A,P	US, A, 4594064 (N.L. ANDERSON) 10 June 1986, see abstract; column 2, lines 5-35	6,10-12,14, 15

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 87/00156 (SA 16422)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 05/06/87

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A- 3384564		None		
US-A- 4481094	06/11/84	None		
US-A- 4415428	15/11/83	None		
EP-A- 0113700	18/07/84	JP-A-	59126237	20/07/84
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		CH-A-	574748	30/04/76
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