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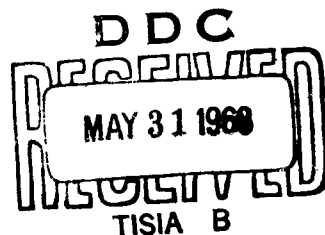
**FLUOROCHROMATIC EXAMINATION  
WITH  
MICROORGANISMS IN LIQUID MEDIA**

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FLUOROCHROMATIC EXAMINATION WITH MICROORGANISMS  
IN LIQUID MEDIA, REPORT 1962

by

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*20 August 1962*  
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## SUMMARY

1. About 15 fluorochromates are suitable for inducing satisfactory fluorescence in bacteria and other microorganisms in a liquid medium, in order to improve the conditions for automatic counting in continuous-flow chambers. This applies to living or dead bacteria.

2. The most efficient terminal concentration for the dyes is 1 : 10,000. Fluorochromate solutions should be alkaline (pH 9) but isotonia is not necessary.

3. Under the selected specific experimental conditions, dead or living bacteria cannot be distinguished from each other on the basis of fluorescent reaction.

4. Differentiation of individual types of microorganisms on the basis of fluorescent reaction is not possible in the present experimental arrangement.

5. The disturbance through inherent fluorescence of the dye solutions can be reduced by elution and, to an even greater extent, by the addition of fluorescence-suppressants, e.g. aqueous 1%-potassium iodide solutions.

6. Non-bacterial organic particles show fluorescence after treatment with most fluorochromates. Even clay particles have a positive reaction with certain dyes. At the present time, the possibility of differentiating inorganic particles from bacteria by means of fluorescence-microscopic media exists only to a limited extent.

## 1. Introduction

The Battelle Institute at Frankfurt was charged with carrying out an investigation of fluorochromatic staining of microorganisms by the CAP Company for the Development of Measuring Instruments in Frankfurt in October 1961. The investigation was intended to identify microorganisms in a liquid medium by fluorescence-microscopic methods and to improve the conditions for automatic bacterial count. It included the selection of suitable fluorescence dyes and processes of fluorochromatic staining which would permit the differentiation of bacteria and other microorganisms from other organic or inorganic particles. In addition, it was to be determined whether it is possible with fluorescence-microscopic methods to demonstrate specific types of bacteria as well as to differentiate whether such organisms are dead or living.

From fluorescent staining a basic improvement was to be expected in the performance of the automatic counter which was developed by the CAP Company under Government Order No. P. T. III 4a/00/PL 509/00/0.

The specifications listed above were processed through an experimental program embodied in our design program of 27 October 1961. The present report on the findings of the investigation is therefore arranged in principle on the basis of this design program.

As agreed, we reported on investigative progress in two intermediate reports of February and of May 1962. After discussion, a third intermediate report was not rendered because the research effort was terminated earlier than originally anticipated.

The present final report summarizes all detail investigations and results. As agreed, two copies of this report have been sent to the Federal Office for Defense Technics and Procurement at Koblenz, att: Dr. Rodewald. We are also transmitting three copies of this final report to the Ministry of Defense, T-III-7, at Bonn, att: Dr. Mutschin (by letter dated 2 July 1962).

## 2. Materials and General Methodology

Six types of microorganisms were used as biological experimentation material:

Gram-positive cocci:	Staphylococcus albus Sarcina lutea
Gram-negative, spore-less, rod-shaped:	Serratia marcescens

Gram-positive spore bacilli:	Bacillus subtilis (w/spores)
Gram-positive anaerobic spore bacilli:	Clostridium botulinum (w/spores)
Fungoid spores	Aspergillus niger (mycelium and spores)

These strains were transferred from solid nutritional media to glucose solution. After centrifugation, the microorganisms were suspended in physiological salt solution and kept refrigerated until processing.

The other organic and inorganic particles utilized for comparative purposes in the experiments are described in Section 7. The greater part of them was furnished by the Particle Bank of Stanford Research Institute, Menlo Park, California, or by Badische Anilin und Soda Fabrik, Farbwerke Hoechst and Norwegian Talc.

Fluorochromatic staining was carried out with a total of 59 staining dyes of different companies. Most of them are true fluorochromates and only a few of them were other types of dyes:

acridine yellow	cyanosine
acridine orange	diamond phosphine
auramine O	"entozon granulate"
aurophosphine O	eosine
berberine sulphate	eosine F
"brilliant dianil" green	erythrosine F
"brilliant sulphoflavin"	euchrysin
chelidonium extract	fluoresceine
chlorophyl extract	fluoresceine F
chromotropic acid	fluoresceine K
coriphosphine-fuchsine	fluoresceine Na
true cresyl violet	fuchsine, basic
true cresyl violet V	fuchsine for staining bacilli



fuchsina	"neutral red" B extra
fuchsine S, (acid)	primuline
fuchsine S-methylene blue	prune, pure
geranine G	pyronine
Coumarin	rhodamine B
"magdala" red, true	rhodamine 3 GO
malachite green	rhodamine 6 GO
methylene blue	rivanol
methyl green	rose, bengale
methyl green OO	"rosol red B"
methyl green-pyronine sicc.	thiazine red R
methyl umbelliferone	thiazol yellow
methyl violet	thioflavin S
morin 1 (Chroma)	thioflavin T
morin 2 (Merck)	thionine
mucicarmine	trypaflavin

At the start of the investigation, we utilized the fluorescence-microscope equipment of the Zeiss Company (high-pressure mercury-vapor tube HBO-200). After 15 January 1962, we used concurrently the new fluorescence-microscope equipment of the Leitz Company (high-pressure mercury-vapor tube CS-150) which is complemented by the automatic micro-camera "Orthomat". As objectives for the former, we utilized planachromates and, for the latter, fluorite systems. In most cases, the excitation-light filters were BG-12, BG-38, UG-1, and UG-5 singly or in various combinations. The selection of the suppression filter depended on the given requirements.

### 3. Preparative and Comparative Investigation on Smears

We began with preparative and comparative investigations of smears. Different microorganisms were to be processed as smears with

all fluorochromates available to us and under variations of the staining conditions. This was done for the purpose of finding the most suitable fluorescent-staining dyes for subsequent investigation through the screening method.

Smears were prepared from the bacterial suspensions. Fluorochromatic staining was made by immersing the heat stabilized slide preparations in freshly prepared solutions (aqueous solution 1 : 1,000; chlorophyll extract was dissolved in alcohol). The exposure to the stain was 2 minutes and the staining time was extended to 20 minutes in most cases. After fluorochromatic staining, the preparations were washed in running water for 10 minutes and stored in the dark in preparation containers, after drying, until examination. From the start of fluorochromatic staining until examination, no more than 30 minutes elapsed in any case.

The findings showed that fluorochromatic staining with the staining dyes listed in Section 2 is possible in an overwhelming number of cases. Differences were shown to exist both in the intensity and in the color of fluorescence. The stainability of the various strains was also quite different, especially in regard to color.

Seven of the approximately 60 fluorochromates proved to be particularly suitable and produced the highest fluorescence. Subdivided by type of organism, the results obtained with these seven dyes are grouped in Table 1. We see from the table that fluorescence is predominately yellow and also red-orange and in some cases green. Pure green or pure blue fluorescence was not observed. In addition to the seven highly suitable fluorochromates, the stains listed below gave good results:

- acridine organe (predominantly red fluorescence)
- aurophosphine 0 (predominantly orange " )
- brilliant dianil green (yellow-green " )
- coriphosphine-fuchsine (red, orange, yellow fluorescence)
- cyanosine (orange and yellow fluorescence)
- entozon granulate (orange, yellow-green fluorescence)
- morin 1 and morin 2 (yellow and yellow-green fluorescence)
- primuline (in some cases, luminous yellow fluorescence)
- rhodamine 3 GO (yellow-green fluorescence)

We should also mention fluoresceine K and fluoresceine Na with which a yellow-green fluorescence was obtained, in particular for sarcina.

Color Table 1 gives a tabular review of all the findings obtained in this experimental series.

Color Table 1

General Staining Effect of Fluorochromates

Fluorochrom (1:1000)	Staphylo- cocc albus		Sarcina lutea		Serratia marces- cens		Bacil- lus subtilis		Clostri- dium botuli- num		Asper- gillus niger	
	2'	20'	2'	20'	2'	20'	2'	20'	2'	20'	2'	20'
acridine yellow												
acridine orange												
auramine O												
aurophosphile O												
berberine sulphate												
"brilliant dianil" green												
"brilliant sulphoflavin"												
chelidonium extract												
chlorophyl extract												
chromotropic acid												
coriphosphine O												
coriphosphine-fuchsine												
true cresyl violet												
true cresyl violet V												
cyanosine												
diamond phosphine												
"entozon granulate"												
eosine												
eosine F												
erythrosine F												
euchrysine												
fluoresceine												
fluoresceine F												
fluoresceine K												
fluoresceine Na												
fuchsine, basic												
fuchsine for staining bacilli												
fuchsine												
fuchsine S, (acid)												
fuchsine S-methylene blue												
geranine G												
Coumarin												
"magdala" red, true												
malachite green												
methylene blue												
methyl green												
methyl green OO												
methyl green-pyronine sicc.												
methyl umbelliferone												
methyl violet												
morin 1 (Chroma)												
morin 2 (Merck)												
mucicarmine												

Color Table 1 (Cont.)

"neutral red" B extra  
primuline  
prune, pure  
pyronine  
rhodamine B  
rhodamine 3 GO  
rhodamine 6 GO  
rivanol  
rose, bengale  
"rosol red B"  
thiazine red R  
thiazol yellow  
thioflavine S  
thioflavin T  
thionine  
trypaflavin

Table 1

## Staining Microorganisms with Selected Fluorochromates

Fluorochromate (1:1000 aqueous)	Staphylococcus albus		Sarcina lutea		Serratia marcescens		Bacillus subtilis		Clostridium botulinum		Aspergillus niger	
	Exposure to Staining min.	2	20	Exposure to Staining min.	2	20	Exposure to Staining min.	2	20	Exposure to Staining min.	2	20
Acridingelb	orange ++	+++	gelb ++	gelb ++	orange ++	orange ++	gelb +++	gelb +++	gelb-grün +++	gelb +++		
Coriphosphin O	orange ++	++	orange +	orange +	orange +++	orange +++	orange +++	gelb ++	gelb-grün +++			
Diamantphosphin	orange +	+++	orange +++	orange +++	gelb +++	orange ++	orange +++	gelb +++	gelb-grün +++	gelb +++		
Euchrysin	orange +++	+++	orange +++	orange ++	gelb +	gelb +++	gelb +	gelb +	gelb-grün +++	gelb +++		
Thioflavin S	orange ++	++	gelb +	orange ++	gelb +++	gelb +++	gelb +++	gelb +++	gelb-grün +++	gelb +++		
Thioflavin T	gelb ++	orange ++	gelb +	gelb ++	gelb +++	orange +++	gelb orange	gelb orange	orange	gelb +++		
Trypflavin	orange ++	gelb +++	gelb ++	gelb +	gelb-grün orange +	gelb orange ++	gelb grün	orange	orange	orange +++		

+ = notable fluorescence

+++ = strongly luminous fluorescence

++ = luminous fluorescence

++++ = very strong fluorescence

On this and on all other color tables, the intensity of fluorescence is indicated by the following symbols:

- no symbol = normal fluorescence
- = very weak fluorescence
- + = notable fluorescence
- ++ = luminous fluorescence
- +++ = strongly luminous fluorescence
- ++++ = very strong fluorescence

For reasons of cost, we eliminated reproduction of the numerous microphotographs of especially selected preparations.

#### 4. Demonstration of Microorganisms in Liquid Media

##### 4.1. Fluorochromatic Film Method

Fluorochromatic staining in liquid media was made initially by the fluorochromatic film method. Here the microorganisms in physiological salt solutions were transferred to slides prepared with various fluorochromates:

Starting with a 1% standard alcohol solution of the respective fluorochromate, diluted solutions were prepared, transferred to carefully cleaned slides and poured off after one minute. An extremely thin, hardly visible fluorochromatic film then remained on the slide. Slides prepared in this manner can be stored for a very long time.

One drop of the respective bacterial suspension was applied to the slide and covered by glass. The staining of the living bacteria was then observed immediately and directly. The microorganisms examined were the same six types listed in Section 1: *Staphylococcus albus*, *Sarcina lutea*, *Serratia marcescens*, *Bac. subtilis*, *Clostridium botulinum*, *Aspergillus niger*.

On the basis of the findings of the first experimental series, we did not utilize all of the fluorochromates then employed but only those which had proved themselves as basically suitable. Sixteen of 35 fluorochromates showed themselves to be suitable for fluorochromatic staining under these conditions.

Selection was based not only on satisfactory fluorescence-microscopic demonstrability of the organisms but we directly eliminated as

unsuitable those fluorochromates which produce strong fluorescence of the ambient liquid medium (inherent fluorescence of the dye in solution). This so-called "background fluorescence" must be kept as low as possible if examination is to be performed in liquid media.

With this condition in mind, the following 16 fluorochromates were selected for utilization or further investigations:

acridine yellow	entozone granulate
acridine orange	euchrysin
auramine O	morin 1 (Chroma)
auraphosphine O	morin 2 (Merck)
"brilliant dianil green"	rivanol
coriphosphine O	thioflavin S
coriphosphine-fuchsine	thioflavin T
diamond phosphine	trypaflavin

With only faint background fluorescence, they induce in living microorganisms notable fluorescence which is either green, green-yellow, yellow, or orange. Especially the acridine dyes produce sharply differentiated pictures. Whether the individual microorganisms are stainable to a different degree could not be decided with certainty.

In view of the extensive material and the following results, we rejected reproduction of the findings in a color table.

#### 4.2. Direct Fluorochromatic Staining in Liquid Media

After arriving at a selection of a limited number of suitable fluorescent dyes with the aid of the fluorochromatic film method, direct fluorochromatic staining of living microorganisms was carried out in simple covered dishes or covered slides.

For fluorochromatic staining, the bacterial suspensions in physiological salt solution were mixed with the respective fluorochromate, also in physiological salt solution, at a ratio of 1 : 1. The initial fluorochromate concentration was 1 : 1,000 and the terminal concentration therefore 1 : 2,000. Immediately after adding the fluorochromate, one drop was transferred to a slide, covered and examined. The fluorochromates morin 1 and morin 2 as well as rivanol were not dissolved in physiological salt solution but in distilled water.

The same experimental procedure was employed in a second series in which the terminal concentration was 1 : 4,000. Findings can be summarized as follows:

All of the fluorochromates investigated notably stained the living bacteria but *aspergillus niger* is demonstrated only very poorly.

In most cases, there again occurs a green, green-yellow, yellow and orange fluorescence and in some cases only a green or yellow fluorescence of the microorganisms. Disregarding minor differences, staining at a concentration of 1: 4,000 is as distinct as in one of 1: 2,000.

In this experimental arrangement, special attention was given to the question whether and to what extent the background fluorescence of the respective dyes influences the demonstration of the corpuscular elements. We found that, with the exception of tryptaflavine, the background fluorescence is so minor that any disadvantageous influence was not noted with our experimental conditions. This is shown particularly well in the weaker concentration.

Color Table 2 groups the findings of this experimental series for a concentration of 1:4,000.

This table also indicates that it is apparently not possible with the fluorochromates presently available and under the experimental conditions described, to achieve a differentiation of the types of microorganisms. This corresponds also to our other experiences during this commission for research. We were not able to observe, in any case, a specific demonstration of certain bacteria or other organisms. This observation applies strictly only for the six microorganisms tested. However, since the microorganisms employed by us represent definite groups, the possibility of a differentiation by type in the experimental arrangement as described is improbable in general.

#### 5. Influence of Dye Concentration, Osmotic Pressure and pH

In a further experimental series, we attempted to achieve optimum demonstration of bacteria by varying given characteristics of the fluorochromatic solutions. Utilization of all six types of microorganisms would have resulted in very extensive experimental series. We employed only staphylococcus albus for this.

##### Variation of Dye Concentration

With the utilization of direct fluorochromatic staining in liquid media (as in 4.2), the fluorochromates were prepared in four terminal concentrations of 1:4,000, 1:10,000, 1:100,000, 1:1,000,000. By "terminal concentration", we here mean the concentration resulting after addition of the bacterial suspension. We utilized 15 fluorochromates but eliminated morin 2 (Chroma) because it offers no advantages over morin 1 (Merck).



Color Table 2

Fluorochromation in Liquid Media (Living)

Fluorochromate  
concentration  
1: 2,000  
Terminal  
concentration  
1: 4,000

Fluorochromate	Staphyl. albus	Sarcina lutea	Serratia marcesc	Bacillus subtilis	Clostrid. botulinum	Asperg. niger
----------------	-------------------	------------------	---------------------	----------------------	------------------------	------------------

Acridine yellow

Acridine orange

Auramin O

Aurophosphin O

"Brilliant dianil" green

Coriphosphine O

Coriphosphine-fuchsine

Diamond phosphine

"Entozon granulate"

Euchry sine

Morin I (Chroma)

Morin II (Merck)

Rivanol

Thioflavin S

Thioflavin T

Trypaflavin

Color Table 3 shows the results obtained in this experimental series. It was shown that a terminal concentration of 1: 10,000 was the most suitable for all further investigations. At this dye concentration, the bacteria were made visible to almost the same extent as with the concentration of 1 : 4,000. However, the background fluorescence was appreciably stronger in the latter. In the lower concentrations, staining was less pronounced which may possibly be due to the longer time of exposure required.

All other investigations were carried out at a terminal concentration of 1 : 10,000.

#### Variation of Osmotic Pressure

The influence of the osmotic pressure of the fluorochrome solution on fluorochromatic staining of staphylococcus albus was tested in two experimental series. The dye solutions were prepared in several concentrations (1 : 4,000 to 1 : 1,000,000) either in distilled water or in a 2% salt solution.

This permitted us to establish the stainability of the bacteria by 15 fluorochromes in hypotonic and hypertonic media.

The findings of these experimental series may be grouped as follows: essential differences in the colors of fluorescence do not occur. However, since the microorganisms are more sharply presented in a hypotonic medium in which background fluorescence is less, the use of hypotonic solutions is preferable for the intended purpose. We eliminated reproduction of the tabular material in this report because of the extent of the former.

#### Variation of Hydrogen-Ion Concentration (pH-value)

In regard to color differentiation and intensity of fluorescence, advantages were to be expected from variation of the pH-value of the fluorochrome solutions. We therefore subjected living staphylococci to fluorochromatic staining with dye solutions of different hydrogen-ion concentrations. We again utilized the 15 fluorochromes listed above at terminal concentrations of 1 : 10,000. The dye solutions were adjusted with a phosphate buffer (secondary sodium phosphate and primary potassium phosphate, 1/5 mol in each case) to the following 9 pH-values: 4.40, 5.33; 5.98; 6.42; 6.78; 7.09; 7.71; 8.39; and 9.07, by means of a "knick" pH-meter (glass electrode).

Color Table 3

Fluorochromation in Liquid Media (staphyl. albus - live)

Terminal concentra- tion	1:4,000	1:10,000	1:100,000	1:1,000,000
Acridine yellow				
Acridine orange				
Auramin O				
Aurophosphin O				
"Brilliant dianil" green				
Coriphosphine O				
Coriphosphine-fuchsine				
Diamond phosphine				
"Entozon granulate"				
Euchrysine				
Morin (Merck)				
Rivanol				
Thioflavin S				
Thioflavin T				
Trypaflavin				

The results are combined in Color Table 4. It will be seen that variation of pH-value, in contrast to experience in histology with bacteria, does not produce a differential bonding of the fluorochromates and any consequent color differentiation. On the contrary, for most fluorochromates, the fluorescent coloring is independent of pH. Only in the alkaline range does it appear that the red fluorescence of the bacteria is less.

In nearly all fluorochromates, an increase of the intensity of fluorescence is notable in the alkaline range and this increase is especially marked at pH 9.07. This finding is not without significance for practical purposes, especially since the background fluorescence of the solutions is generally less in the alkaline than in the acid range.

On the basis of the findings of this experimental series, we can state that a dye concentration of 1:10,000 at a pH value of about 9 produces optimum staining.

#### 6. Differentiation of Dead and Living Bacteria

A further experimental series was intended to answer the question whether it is possible, under the experimental conditions described above and nearly equivalent to the conditions existing in practice, to differentiate between dead and living bacteria. We again used staphylococci as experimental subjects and investigated both living as well as heat-killed bacteria (autoclave; 120° C; 1.2 at; 30 minutes). Fluorochromatic staining was again carried out with 15 dyes at terminal concentrations of 1: 10,000. On the basis of the experience of the experimental series immediately preceding, the solutions were prepared in each case once with distilled water and the second time with a phosphate buffer at a pH of about 9.

Color Table 5 shows the results in simplified form. It indicates that it is not possible to speak of a systematic change of fluorescent coloring of dead as compared to living staphylococci. In particular, acridine dyes are apparently not suitable for the differentiation of dead from living bacteria. Although these findings do not appear to permit differentiation between dead and living bacteria, we believe that this might be possible under different experimental conditions. However, examination of this question would be extensive and expensive.

Color Table 4

Fluorochromation in Liquid Media (staphyl. albus - live)

Concentration  
1:10,000 in  
phosphate  
buffer

pH                    4.40    5.33    5.98    6.42    6.78    7.09    7.71    8.39    9.07

Acridine yellow

Acridine orange

Auramin O

Aurophosphin O

"Brilliant dianil" green

Coriphosphine O

Coriphosphine-fuchsine

Diamond phosphine

"Entozon granulate"

Euchry sine

Morin (Merck)

Rivanol

Thioflavin S

Thioflavin T

Trypaflavin

Color Table 5

Fluorochromation in Liquid Media  
(Staphylococcus albus - live and dead)

Concentration  
1:10,000

pH 9.06  $\nabla$  pH 9.06 + Aqua dest.  $\nabla$  Aqua dest +

Acridine yellow

Acridine orange

Auramin O

Aurophosphin O

"Brilliant dianil" green

Coriphosphine O

Coriphosphine-fuchsine

Diamond phosphine

"Entozon granulate"

Euchrysine

Morin

Rivanol

Thioflavin S

Thioflavin T

Trypaflavin

## 7. Differentiation of Organic and Inorganic Particles

The differentiation of bacterial particles from other organic or inorganic particles of equivalent magnitude is of considerable importance. We therefore carried out one experimental series which was intended to determine the fluorochromatic stainability of such substances with the selected 15 fluorochromates on 10 organic and inorganic particles:

### A) Organic Particles

#### Spores:

Lycopodium  $\phi \sim 32.0 \mu$

Puffball  $\phi \sim 2.5 \mu$

#### Synthetic organic particles

Latex emulsion  $\phi \sim 1.17 \mu$

Ilmenite  $\phi < 20.00 \mu$

Emu-powder 120-FD, BASF  $\phi < 40.00 \mu$

Mowilith DM-3 Hoechst  $\phi \sim 0.80 \mu$

### B) Inorganic Particles

Micro-talcum, Norwegian talc IT extra  $\phi 5 \mu$

Clay (Putman)  $\phi \sim 0.1 - 7.0 \mu$

Soot (Carbon black)  $\phi \sim 0.47 \mu$

Fly ash  $\phi < 80.0 \mu, 10\% < 5.0 \mu$

All substances were processed with aqueous fluorochromate solutions at 1:10,000 and examined immediately.

Color Table 6 shows the results obtained in this experimental series and indicates that most of the fluorochromates will stain organic particles, e.g. spores. Other organic particles, e.g. emu-powder (a mixed styrol polymeride) and mowilith DM-3 (polyvinyl acetate) also acquire notable fluorescence.

However, in principle, it does not appear that inorganic particles can be demonstrated with fluorochromates. As an exception to this, we should note that clay particles react positively to fluorochromatic staining, especially with acridine dyes, by predominantly red fluorescence. Acridine orange also induces a positive reaction of talcum.

These fluorescent effects on inorganic particles are probably due to absorption. In the practical differentiation of bacteria from certain minerals through fluorescence-microscopic techniques, however, the type of bonding has no importance. We can definitely note that a positive effect of fluorescence is not proof of the presence of organic substances. We must consider the possibilities that certain organic particles may have a positive reaction. Further investigation will be necessary to settle this question for all pertinent matter and to eliminate the sources of error by the selection of suitable dyes, e.g. auramine O, aurophosphine, rivanol, tryptaflavin (cf. Color Table 6).

## 8. Lessening of Background Fluorescence

As already stated above, it is important, during fluorochromatic staining in liquid media, to keep background fluorescence, (i.e. inherent fluorescence of the dye in solution) as low as possible. This is especially true in the case where fluorochromatic staining is intended to improve the possibility of counting corpuscular elements in continuous-flow chambers.

Although we had already selected, on the basis of earlier experimentation, those fluorochromates which produce a relatively low background fluorescence (5.2), we felt that it would be pertinent to attempt to lower the background fluorescence of the solution also in the finally selected 15 fluorochromates.

For this were available in principle two methods, that of suppression of fluorescence and that of elution of the fluorochromates.

### 8.1. Suppression of Fluorescence

We know that there are a number of chemical substances which are able to lower the intensity of fluorescence. This is in particular true for heavy-metal ions, for bromine and iodine and also for certain organic molecules. Hemoglobin also has a fluorescence-suppressing effect. We therefore tested six fluorescence-suppressing substances in an investigative and semi-quantitative experiment. The reaction of these substances to solutions of 15 fluorochromates is embodied in Table 2.



Color Table 6

Organic and Inorganic Particles

Spores of Lycopodium	Spores of Puffball	Latex Emul- sion	limenite Emul- sion	Emu- powder	Poly- vinyl ace- tate	Talcum Clay	Putman Clay	Carbon Fly black ash
-------------------------	-----------------------	------------------------	---------------------------	----------------	--------------------------------	----------------	----------------	-------------------------

Acridine yellow

Acridine orange

Auramin O

Aurophosphin O

"Brilliant dianil" green

Coriphosphine O

Coriphosphine-fuchsine

Diamond phosphine

"Entozon granulate"

Euchrysine

Morin (Merck)

Rivanol

Thioflavin S

Thioflavin T

Trypaflavin

All suppressants were always utilized as 1% aqueous solutions. The best results were obtained with iron and iodine compounds. Both partially suppress the fluorescence of most of the fluorochromatic solutions. Iodine compounds are apparently most effective. The addition of a potassium-iodide solution to the respective fluorochromatic solution produced a lessening of background fluorescence to about one-half of its original intensity. In an experiment with the counter of the CAP company, the favorable influence of this fluorescence suppressant was very marked. Quantitative tests would probably result in even more favorable findings.

Table 2

	Fe <sup>++</sup> (FeSO <sub>4</sub> )	Cu <sup>++</sup> (CuSO <sub>4</sub> )	Mn <sup>++</sup> (MnCl <sub>2</sub> )	I <sup>-</sup> (KI)	Br <sup>-</sup> (KBr)	Hb
Acridine yellow	-	-	-	+	±	+
Acridine orange	+	-	-	+	±	
Auramin O	+	-	-	+	+	
Aurophosphin O	+	-	-	+	+	+
Coriphosphine O	+	-	-	+	-	±
Coriphosphine-fuchsine	-	-	-	+	-	
"Brilliant dianil" green	++	-	-	-	-	
Diamond phosphine	+	-	-	+	±	
"Entozon granulate"	+	-	-	+	-	
Euchrysine	+	±	-	+	±	
Morin (Merck)	+	-	-	+	-	
Rivanol	+	-	-	+	-	
Thioflavin S	++	-	-	-	-	
Thioflavin T	-	-	-	+	+	
Trypaflavin	-	-	-	-	±	

## 8.2. Elution of Fluorochromate

Experimentation for this purpose was begun by fluorochromatic staining of bacteria with the 15 selected agents in the customary manner. After exposure (to staining) for three minutes, the bacterial suspension was centrifuged (2,000 rpm for 10 minutes), the supernatant discarded and the sediment dissolved in physiological salt solution by shaking. This process was repeated three times. After each elution, the sample was examined under the fluorescence-microscope for evaluation of the intensity of particle fluorescence.

The results of these experiments can be summarized by noting that the background fluorescence is practically eliminated after a single elution whereas bacterial fluorescence is fully retained. Further elution has no advantages; on the contrary, the intensity of the fluorescence of the bacteria decreases increasingly.

We are therefore inclined to favor one single elution of the fluorochromate, provided that conditions in practice permit this. Otherwise, suppression of fluorescence (with potassium-iodide) is preferable.