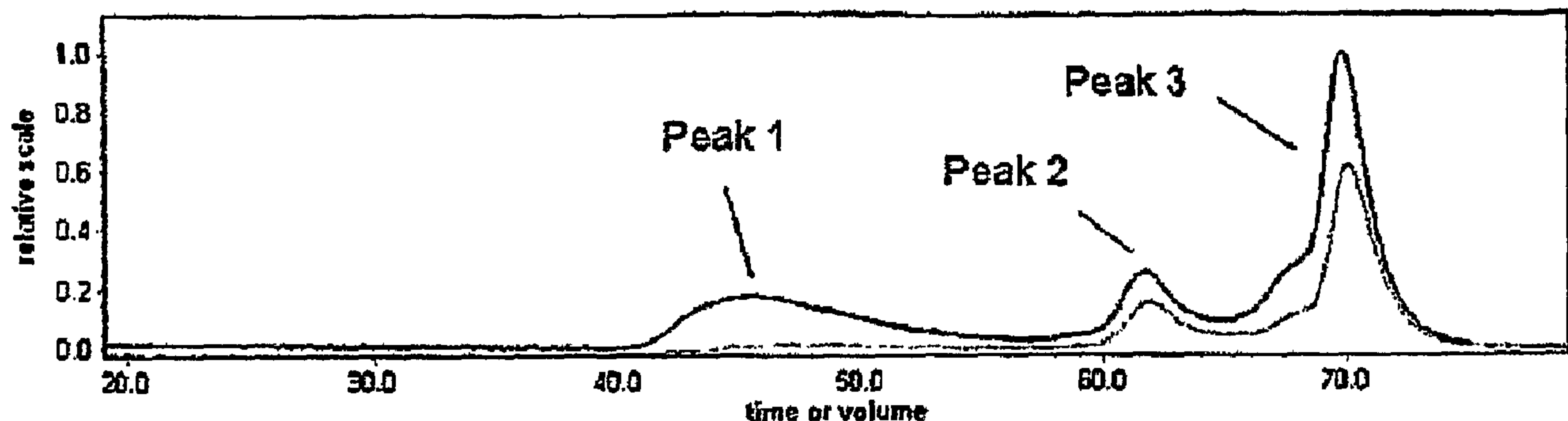




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(71) Demandeur/Applicant:
VIVOLUTION A/S, DK
(72) Inventeur/Inventor:
HOLM, NIELS ERIK, DK
(74) Agent: OSLER, HOSKIN & HARCOURT LLP

(54) Titre : COMPOSITION AMELIOREE DE COLLE A LA FIBRINE ET UTILISATION DE CETTE DERNIERE
(54) Title: IMPROVED FIBRIN SEALANT COMPOSITION AND ITS USE



(57) Abrégé/Abstract:

The wound healing process may be augmented by the addition of fibrin sealant. Dependent on the sealant composition and way of application the wound healing process and tissue regeneration may be further accelerated. The present invention provides for an improved sealant composition and its use.

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(71) Applicant and

(72) Inventor: HOLM, Niels, Erik [DK/DK]; 10B Julmosevej, DK-3460 Birkerød (DK).

(74) Agent: CHAS. HUDE A/S; H.C. Andersens Boulevard 33, DK-1780 Copenhagen V (DK).

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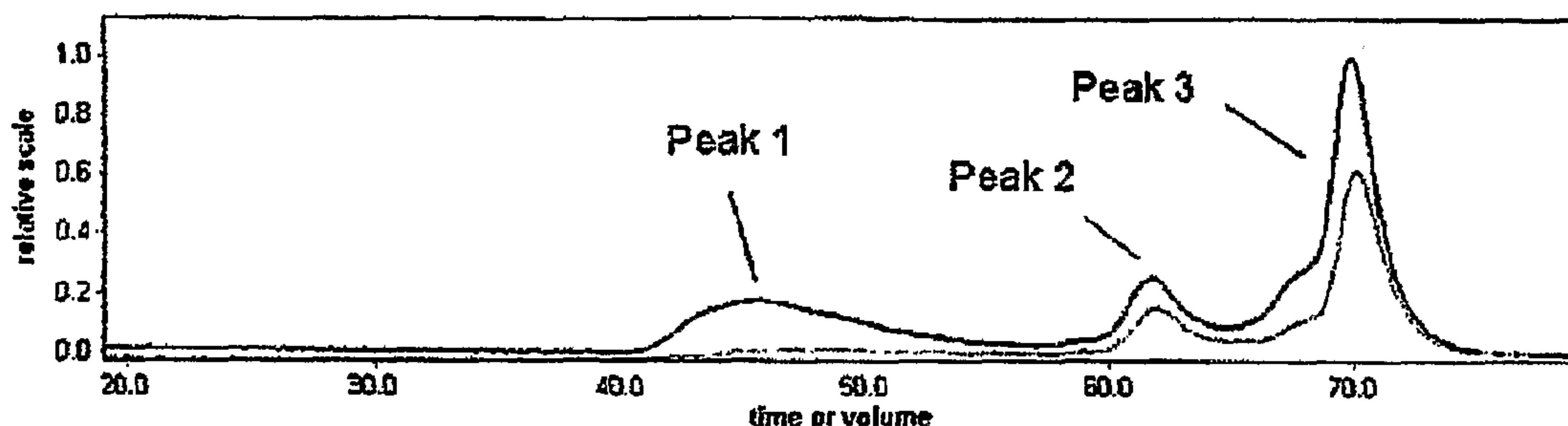
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(57) Abstract: The wound healing process may be augmented by the addition of fibrin sealant. Dependent on the sealant composition and way of application the wound healing process and tissue regeneration may be further accelerated. The present invention provides for an improved sealant composition and its use.



WO 2007/121748 A3

Title: IMPROVED FIBRIN SEALANT COMPOSITION AND ITS USETechnical Field

The present invention relates to the field of homeostasis and tissue regeneration.
5 More specifically, the invention pertains to improved fibrin sealant compositions and their application and uses.

Background of the Invention

The entire wound healing process is a complex series of events that begins at the
10 moment of injury and may continue for months to years. The first 2-5 days is called the inflammatory phase, during which first hemostasis is taking place by vasoconstriction, platelet aggregation and clot formation by thromboplastin, followed by inflammation characterized by vasodilation and phagocytosis. The next approximately 3 weeks is called the proliferative phase, starting with granulation where fibroblasts
15 lay bed of collagen, and defect capillaries are filled and new produced. During contraction wound edges pull together, and at epithelialization surfaces are covered with cells. The remodeling phase may last for 3 weeks to 2 years. Here new collagen forms which increases tensile strength .

20 Conventional fibrin sealants also called fibrin adhesives or fibrin glues are based on the polymerization of fibrinogen monomers to an insoluble network (clot). The process is initiated when thrombin is cleaving fibrinogen to fibrin II acid-soluble monomers, and when thrombin in the presence of Ca^{++} is activating Factor XIII to Factor XIIIa. Fibrin II acid-soluble monomers are then converted by Factor XIIIa
25 into an acid-insoluble cross linked fibrin II polymer.

Uses of fibrin sealant have been described for a variety of medical procedures, such as hemostatic agents, absorbent of excess fluid, surgical adhesion barrier, wound dressing and delivery vehicle of biologically active substances.

Numerous ways of applying fibrin sealant are known in the art. They are all developed with respect to the rapid polymerization of the fibrin sealant following mixing of the fibrinogen component and the thrombin component. In one method the mixture is drawn into an appropriate size syringe and must be rapidly applied to the desired site. In another method double barrel syringe is used. Other methods employ microdrop delivery systems or spray application systems. Some of the methods require special applicators. In yet other methods the components are fixed to a carrier material, alternatively one or more of the components may be in a non-liquid form, such as e.g. powder.

10

Biocompatible and biodegradable polymers have been identified as suitable carrier material for fibrin sealant components. Such polymers confer the advantages that a fibrin sealant fabric may be prepared in beforehand and is activated when placed on a wound site (US 6,503,527). Examples of useful polymers are polyglucosamin based material such as hyaluronic acid (HA) and chitin/chitosan. HA has also been described as a preferred viscosity enhancing polymer to keep fibrin sealants from dislocating from the application site (EP 0590015).

Polyglucosamin based material may in addition to the above mentioned uses also possess other advantages. For example HA is used in plastic surgery for removing of wrinkles, and physicians have for the past 20 years injected HA directly into the synovial fluid of the knee as a treatment for osteoarthritis. However, the bioavailability of HA per se is limited due to its rapid turnover and short half life. Chitosan, which has been used as a dietary supplement for obtaining weight loss, has also been found to be useful in wound healing (US 4,532,134).

In addition to controlling hemostasis it is furthermore desirable to stimulate and thereby accelerate wound healing by providing the best possible conditions for tissue regeneration. Cell therapy and the use of topical growth factors are increasingly used methods for modern treatment of difficult to heal wounds. Growth factors may work synergistically with surgical debridement in the healing of wounds.

Several types of material have been tested as media for cell or growth factor delivery, and none have been shown to be optimal. However autologous fibrin appears to be useful for delivery of autologous keratinocytes (Grant 2002 Br J Plast Surg vol. 55:219-227). Moreover autologous fibrin has now been developed to include and deliver the family of platelet-derived growth factors.

Conversion of fibrin monomers by fibrinogen-cleaving agents to an insoluble network of polymerized fibrin is a rapid process, thus necessitating the preparation of fibrin sealant mixture just prior to use. The fast polymerization may cause problems in application by clogging of the applicator and furthermore difficulty in applying the correct dosage for obtaining the desired tensile strength of the fibrin sealant. Therefore, fibrin sealant compositions have been employed wherein one or more of the components comprised are in a solid phase, like e.g. fiber, sheet or powder, or delivered by means of a solid phase carrier material. Such partly solid phase compositions may be prepared in good time before use due to the delay in initiation of the polymerization process. Although these partly solid phase fibrin sealant compositions may appear more easily handled by the medical professionals, they are also confined with certain limitations. For example, use of fibrin sealants as a vehicle for other components suitable for tissue regeneration such as e.g. cells may not be compatible with such a semi-solid phase or a non-aqueous environment. In addition, liquid phase fibrin sealant compositions conform more easily than film or sheets to wound edges. Thus, there is still a strong need for liquid fibrin sealant compositions and convenient methods of their application.

Brief Summary of the Invention

In a first aspect, the present invention relates to a liquid phase composition, wherein the liquid components of said composition upon application to a desired site is forming a fibrin sealant, comprising fibrin monomer or polymer, fibrinogen-cleaving agent and polyglucosamine based material.

In a second aspect, the present invention relates to a method of preparing the composition, wherein the components of the composition are prevented from polymerizing by storage in a low pH buffer until use.

5

In a third aspect, the present invention relates to a method of applying the composition comprising the steps of providing the components of the composition, providing an applicator device, wherein said device comprises at least two outlets for reagents and at least one outlet for air, and applying said components of the composition to
10 the desired site using the applicator device.

In a fourth aspect, the present invention relates to use of the composition for supporting cell growth.

15 Brief Description of the Drawing(s)

The invention is explained in detail below with reference to the drawing(s), in which

Figure 1 is a chromatogram showing the result from SEC-MALLS-VISC analyses for extracts from clot with and without HA added (sample C and A, respectively).

20 Legend: Sample A; lower line and sample C; upper line.

Figure 2 is a chromatograms showing the result from SEC-MALLS-VISC analysis for extract from clot with HA added (sample B).

25 Figure 3 is a picture of the ring for making the polymerized fibrin or platelet rich fibrin tablet.

Figure 4, 5 & 6 are pictures of the Mueller Hinton agar plates plated with either S. aureus or E. coli

30

Figure 7 is an illustration of moulded clots.

Figure 8 is a figure illustrating the weight loss of moulded clots within the first 18 hours.

5 Detailed disclosure of the Invention

The present invention is described in detail below.

One embodiment of the present invention relates to the liquid phase composition, wherein the liquid components of said composition upon application to a desired site
10 is forming a fibrin sealant comprising fibrin monomer or polymer, fibrinogen-cleaving agent and polyglucosamine based material, that is partially cross-linked or partially polymerized for at least 1 minute after application to a desired site.

The performance of a liquid fibrin sealant composition is determined by its physical
15 properties, such as the ability to adhere to the site of application without dislocating, elasticity and tensile strength, which are formed during polymerization. In clinical use instant polymerization of fibrin sealant with defined physical properties is required. This must of cause be balanced with the time needed for handling the fibrin sealant.

20

“Partially cross-linked or partially polymerized” as used herein, means that the process of clot formation has not been fully completed.

The liquid composition of the present invention is suitable for most uses, as the fi-
25 brin sealant conforms to the site of application. The rapid polymerization secures that the fibrin sealant remains at the site of application and is not removed by bodily fluid or target movements. Therefore, the present invention does not need addition of components for providing an increased viscosity or a carrier material. By one or more applications of said liquid phase composition it is possible to build up a fibrin
30 sealant structure having the desired elasticity and tensile strength.

“Fibrin” as used herein, means fibrin, fibrin I or fibrin II.

“Fibrinogen cleaving agent” as used herein, means an enzyme capable of cleaving either peptide A or peptide B or both from fibrinogen. Fibrinogen-cleaving agents
5 commonly used in fibrin sealants are thrombin, which may be obtained from any suitable source such as e.g. human or bovine, enzymes from snake venom such as e.g. batroxobin, calobin, fibrozyme, and enzymes from the venom of Bothrops jararacussu.

10 “Polyglucosamine based material” as used herein, means a molecule where the glucosamine is a repeated part of the molecule. It comprises biocompatible and bioabsorbable materials such as hyaluronic acid, chitin/chitosan and derivatives thereof.

Chitin is widely distributed in both plant and animal kingdom, where its main func-
15 tion is to provide structural and skeletal support. Chitin is a linear homopolymer of beta-D(1-4) linked 2-acetamido-2-deoxy-D-glucopyranose (N-acetylglucosamine) units, of which a portion, typically about 15%, is N-deacetylated. The fully acetylated polymer is called chitan and the fully deacetylated polymer is called chitosan.

20 Hyaluronic Acid (HA) also called hyaluronan is a linear polymer of the disaccharide repeat unit, D-Glucuronic acid and N-Acetyl D-Glucosamine. This mucopolysaccharide has shown to possess different biological properties depending on the molecular weight. HA has an extraordinarily high rate of turnover in vertebrate tissues, with a half life of 2-5 minutes, and is being removed by degradation with time, even with
25 the highest molecular weight. Furthermore, viscosity of the high molecular weight solution confers a major problem during application.

The present invention demonstrates in example 1 that a polyglucosamin, such as HA surprisingly was trapped within fibrin sealants clots whereby its rapid turn-over and
30 short half life were delayed. This provides for a broader employment in the selection and use of various molecular weight polyglucosamines. The retention of HA in fi-

brin clots may allow for use of low molecular weight polyglucosamines in combination with fibrin sealants, thereby making the delivery more convenient due to the lower viscosity of the components.

5 Infection is an issue in the treatment of chronic wounds and the use of an antimicrobial agent is thus desirable. Example 2 of the present application demonstrates the antibacterial effect of chitosan. Other investigations have also reported this property of chitosan (US 4,532,134). Furthermore, they found that chitosan when applied alone decreased the tensile strength of the wound at an early stage of the wound
10 healing process and did not provide any significant effect at a later stage. An initial study has indicated that HA may help to decrease the incidence of bacterial infection in bronchitis (Venge, P., 1996 Am J Respir Crit Care Med 153(1):312-6). Therefore, the co-application of chitosan, HA or other polyglucosamines together with fibrin sealant, as proposed in the present invention, may confer the composition the additional feature of anti-microbial activity.
15

High fibrin density may affect the proliferation and mobility of cells in the fibrin matrix, and thus affect the wound healing process. Initial studies have shown that high fibrin density results in a compact network in which the in-growth of cartilage
20 cells is prevented, whereas a relative lower fibrin density resulted in an HA expanded and thus more loose network in which said cells may grow. In order to provide the best possible cell growth environment it is, therefore, desirable to expand the fibrin sealant network by incorporating a cellular compatible media such as polyglucosamine.

25

In one embodiment of the invention the polyglucosamine based material comprises at least 50% glucosamine monomers.

In one embodiment of the invention the polyglucosamine based material is selected
30 from the group consisting of hyaluronic acid material, hyaluronic acid derivative

material, chitin material, chitin derivative material, chitosan material and chitosan derivative material.

It should be understood that polyglucosamine based material also embraces materi-
5 als comprising at least one modified monomer. The modification may for example be chemically and non-limiting examples of the principal targets for modification is the carboxyl group present at N-Acetyl D-Glucosamine and the hydroxyl group present at D-Glucuronic acid, additional sites may be reducing end modification or N-acetyl. Furthermore, monomers may be modified by numerous cross-linking strate-
10 gies which are known in the art, many of which are described at www.glycoforum.gr.jp.

In one embodiment of the invention the polyglucosamine based material comprises a mixture of different materials.

15

In one embodiment of the invention the polyglucosamine based material comprises a mixture of different molecular sizes.

In one embodiment of the invention the polyglucosamine based material comprises a
20 mixture of both different materials and different molecular sizes. The combined use of chitosan and HA results in a protection of HA from enzymatic hydrolysis, and thus different material of different sizes may be selected for use depending on the requirement for resistance against degradation of the polyglucosamine based material.

25

In one embodiment of the invention the degree of acetylation of the polyglucosamine based material is from 0 to 100%.

One embodiment of the invention relates to the composition further comprising
30 cells. The cells may be included in a solution together with any other component or the cells may be kept in a separate solution. This may be determined depending on

the origin of the cells and the selection of the best environment for viable cells. For example platelets may be isolated from the same source as the fibrin and is therefore already present in the fibrin containing solution. If cells are cultured the cells may be provided suspended in cell growth media, alternatively cells may be suspended in
5 other suitable solutions further comprising one or more other components.

In one embodiment of the invention the cells are selected from the group consisting of platelets, stem cells, fibroblasts, keratinocytes, cartilage cells and bone cells.

10 One embodiment of the invention relates to the composition further comprising growth factors. The growth factors may be included in a solution together with any other component or may be supplied in a separate solution. Such growth factor may aid in creating the best possible condition for the effector cells delivered to or already present at the wound site. Application of growth factors together with the fi-
15 brin sealant saves a step in the wound treatment procedure.

In one embodiment of the invention the growth factors are selected from the group consisting of Epithelial Growth Factor (EGF), Fibroblast Growth Factor (FGF), Platelet Derived Growth Factor (PDGF), Transforming Growth Factor alpha
20 (TGF α), Transforming Growth Factor beta (TGF β) and Interleukin-1.

One embodiment of the invention relates to a method of preparing the composition, wherein the components of the composition are prevented from polymerizing by storage in a low pH buffer until use, wherein the pH is 4.

25

In an alternative embodiment of the invention the components of the composition are prevented from polymerizing by storage in two or more separate reservoirs until use.

30 One embodiment of the invention relates to a method of applying the composition to the desired site, comprising the steps, providing the components of the composition,

providing the applicator device, wherein said device comprises at least two outlets for reagents and at least one outlet for air, and applying said components of the composition using the applicator device,

- 5 In one embodiment of the invention the device comprises three, four or five outlets for reagents and at least one outlet for air. The use of a spray applicator device such as e.g. Vivostat® application system (Dodd, R. A., 2002 Technology and Health Care 10:401-11) provides easy application and dosage, and a homogenous mixture of the components.

10

One embodiment of the invention relates to the use of the liquid phase composition for supporting cell growth. This may for example be done by adding to said composition additional (support) cells, growth factors, and the like for improving the conditions for cell proliferation, cell activation, cell migration or cell differentiation.

15

In one embodiment of the invention the composition is used as a delivery vehicle for biologically active substances. Non-limiting examples may be anti-bacterial substances, drug substances, cells, growth factors, moist substances etc.

- 20 In one embodiment of the invention the composition is used as a surgical adhesion barrier.

- In one embodiment of the invention the composition is used as an absorbent of excess fluid. Hyaluronic acid is found in all connective tissue of the body where it performs special functions of lubrication. HA is also called hydrogel due to its ability to bind and retain large amount of water.
- 25

- In one embodiment the composition is used for reducing scar tissue formation. Application of chitosan alone has been shown to prevent fibrosis formation by fibroblasts, which is considered as scar tissue, and instead endothelial lining were observed.
- 30

served (US 4,532,134). Thus inclusion of polyglucosamines, such as e.g. chitosan, its derivatives or other polyglucosamines, may reduce the formation of scar tissue.

Examples

- 5 In the following the invention is described in more detail by way of non-limiting examples.

Example 1: Retention of Hyaluronic Acid (HA) in fibrin sealant.

- 10 The purpose was to determine the amount of HA extractable from the fibrin clot and thereby determine how much was retained. Furthermore, the molecular weight of the extractable HA was determined.

Materials

- 15 Three fibrin clots were prepared:

Sample A labeled "0,484g Fibrin" contained 20mg/ml fibrin.

Sample B labeled "0,568g Fibrin" contained approx. 10mg/ml fibrin and 1% HA (v/v).

Sample C labeled "0,535g Fibrin" contained 20 mg/ml fibrin and 1% HA (v/v)

20

HA (MAG 30014 supplied by NovoZymes) with a molecular weight at 800 kDa was co-applied in a 1% solution with fibrin (fibrin:HA ratio 1:8). HA was extracted on the same day as preparation of clot. All samples were obtained from the same donor.

- 25 Extraction method

Clots were crushed with a spatula against a glass wall before extraction in 10ml PBS buffer for 4x24h at 4°C with slight agitation. Extracts were collected by centrifugation (10min, 3000rpm, 4°C), 5ml deionized milliQ H₂O was added before passing through a 0.45µm syringe filter. Extracts were stored at 4°C prior to analysis after 2

30 days.

Quantification and characterization.

The extracts were analyzed by SEG-MALLS-VISG (mobile phase: 150mM NaCl, 50mM NaH₂PO₄, pH 7.0, 0.5ml/min) on a PL aquagel OH-40/OH-50/OH60 column. System: Waters Alliance HPLC system Waters 2410 RI detector and Wyatt MALLS

5 detector.

Quantification was done by evaluating the RI signal. 0.5ml of each sample was injected on the column. The data was processed using the ASTRA V software from Wyatt Technology Corp. The samples were labeled: A: 14919-037-1, B: 14919-037-2, and C: 14919-037-3.

10

Results

The chromatograms (see figure 1 and 2) showed three distinct peaks for HA containing samples B and C, whereas peak 1 was absent from sample A devoid of HA.

15 Results from the SEG-MALLS-VISG analysis of the extracts are presented in Table 1 to 5.

TABLE 1. Results from extraction in PBS (g/g injected) determined from RI.

Sample	Peak 1	Peak 2	Peak 3
A	-	9.9 x 10 ⁻⁷	4.0 x 10 ⁻⁵
B	1.2 x 10 ⁻⁵	1.8 x 10 ⁻⁷	1.8 x 10 ⁻⁵
C	8.1 x 10 ⁻⁶	1.9 x 10 ⁻⁶	5.6 x 10 ⁻⁵

20 TABLE 2. Results from extraction in PBS, MW determined by SEC-MALLS.

Sample	Peak 1	Peak 2	Peak 3
A	-	710 kDa	100 kDa
B	260 kDa	760 kDa	92 kDa
C	710 kDa	660 kDa	79 kDa

TABLE 3. Results from extraction in PBS, weight average intrinsic viscosity ([η]_w) determined by SEC-MALLSVISC all values are given in ml

Sample	Peak 1	Peak 2	Peak 3
A	-	ND	ND
B	350	140	ND
C	1500	840	39

TABLE 4. Results from extraction in PBS, z-average radius of gyration (Rz) determined by SEC-MALLSVISC (all values are given in nm).

Sample	Peak 1	Peak 2	Peak 3
A	-	ND	ND
B	100	33	21
C	98	19	ND

- 5 The high molecular weight peak (peak 1) was identified as HA since it only appeared in sample B and C containing HA. Further, comparing molecular weight (Table 2), intrinsic viscosity (Table 3) and radius of gyration (Table 4) all indicate that peak 1 is indeed HA. Peak 2 and 3 are either proteins or protein fragments from the clots. Calculated quantities are summarized in Table 5.

10

TABLE 5. Results from extraction in PBS determined from RI.

Sample	Peak 1 (mg)	Peak 2 (mg)	Peak 3 (mg)	ExHA / HA (%)
A	-	0.03	1.20	0
B	0.42	0.01	0.51	9
C	0.24	0.06	0.30	5

Extracted HA (ExHA), Total HA (HA)

- 15 Total amount of HA extractable seems to be proportional to the amount of fibrinogen used. Apparently, a considerable amount of HA is contained/bound in the cloth (approx. 90-95%).

Conclusion

These results show that approximately 90-95% of the HA is retained in the fibrin clot. Some degradation of HA was observed in extracts from clots prepared in the presence of 10mg/ml fibrinogen, whereas no degradation could be observed in extracts prepared in the presence of 20mg/ml HA.

5

Example 2: Antibacterial properties of fibrin or platelet rich fibrin compositions further comprising chitosan.

The purpose of this study was to investigate the anti-bacterial effect of fibrin and platelet rich fibrin (PRF) on *Staphylococcus aureus* or *Escherichia coli* bacteria plated on Mueller Hinton agar. Different concentrations of chitosan mixed with fibrin or PRF were tested.

The agar diffusion method used in this study is based on the diffusion of antimicrobial substances from a tablet or alike placed directly on agar plated with bacteria. The plated microbes will grow on the entire plate, except for areas with microstatic concentrations of anti-microbial substance(s). This will lead to the formation of a ring around the tablet or substance, the diameter of this ring correlating with the potency of the added anti-microbial substance against the plated microbe. A limitation of this method is the need for the anti-microbial substance to be able to diffuse in the used media (e.g. agar).

Experimental

Fresh colonies of the bacteria *S. Aureus* strain S29 (sensitive to antibiotic originating from KVL) and *E. coli* strain Top10/F^{Tet}^R (resistant to tetracycline) were diluted in sterile 0,9% NaCl (Nycomed) to an OD₆₂₅ of approx. 0,1 (Spectrophotometer Cecil 2040) and seeded on agar plates by dipping a sterile cotton bud in the bacteria solution and wiping it several times across the agar plate. Test substances were added to the plates within 30 min.

30

Fibrin and PRF were obtained from donor BX051901 using RC Preparation Unit 05.17.003 or PRF Preparation Unit 05.17.003 (filters removed) respectively with Citrate buffer 04.41.002 (PRF) and 02.23.007 (RC). Fibrin or PRF was sprayed into a ring (diameter 10mm, and height 3mm => internal volume 235 mm³, see figure 3) made for the purpose and autoclaved using Automated Processor Unit 75002 with short tubing. For pH shift pH 10 buffer 03.11.001 was used. The polymerized Fibrin or PRF tablets were transferred to a Mueller Hinton agar plate (batch no. 2105008) plated with either *S. aureus* or *E. coli*.

10 Addition of 1% or 2% (w/v) chitosan (0.02g or 0.04g Protasan B 0/500, Novamatrix batch no. FP-412-03 respectively dissolved in 2ml pH 4buffer) was performed using a 3rd syringe. The syringe was connected to the free valve/port in the Vivostat® application system. This lead to the addition of chitosan at an amount of approximately one seventh of the final fibrin/PRF (0.14% and 0,28 % of the final concentration).

15 The chitosan containing fibrin/PRF clots were made as described above and placed on the agar plates. Additional controls were made by adding pure 2% chitosan to the surface or into a well on the agar. All plates were made in duplicate. The plates were incubated at 37°C for approximately 20 hours followed by photographing (see figures 4-6).

20

Conclusion

The experiment confirms earlier results demonstrating bacterial inhibition by fibrin or PRF clots. At the concentration tested chitosan alone was also inhibitory to bacterial growth. No significant synergistic effect of mixing fibrin or PRF with chitosan could be detected.

The controls for complete resistance (*E. coli* with Vancomycin or Tetracycline) showed no inhibition at all – suggesting some antibacterial effect of PRF, fibrin and chitosan alone on both bacterial strains. Whether this is due to diffusion or simple “spreading” of the substances on the surface is unknown.

Example 3: Platelet rich fibrin with addition of chitosan or Hyaluronic Acid.

The purpose was to test the water absorption properties of compositions comprising autologous platelet rich fibrin (PRF) further containing Hyaluronic Acid (HA) or
 5 Chitosan.

Experimental

Day 1. Automated Applicator Unit no. 30002 with short tubing line was used to create moulded fibrin and fibrin containing either 2% HA (0,08g Hyaluron from No-
 10 voZymes was dissolved in NaCl batch no. R052002) or 2% chitosan (0,08g Protasan batch no. FP-412-03, NovaMatrix dissolved in 4ml pH 4 buffer 04.49.001). The fibrin was obtained from plasma donor AX052001 and AX052002. For pH shift a pH 10 buffer 03.11.001 was used.

The mass of moulded clots was determined (0 hours), and clots were immersed in
 15 saline buffer (see figure 7) and mass were determined at intervals given in table 6 and figure 8.

Day 2. After approximately 18 hours in saline buffer fibrin samples from the first plasma were intact, whereas samples from the second plasma were completely dissolved,
 20 possibly due to difference in fibrinolysis activity. After the mass of run 1 was recorded, the clot was put in 100% humidity. There was no apparent difference between the samples.

Day 3. The clots were all clear and partly dissolved.

25

Day 4. All clots were dissolved with the exception of a few jelly-like Chitosan containing clots.

TABLE 6. The weight of fibrin clots.

Hours	Fibrin	Fibrin	Fibrin + Chitosan	Fibrin + Chitosan	Fibrin + HA	Fibrin + HA
0	0.290	0.342	0.345	0.296	0.299	3.356

2	0.275	0.321	0.303	0.207	0.255	0.309
18	0.268	0.308	0.293	0.211	0.224	0.287
24	0.223	0.275	0.274	0.196	0.172	0.257

Conclusion

The experiment indicated that addition of 2% Chitosan or 2% HA did not affect the
5 water absorption properties of fibrin clots, neither did it influence fibrinolysis of the
clots.

Claims

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My ref: PA 83506 ZEU/LS

5 Date: 8 July 2008

Proposed amended claims

1. A method of applying a liquid phase composition, comprising as separate
10 components a – c each in liquid form

- a. fibrin monomer or polymer;
- b. fibrinogen-cleaving agent; and
- c. polyglucosamine based material

15

on a desired site using an applicator device comprising at least two outlets for reagents
and at least one outlet for air, whereby at least component a and component c are ap-
plied from different outlets, which composition upon application to the desired site is
forming a fibrin sealant, which fibrin sealant is partially cross-linked or partially polymer-
20 ized for at least 1 minute after application to the desired site.

2. The method according to claim 1, wherein the polyglucosamine based mate-
rial comprises at least 50% glucosamine monomers.

25 3. The method according to claim 1 or 2, wherein the polyglucosamine based
material is selected from the group consisting of hyaluronic acid material, hyaluronic
acid derivative material, chitin material, chitin derivative material, chitosan material, and
chitosan derivative material.

30 4. The method according to any of claims 1-3, wherein the polyglucosamine
based material comprises one or more different materials.

5. The method according to any of claims 1-4, wherein the polyglucosamine
based material comprises materials of one ore more different molecular sizes.

35

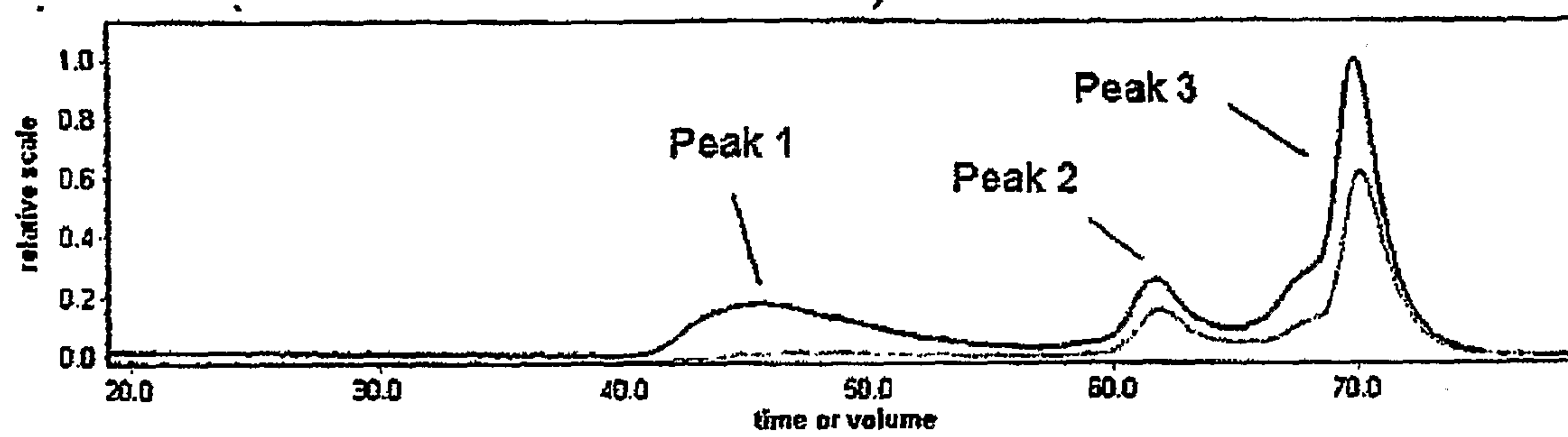
6. The method according to any of claims 1-5, wherein the polyglucosamine based material comprises one or more different materials having one or more different molecular sizes.
- 5 7. The method according to any of claims 1-6, wherein the degree of acetylation of the polyglucosamine based material is from 0 to 100%.
8. The method according to any of the preceding claims further comprising cells.
- 10 9. The method according to claim 8, wherein the cells are selected from the group consisting of platelets, stem cells, fibroblasts, keratinocytes, cartilage cells, and bone cells.
10. The method according to any of the preceding claims further comprising
15 growth factors.
11. The method according to claim 10, wherein the growth factors are selected from the group consisting of Epithelial Growth Factor (EGF), Fibro-blast Growth Factor (FGF), Platelet Derived Growth Factor (PDGF), Transforming Growth Factor alpha
20 (TGF α), Transforming Growth Factor beta (TGF β), and Interleukin-1.
12. A method according to any of claims 1-11, wherein the components of the composition are prevented from polymerizing by storage in a low pH buffer until use.
- 25 13. The method according to claim 12 wherein the pH is 4.
14. A method according to any of claims 1-11, wherein the components of the composition are prevented from polymerizing by storage in two or more separate reservoirs until use.
- 30 15. A method according to any of the preceding claims using an applicator device comprising three, four or five outlets for reagents and at least one outlet for air.
16. Use of the method according to any of claims 1-15 for supporting cell growth.

3

17. Use of the method according to any of claims 1-15 as a delivery vehicle for biologically active substances.
18. Use of the method according to any of claims 1-15 as a surgical adhesion barrier.
19. Use of the method according to any of claims 1-15 as an absorbent of excess fluid.
20. Use of the method according to any of claims 1-15 for reducing scar tissue formation.

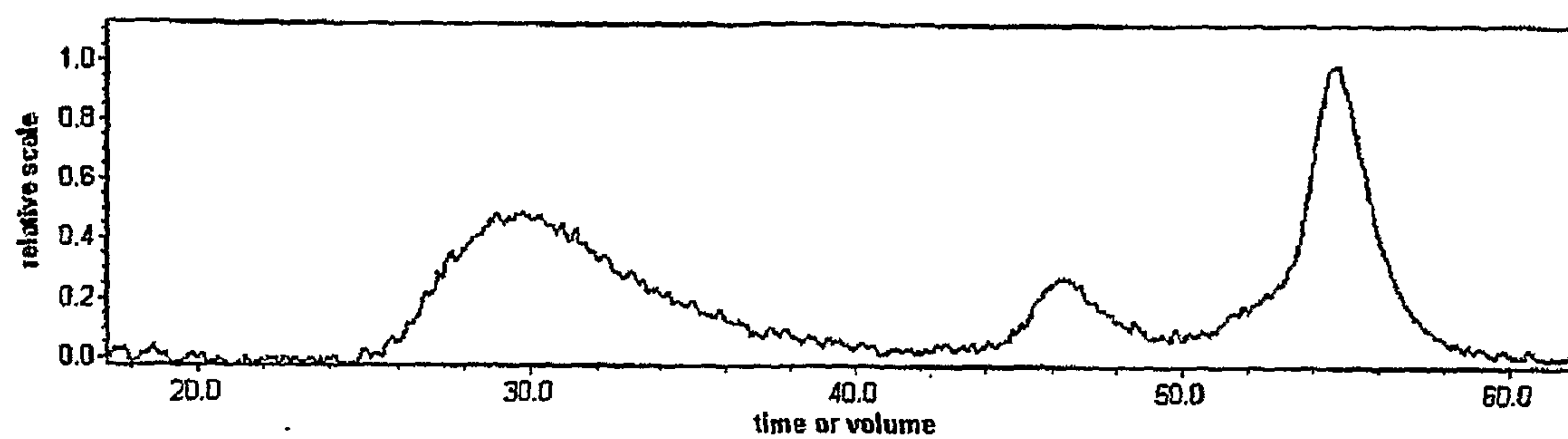
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FIGURE 1



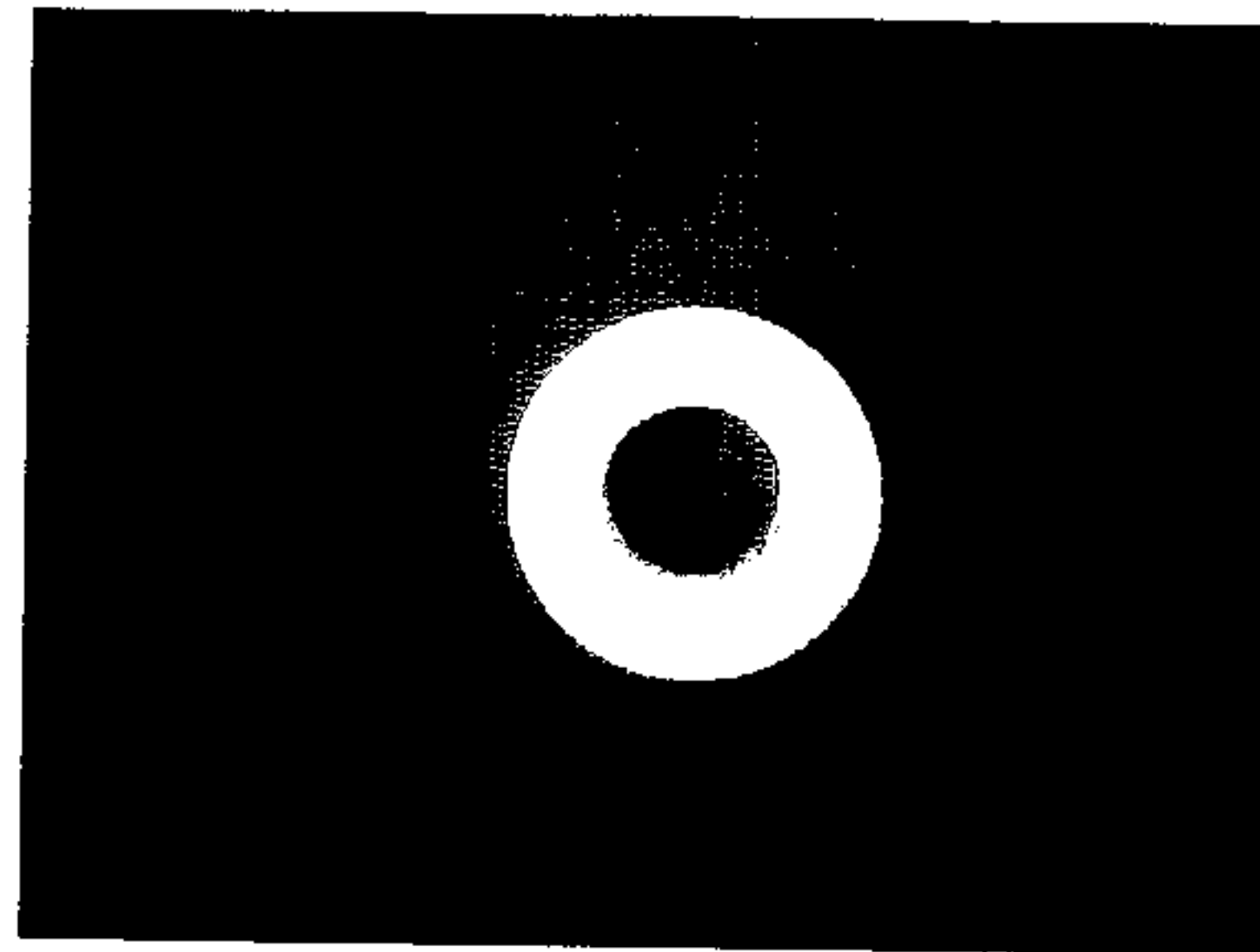
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FIGURE 2



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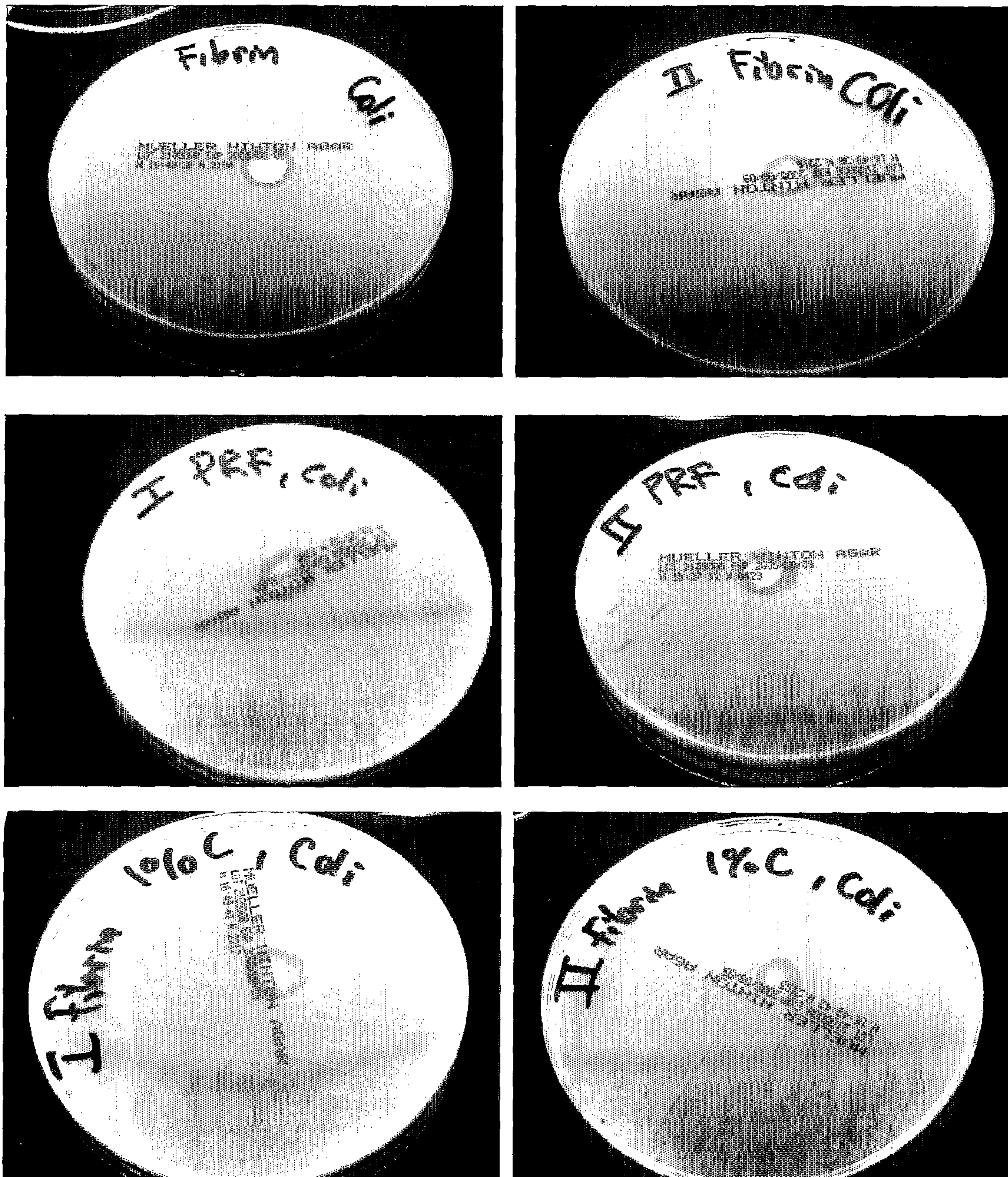
FIGURE 3



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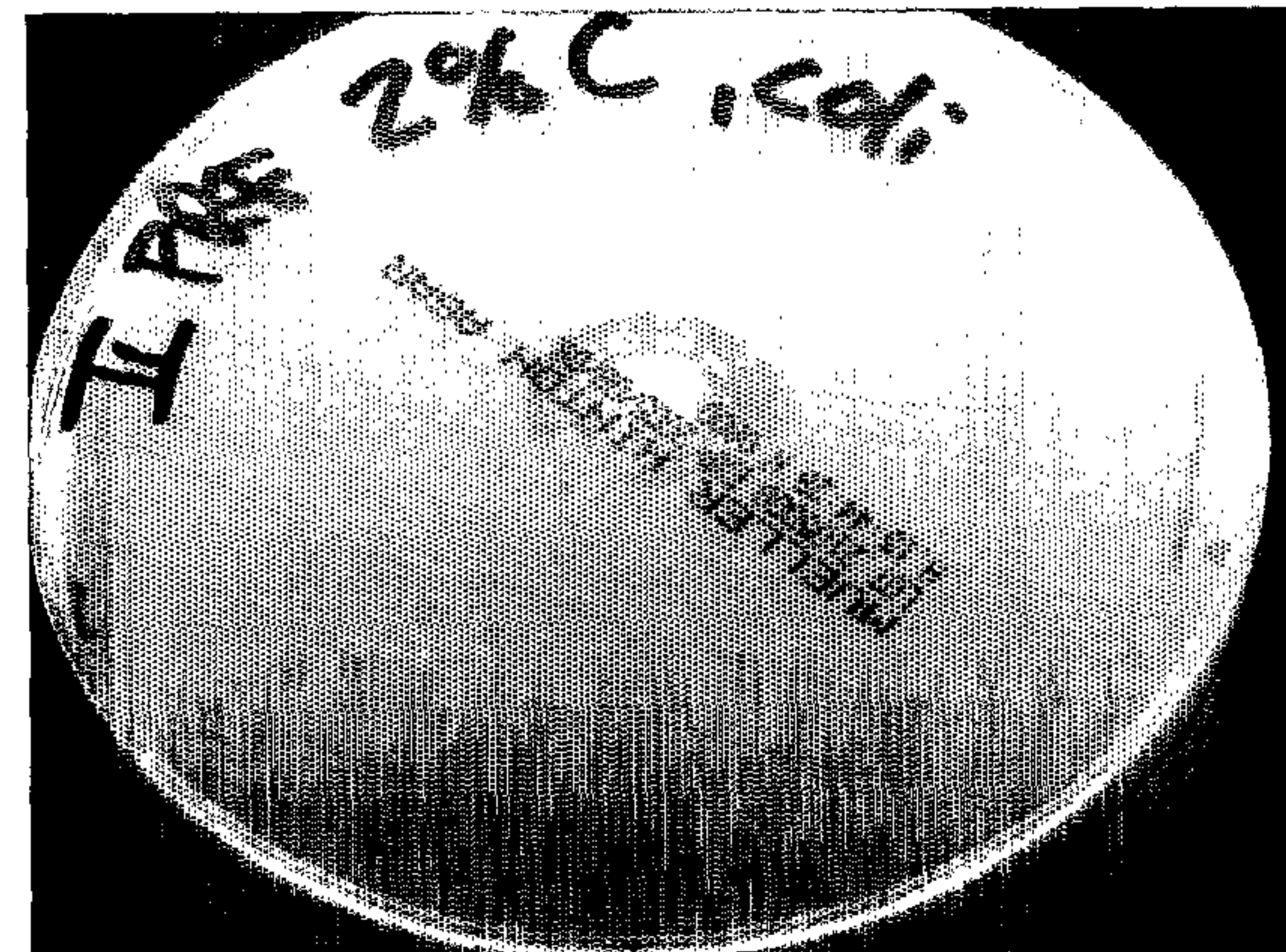
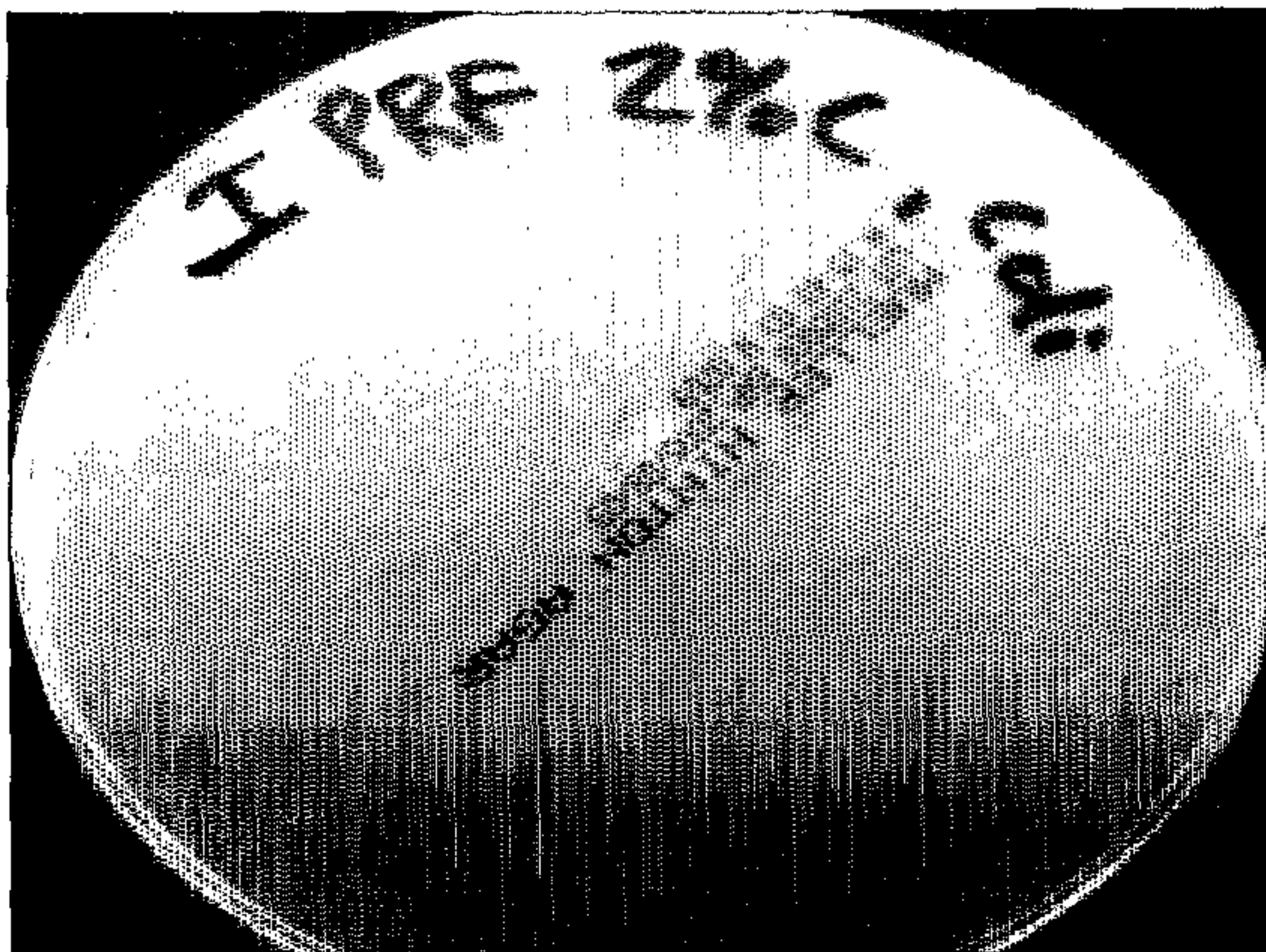
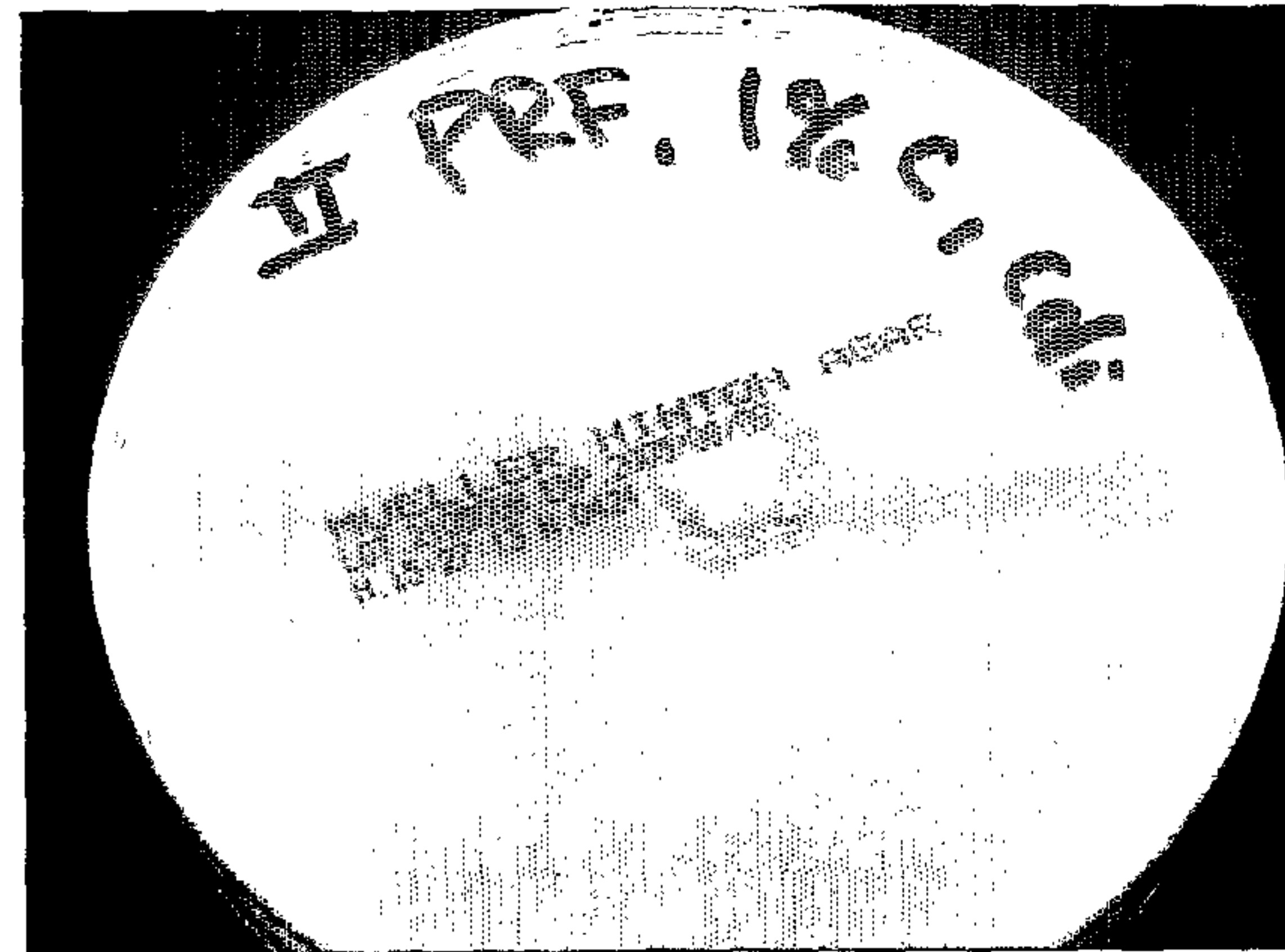
FIGURE 4

E Coli:



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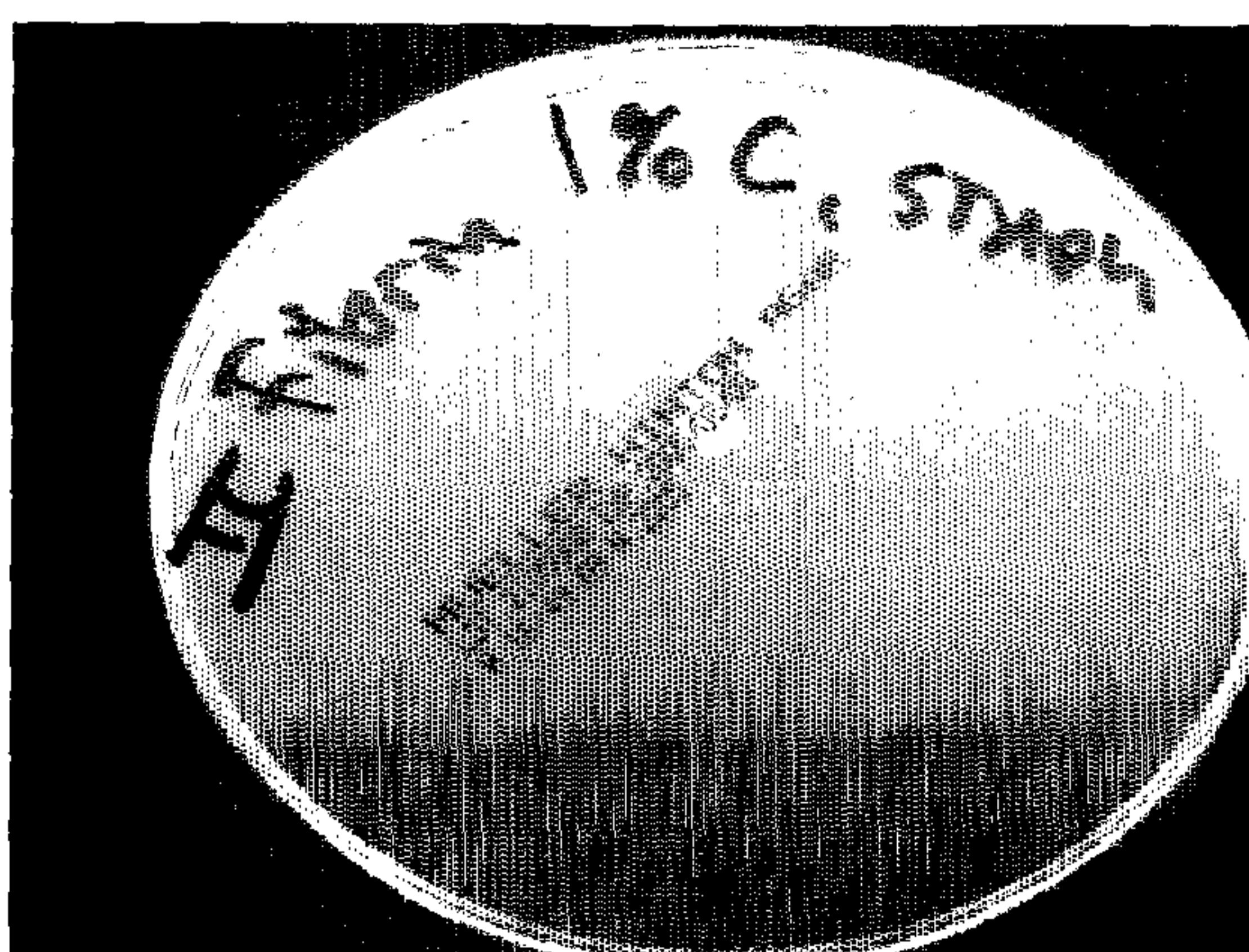
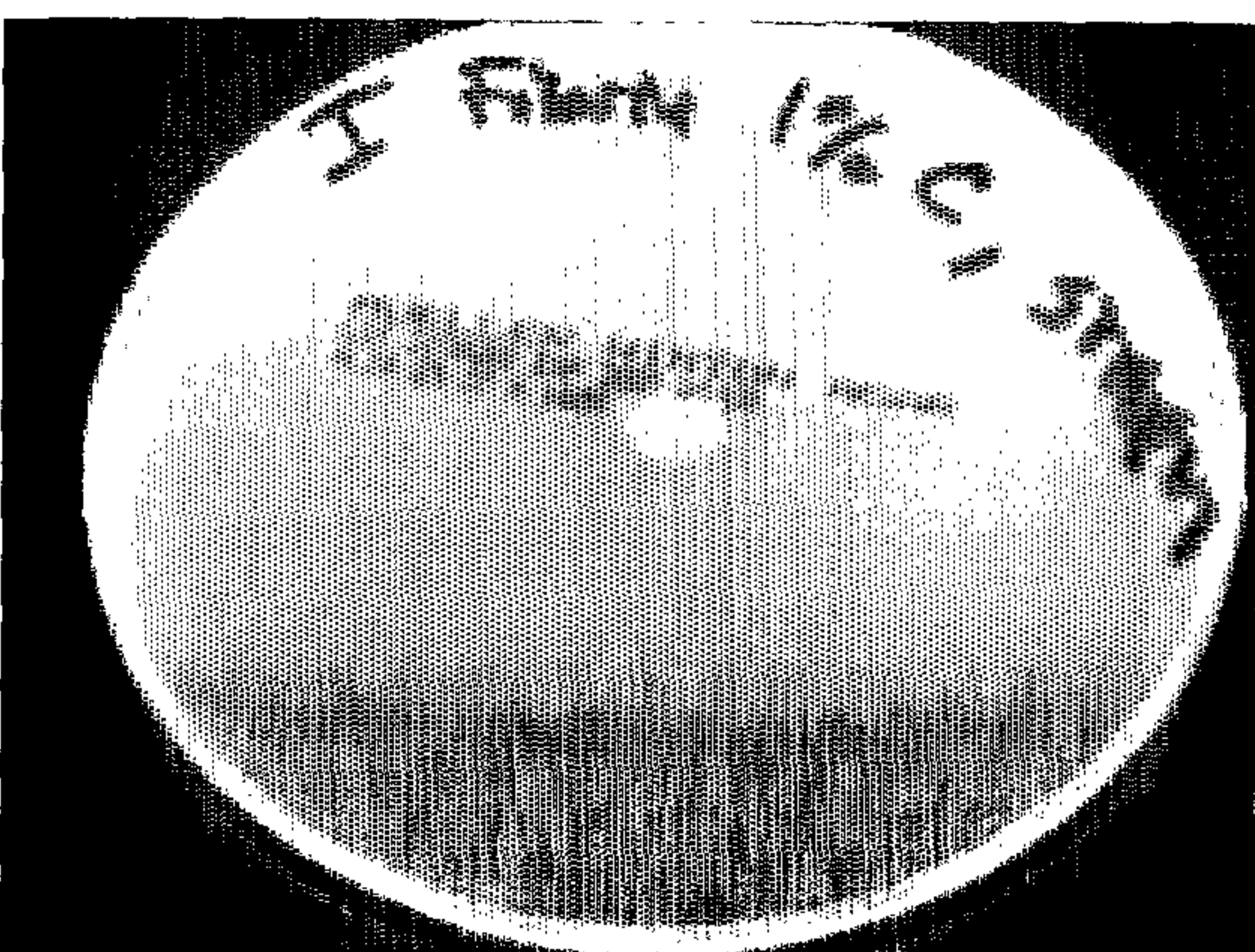
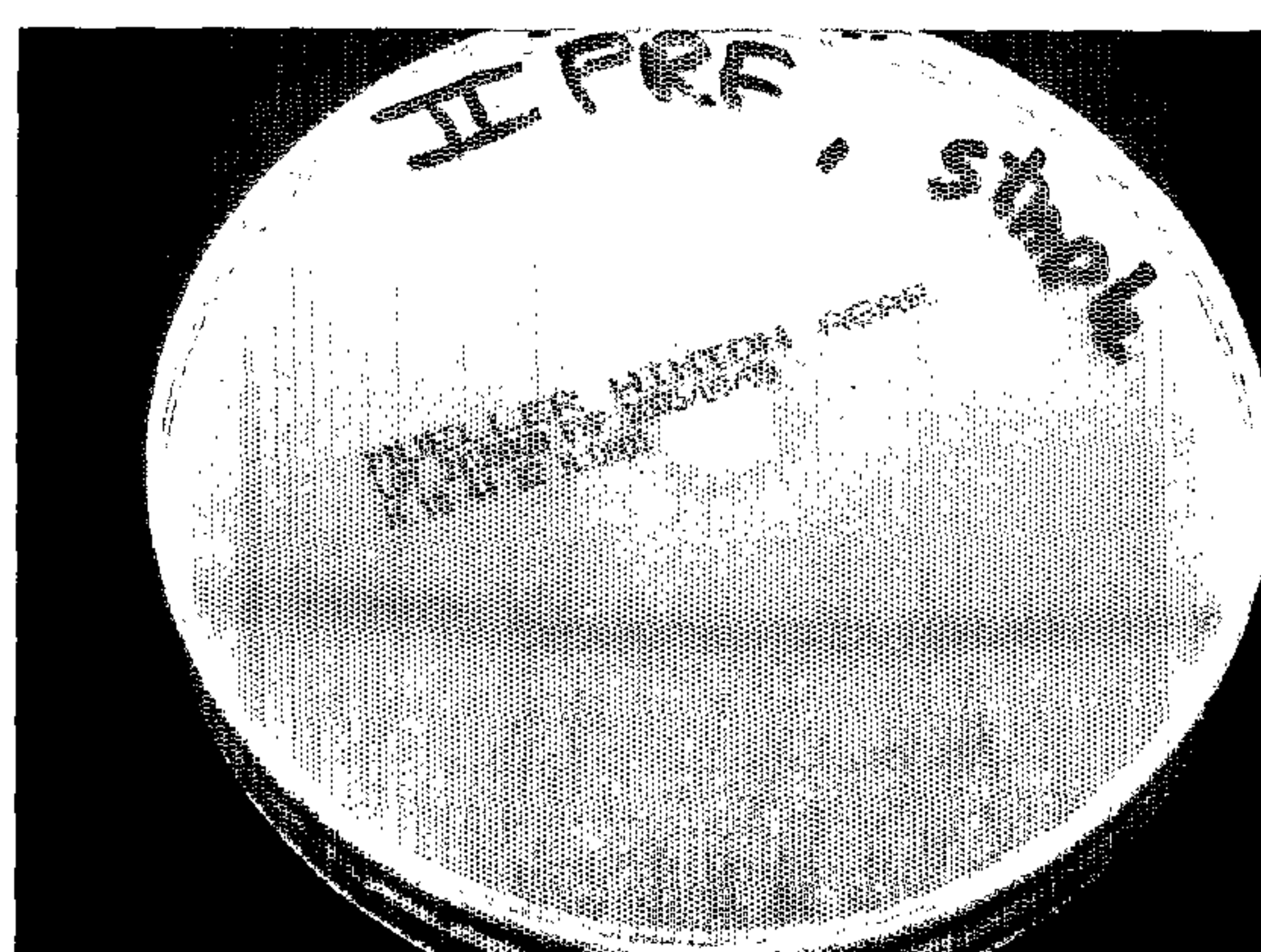
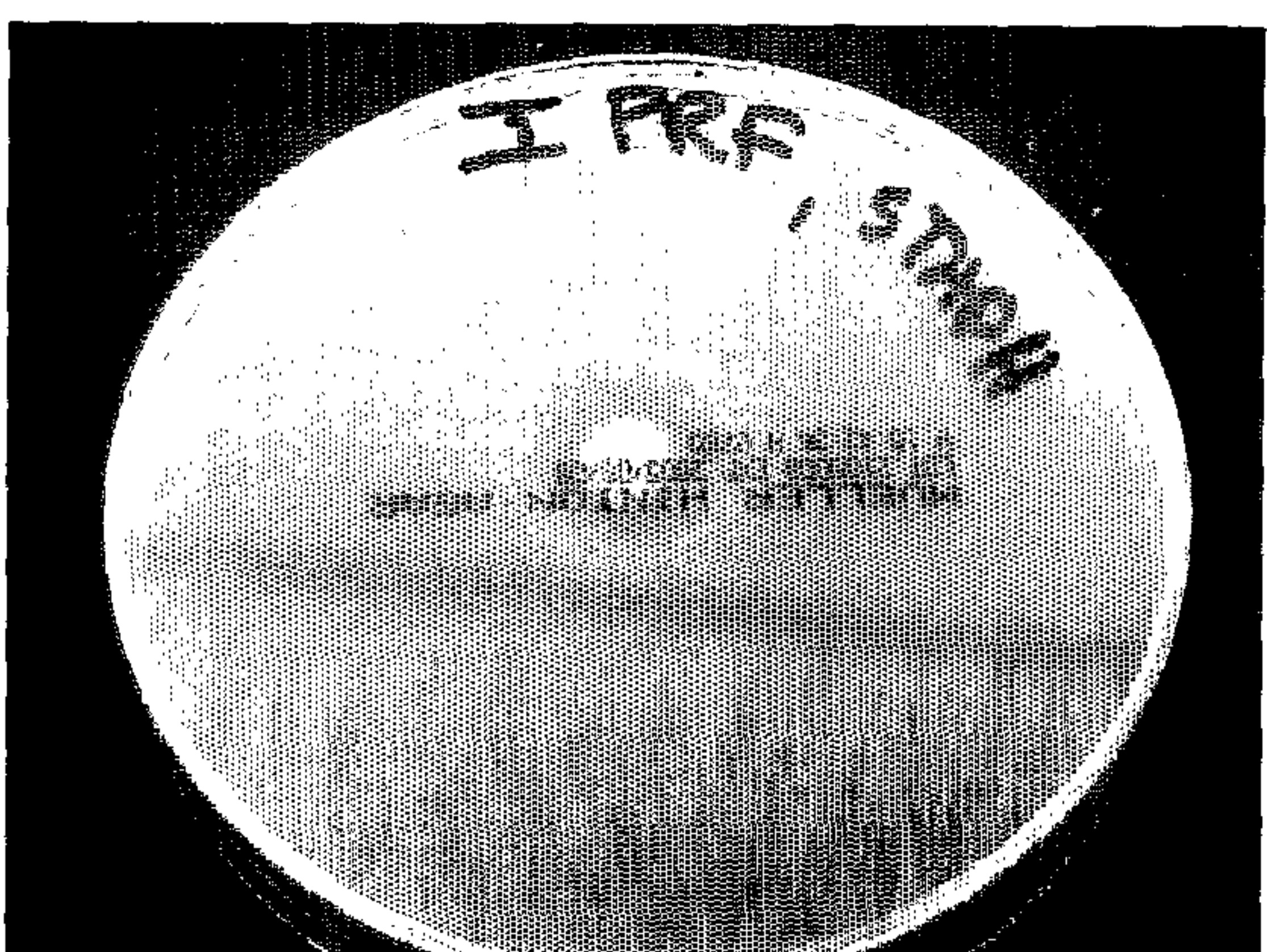
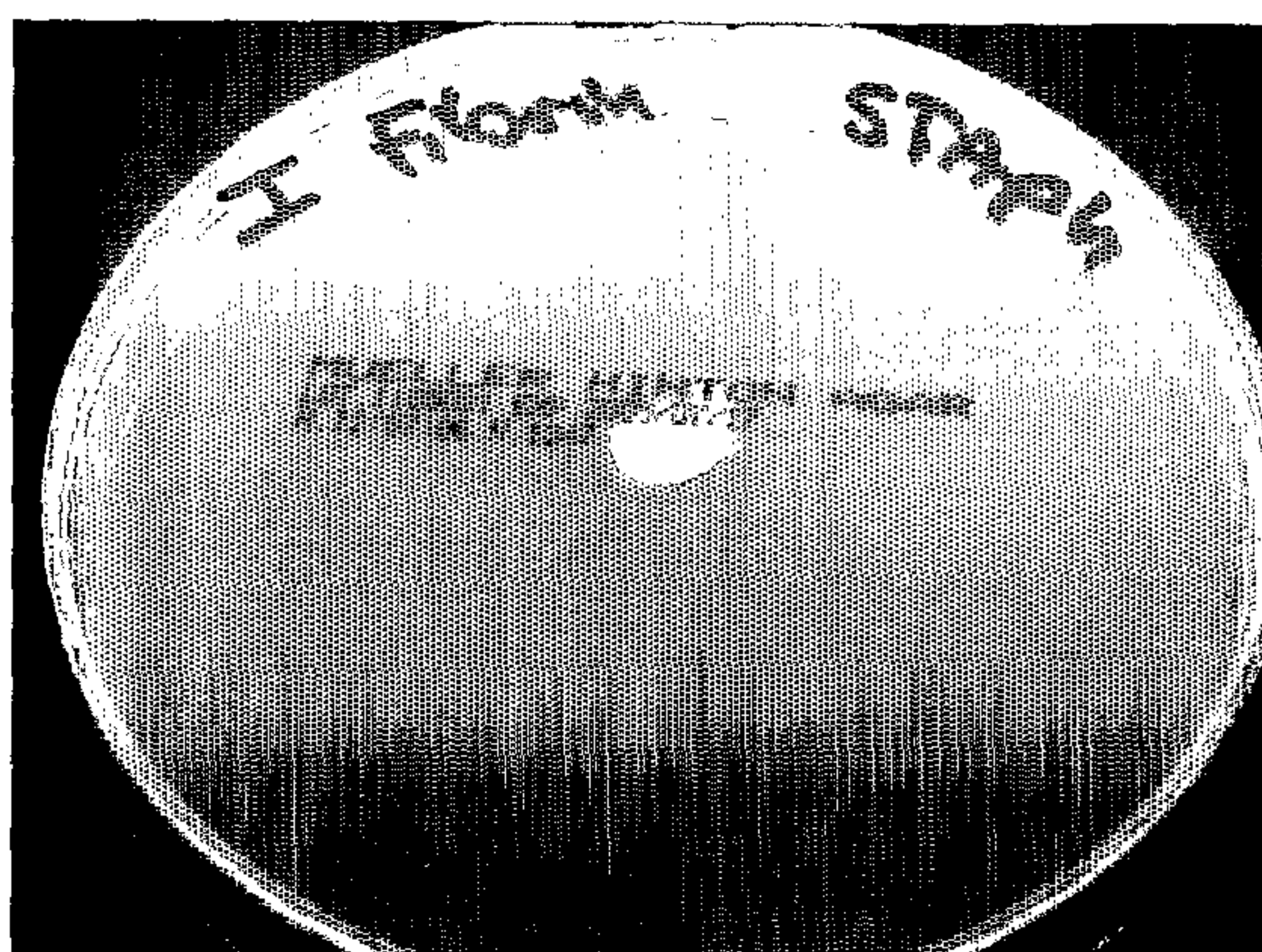
FIGURE 4 continued



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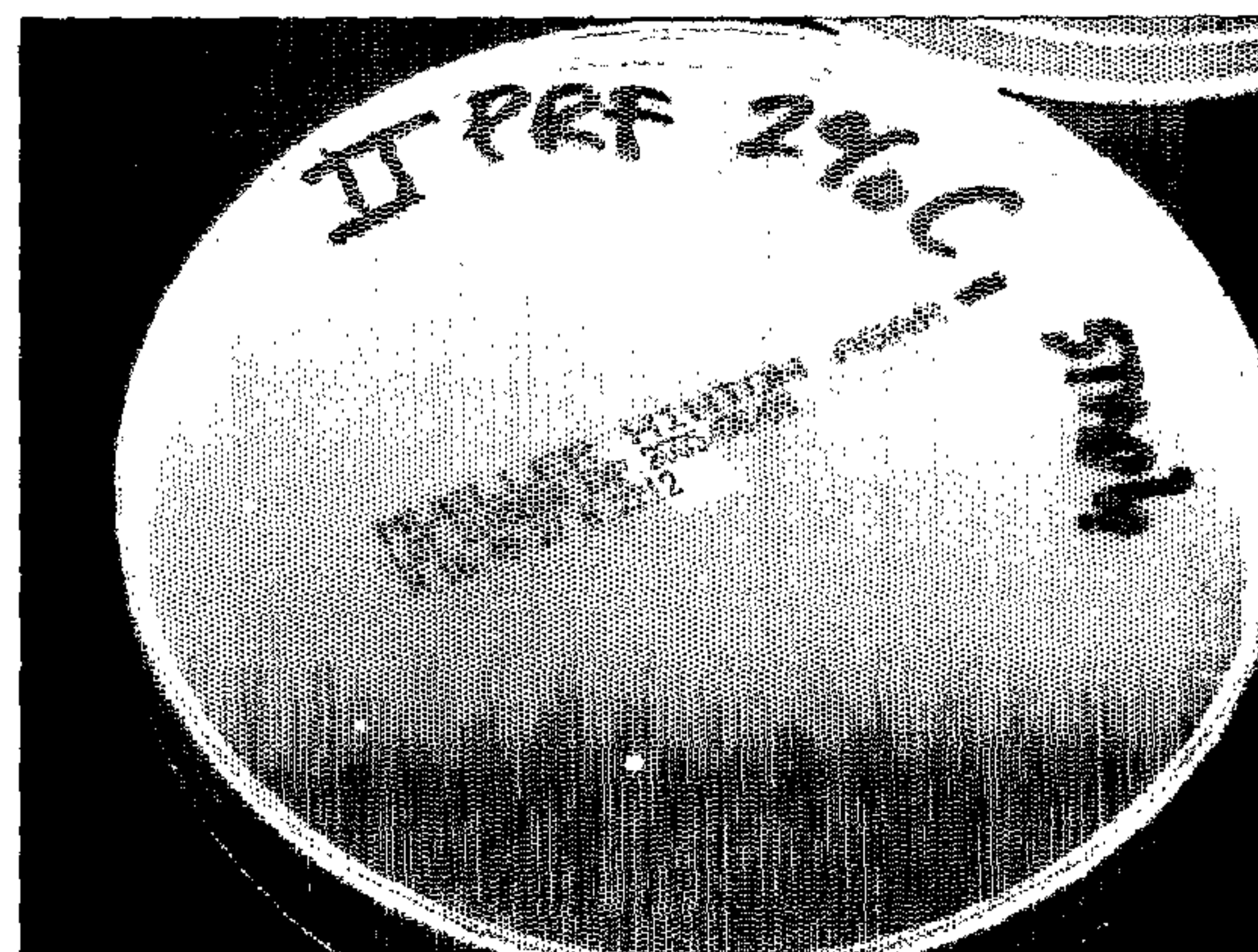
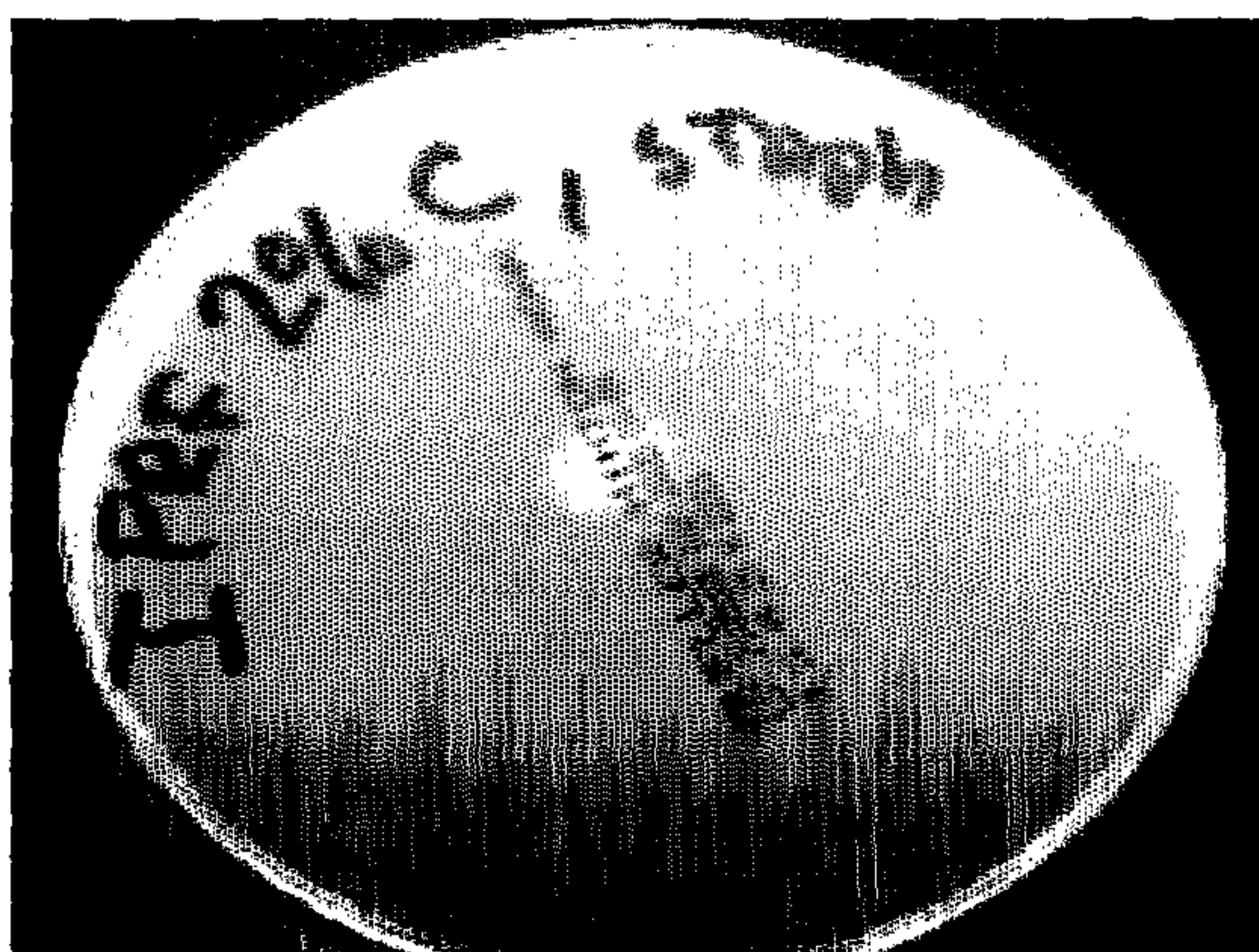
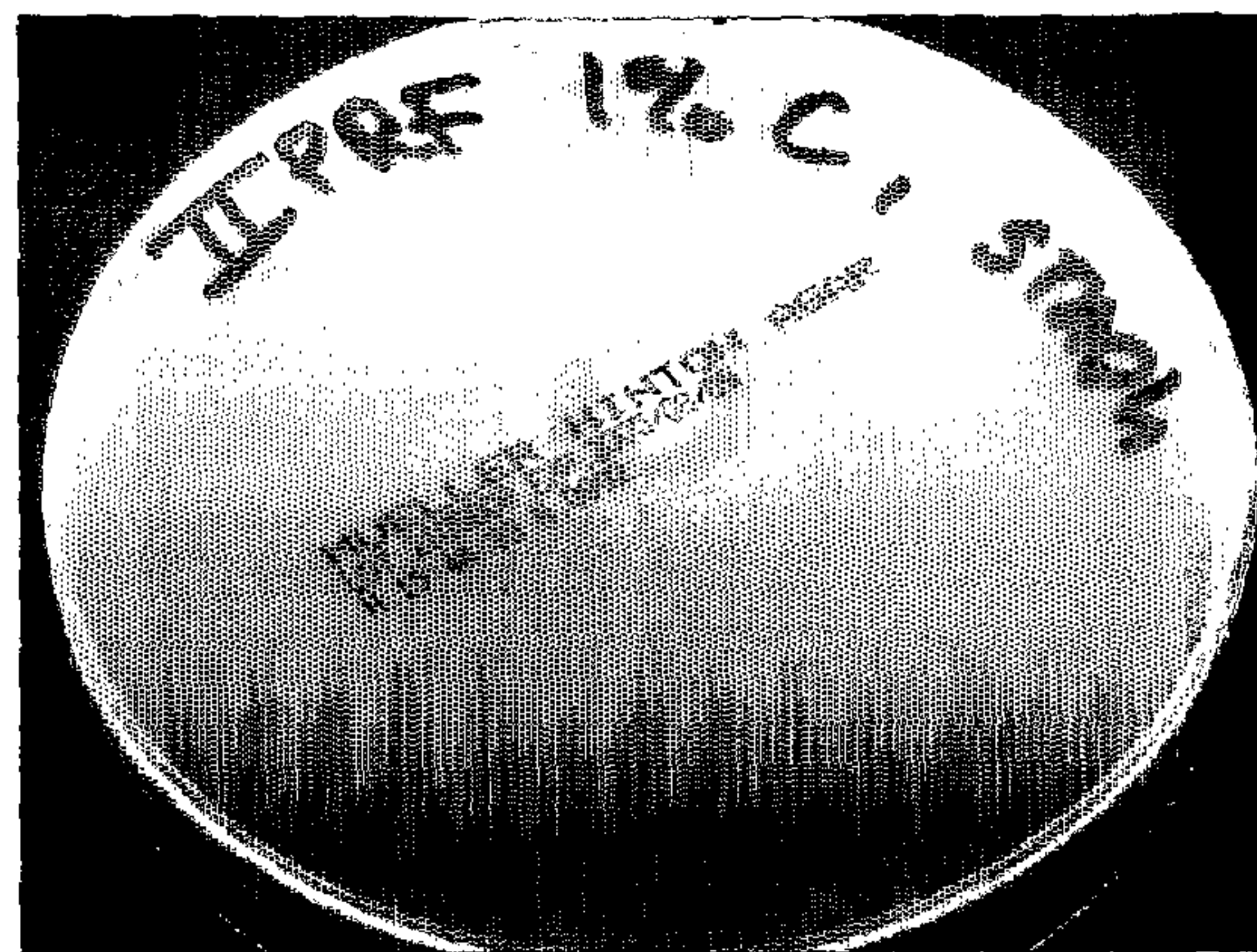
FIGURE 5

Staphylococcus:



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FIGURE 5 continued

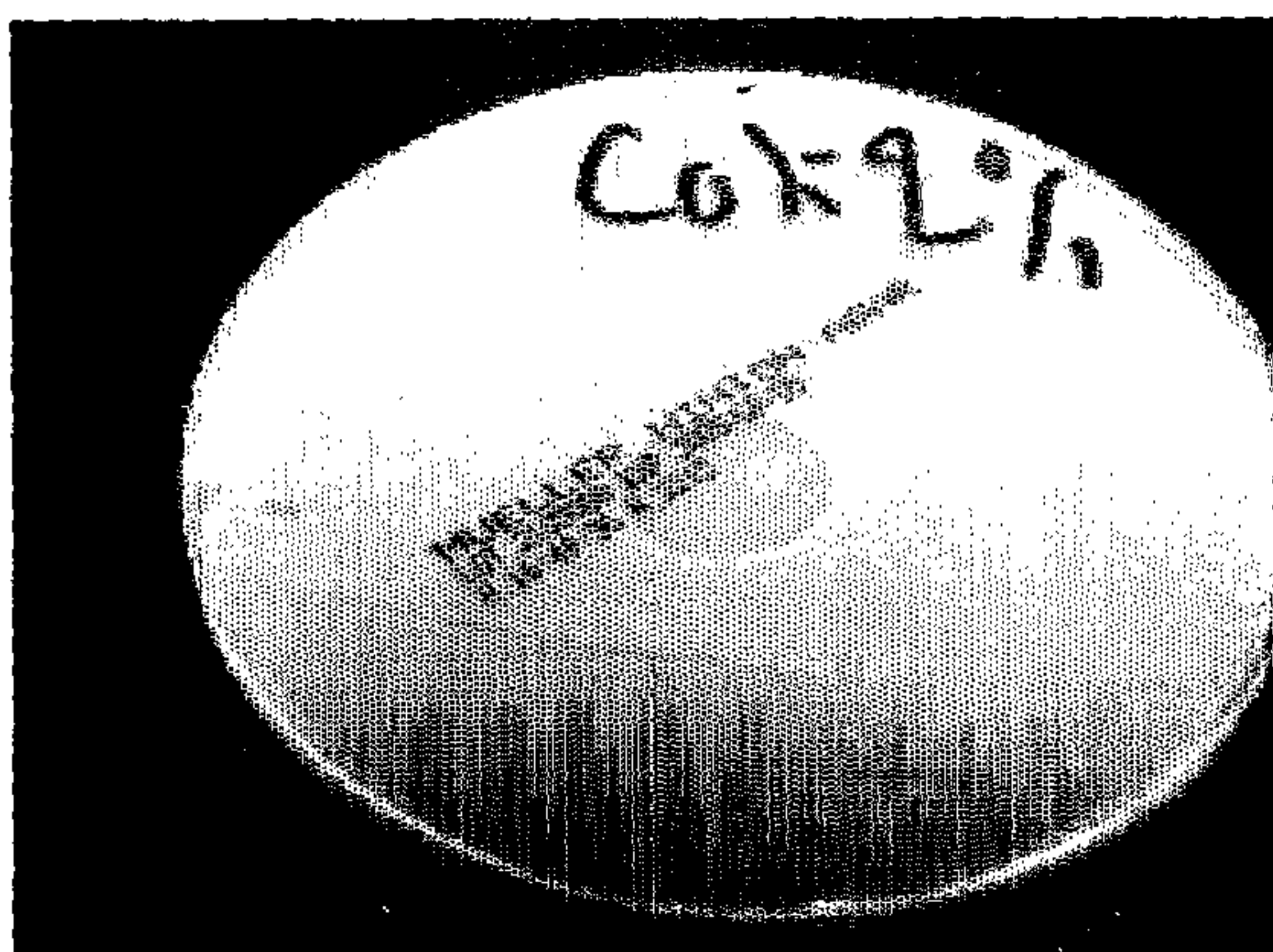


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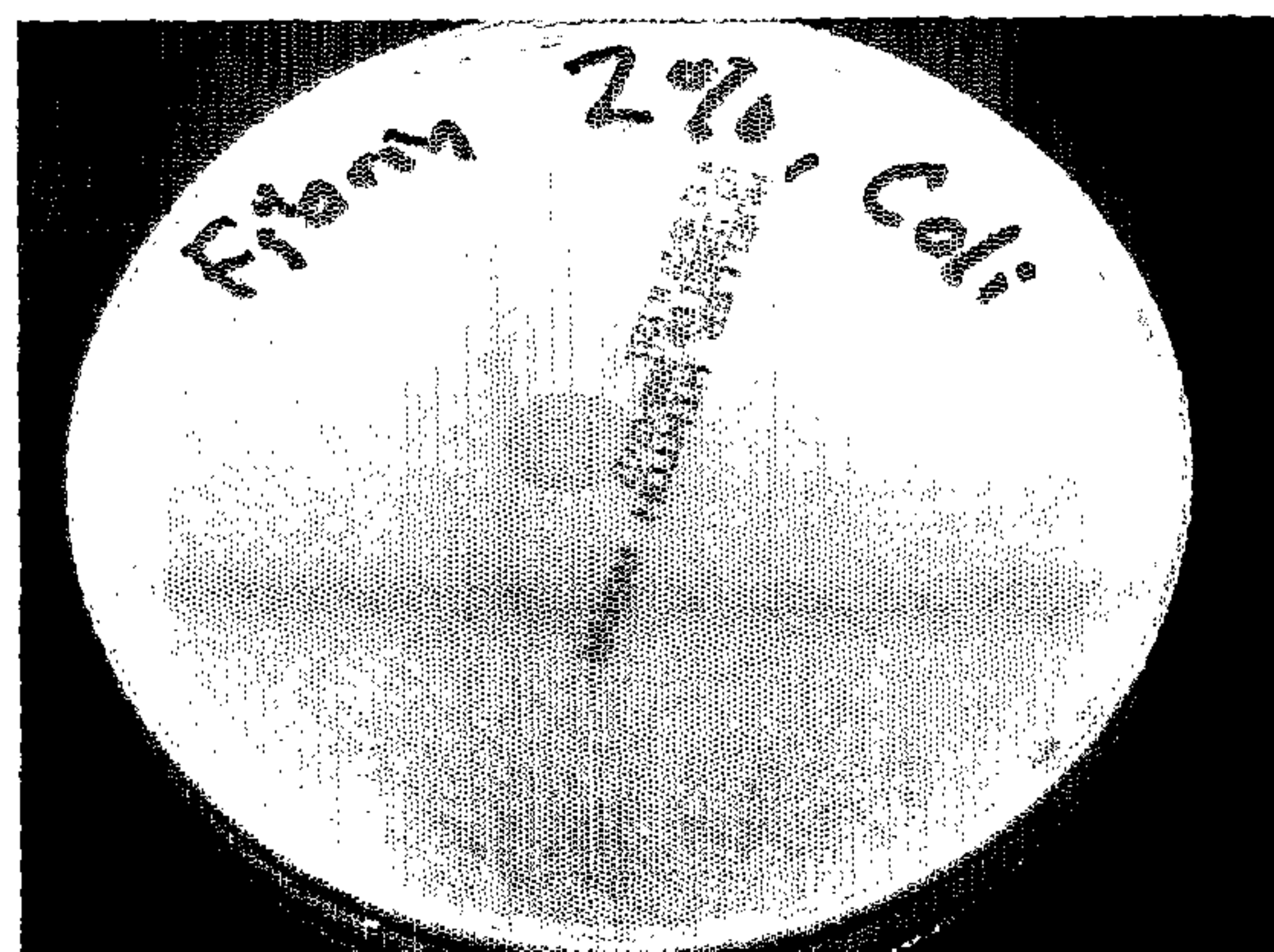
FIGURE 6

Chitosan:

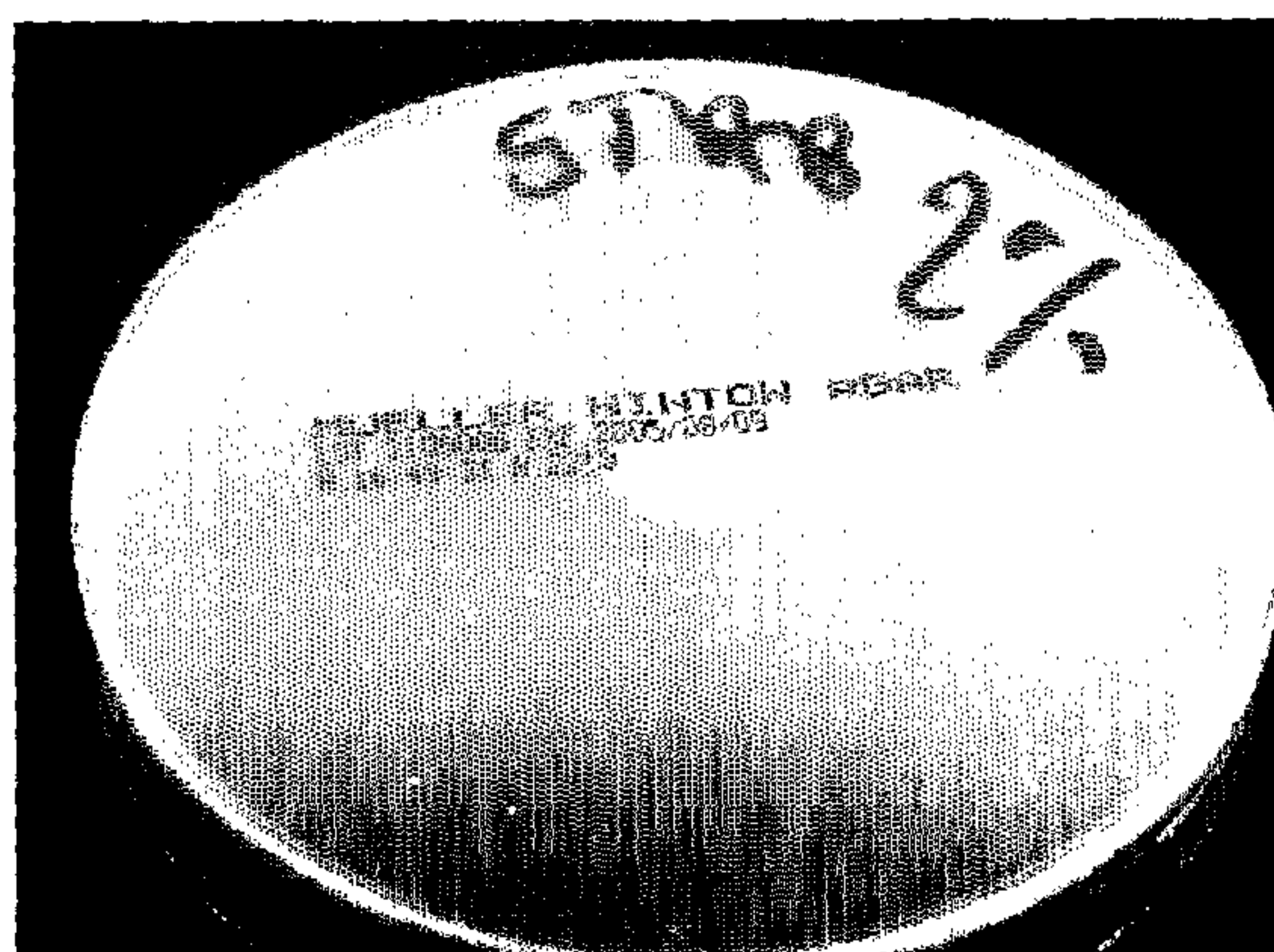
2% Chitosan.



1% Chitosan without Fibrin.



2% Chitosan.



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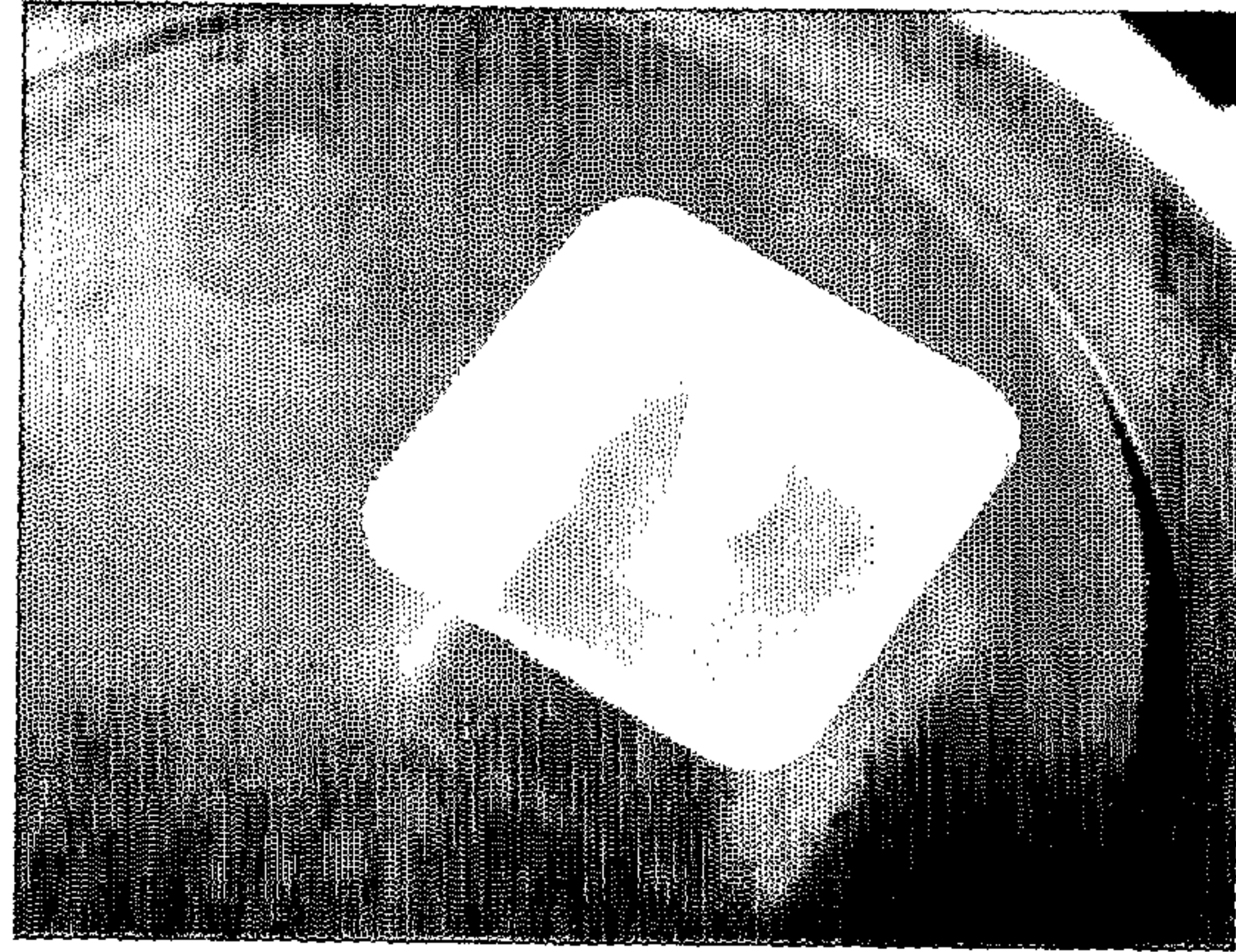
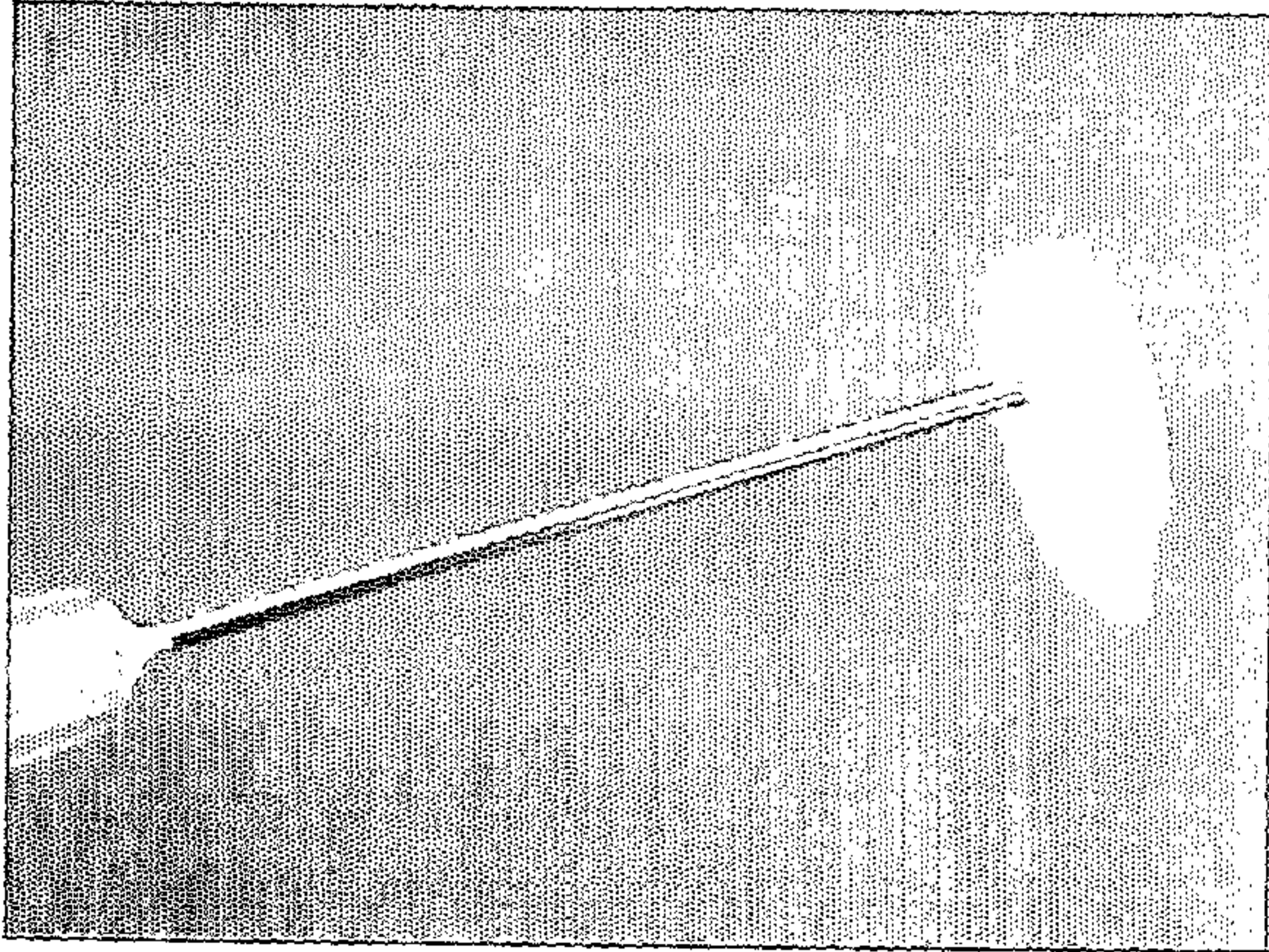
FIGURE 6 continued

1% Chitosan without fibrin.



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FIGURE 7



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FIGURE 8

