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The Effect of pH on Intensities of Histological Staining of Picric Acid, Acid Fuchsin, as Ordinates and Coordinates

Irvin M. Gerson

THE EFFECT OF pH ON INTENSITIES OF HISTOLOGICAL STAINING OF PICRIC ACID, ACID FUCHSIN, AS ORDINATES AND COORDINATES.

This paper is submitted to the Faculty of Ursinus College in partial fulfillment of requirements for honors in the department of Biology.

Approved by,

May 1, 1940.

Submitted by,

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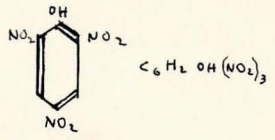
In addition to this paper twelve histological slides have been submitted representing each experiment. These are on file with the department of Biology and may be examined by permission of Committee on honors or the department head.

THE EFFECT OF pH ON INTENSITIES OF HISTOLOGICAL STAINING OF PICRIC ACID - ACID FUCHSIN, AS ORDINATES AND COORDINATES.

The main theory upon which this thesis is based, is the ability of certain ions of organic dyes to penetrate non-living fixed and paraffin imbedded tissues with varying degrees of intensity at different hydrogen ion concentrations. My problem seeks to establish the optimum pH of this stain reaction and differentiation for but one pair of dyes, but it serves to illustrate the importance of the control of hydrogen ion concentration in all similar situations.

The dyes I selected to illustrate my case were acid fuchsin and picric acid. Picric acid gives useful cytoplasmic stains both as a rough general stain and as a differential counterstain. It may be used either as an alcoholic stain, or as an aqueous one depending on the pH desired. The place introduced, i.e. hydrated or dehydrated has little effect if stained sufficiently long enough. Picric acid is soluble in water to about 1.18 per cent, while in alcohol it is about 8.96 per cent soluble. We see that roughly picric acid is seven times as soluble in alcohol as in water, so if the stain is introduced in H_2O , it must be heavily overstained in order to prevent washing out of the stain in the dehydrating alcohols. Though picric acid is a useful "whole object" stain, being very diffuse at all pH's, it is a rough one. However, it is one of the few that stain vigorously horn, chitin, muscle, and erythrocytes.

Picric acid is a synthetic organic dye, belonging to the class of nitrophenols and is itself tri-nitro phenol.



The presence of the OH group does not mean basicity, but on the other hand makes it more acidic due to the presence of the nitrous (NO_2) groups.

I have stated that I am seeking to establish an optimum staining reaction for the picric acid, the acid fuchsin, and the establishment of the best combination of these. I must however, state what I classify as optimum staining. There are two ways of looking at it. In a gross way, one might consider cytoplasm, nuclei, and inter-relationships between these. On the other hand inter-cellular substances are often sought, such as, collagen, elastin, cytosomes and mitochondria. Firstly, any solution containing acetic acid will dissolve collagen. This is the case in experiment # 1 , under acid fuchsin below. Collagen consists of exceedingly delicate fibrills which usually present a wavy appearance. They may be more or less widely seperated from each other or united in thick coarse strands. Collagen fibrils retain their characterictic staining properties when fixed in Zenker's in particular. Hence Zenker's is used below. Collagenous fibers are found in cartilage, loose connective tissue, and most epithelial tissue. Care must be taken to keep any alkali away from the tissue in as much as collagen is highly soluble in alkaline medium. Elastic fibers on the other hand are very long and run in various directions. They appear as brilliant, highly refractive threads, much thinner than the collagenous fibers. Furthermore, they are homogeneous in constrast with the collagenous fibers. The principal constituent of these fibers is elastin, which is an albuminoid substance; consequently, they are very easily stained in basic fuchsin or orsein. Since the entire process that I am carrying out is either neutral or acidic, the elastic fibers must be sacrificed. Mucin requires a special stain of hematin, but owing to the goblet cells in the villus epithelium, too much mucous is present to prevent the entire tissue from becoming black. In summary then, the values to look for are:(1) nucleur (2) cytoplasmic (3) collagenous fibers (4) mitochondria and (5) centrosomes.

Before considering the action of the acid, the method in which the tissue was fixed and its potentials must be dealt with. I chose Zenker's to fix the frog stomach because of its good penetration and non-shrinking properties. The composition of the Zenker's is:

5 % $HgCl_2$
5 % CH_3COOH GLACIAL
2½ % $K_2Cr_2O_7$
1 % Na_2SO_4
87 % H_2O
} MULLERS SOLUTION
100 % ZENKERS.

The Na_2SO_4 is a hardening agent, and the remaining ions cause a high acidity.

$$CH_3COOH \rightleftharpoons CH_3COO^- + H^+$$

$$\left.\begin{matrix} HgCl_2 \\ H_2O \end{matrix}\right\} \rightleftharpoons HgCl + HgClOH + H^+ \quad (\text{CAUSES ACIDITY})$$

$$\text{OR} \rightleftharpoons (HgCl)_2O + Cl^- + H^+$$

Obviously now, crystals of $HgCl_2$ would distort the tissue if allowed to remain, so an iodine solution is used to dissolve out the crystals. The solution employed is Lugol's, and the reaction involved is:-

FORMULA FOR LUGOL'S
5 PARTS KI
5 PARTS I
100 c.c. 95% C_2H_5OH

$$4\,KI + 2\,HgCl_2 \longrightarrow 2\,KHgI_2 + 2\,KCl$$

all of which are soluble and dissolve out.

The pH of various solution of picric acid are represented on chart #1. The dissociation of picric acid is as follows:

$$(C_6H_3O_7N_3) \rightleftharpoons C_6H_2O_7N_3^- + H^+$$

$$K = \frac{C_{C_6H_2O_7N_3} \times C_{H^+}}{C_{C_6H_3O_7N_3}} \qquad \pi = \frac{(M\alpha)(M\alpha)}{C_{C_6H_3O_7N_3}} = \alpha^2$$

GIVEN
M = 1
α = % OF IONIZATION
C = CONCENTRATION

$\alpha^2 = .16 = 4 \times 10^{-1}$ = DEGREE OF IONIZATION

1. J. Baker, Cytological Technique 1933.

Four tenths is a very diffuse dissociation, therefore at ideal conditions we should have a pH of 2.5

$$pH = \frac{1}{C_{H^+}} \simeq \frac{1}{.4} = 2.5$$ RECIPROCAL LOG OF CONCENTRATION.

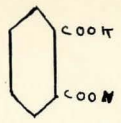
This is indeed very acidic and contains many free ions for active service.

PH READINGS PICRIC ACID			AV.	PH READINGS ACID FUCHSIN			
PICRIC CONC. AQUEOUS	1.54	1.55	1.55	AQUEOUS SOLUTION	3.31	3.49	3.49
	1.55	1.55			3.40	3.49	
	1.55	1.55			3.47	3.51	
	1.56	1.55			3.47	3.51	
					3.48	3.50	
" IN CONC ALCOHOL	2.64	2.63	2.64	ALCOHOLIC "	3.01	3.02	3.01
	2.71	2.62			3.01	3.01	
	2.64	2.64			3.02	3.01	
	2.64	2.64			3.01		
	2.63	2.64			3.01		
" PH 1.55 CONC BUFFER 3.5	2.11	2.12	2.12				
	2.12						
	2.12						
	2.12						
	2.12						
" + N HCl 50% 50%	.20	.29	.28				
	.28	.28					
	.28	.28					
	.30	.28					
		.28					
" NEUTRALIZED	7		NO VALUE				

Generally speaking, the intensities of staining of picric acid on tissue (i.e. frog stomach) is for a relatively dilute solution; muscle→is greater than villi (epithelium)→is greater than submucosa (connective tissue). However, in my experiments many peculiarities and alterations were noted.

Ex. - 1. When I took a picric acid solution of pH 7 and buffered it to 3.5 with a potassium acid phthalate solution, I brought about a different relationship. This new relationship was: villus (epithelium)→is greater than connective tissue→is greater than muscle. Here we see that the status of the submucosa remains rather constant, while the muscle and the epithelium change positions. See slide # I for the first reaction and slide # II for the buffered reaction.

FORMULA: FOR K-H-PHTHALLATE

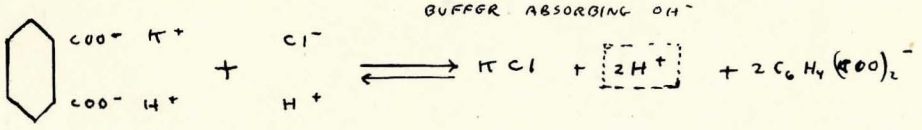


$C_6H_4C_2O_4KH$

COMPOSITION OF BUFFER SOL.

5 CC. .2 M $C_6H_4COOK\ COOH$.
7 C.C. .2 M HCl
188 C.C. NEUT H_2O

DISSOCIATION ACTION:



Ex. - 2. Concentrated picric acid has a pH of 1.55 . When the fixed tissue is stained in a solution of this type, we receive the general reaction (which is opposite to the buffered reaction) of, muscle is greater than submucosa is greater than epithelium.

Ex. - 3. Then again, it may be desireable to introduce the picric acid in an alcoholic solution. An alcoholic solution of 50 per cent concentrated aqueous picric acid,and 50 per cent of 95 per cent C_2H_5OH (neutral), give a pH of 2.64. While advantage is gained in keeping the tissue dehydrated, staining penetration is not so good because of the greater solubility of picric acid in alcohol. Reaction- Epithelium villi = muscle > submucosa (very poor). See slide # III.

Ex. - 4. Very acid picric acid should have a greater intensity of staining theoretically, therefore I conducted several experiments along those lines. I found that at a pH of .55, the crystals of picric acid are precipitated out, but, the intensity of the stain continues to increase even till .28. After that, the tissue is merely turned brown by the strong acid, and the concentration of the picric acid is so weak that it is useless. At this point the degree of staining is epithelium villi = musculature > submucosa, all of which are very vivid. I therefore assume pH .28 to be the optimum with picric acid. My main reasons for this conclusion are the reasons that when treating with acid fuchsin later, the submucosa becomes pink, and only two layers which are penetrated by the picric acid

will be the musculature and the villus epithelium, both being very vivid here. See slide # IV. I might further mention here, that a slight differentiation may be brought about between nucleus and cytoplasm at .28 pH because of a certain blackening of the nucleic substance. However, the chromosomic substances are injured and this differentiation is of use only as a rough estimate. Hematoxylin should be used. See slide # IV.

The actual diffusion through the cell walls depends on the phenomenon of permeability. The contributing factors are many, but in my investigation I have taken but one point, pH, therefore the only phase I am concerned with of the membrane is that of its own potential. The following outline represents the factors governing permeability and penetration of stains:

I. The Membrane.

a) porosity of cell wall.

b) thickness.

c) charge upon (+ or -) potential (Donnan).

d) composition of the membrane.

1) arrangement of the particles.

2) solubility of the penetrating substance in the membrane.

3) age of the membrane (includes also condition, that is, fixed or vitro.),

II. The penetrating stain.

a) monovalent ions penetrate more than divalent.

b) charge on particle.

1) if an ion, must be opposite to charge on membrane.

2) if a molecule, the stricter the neutrality, the more rapid the penetration.

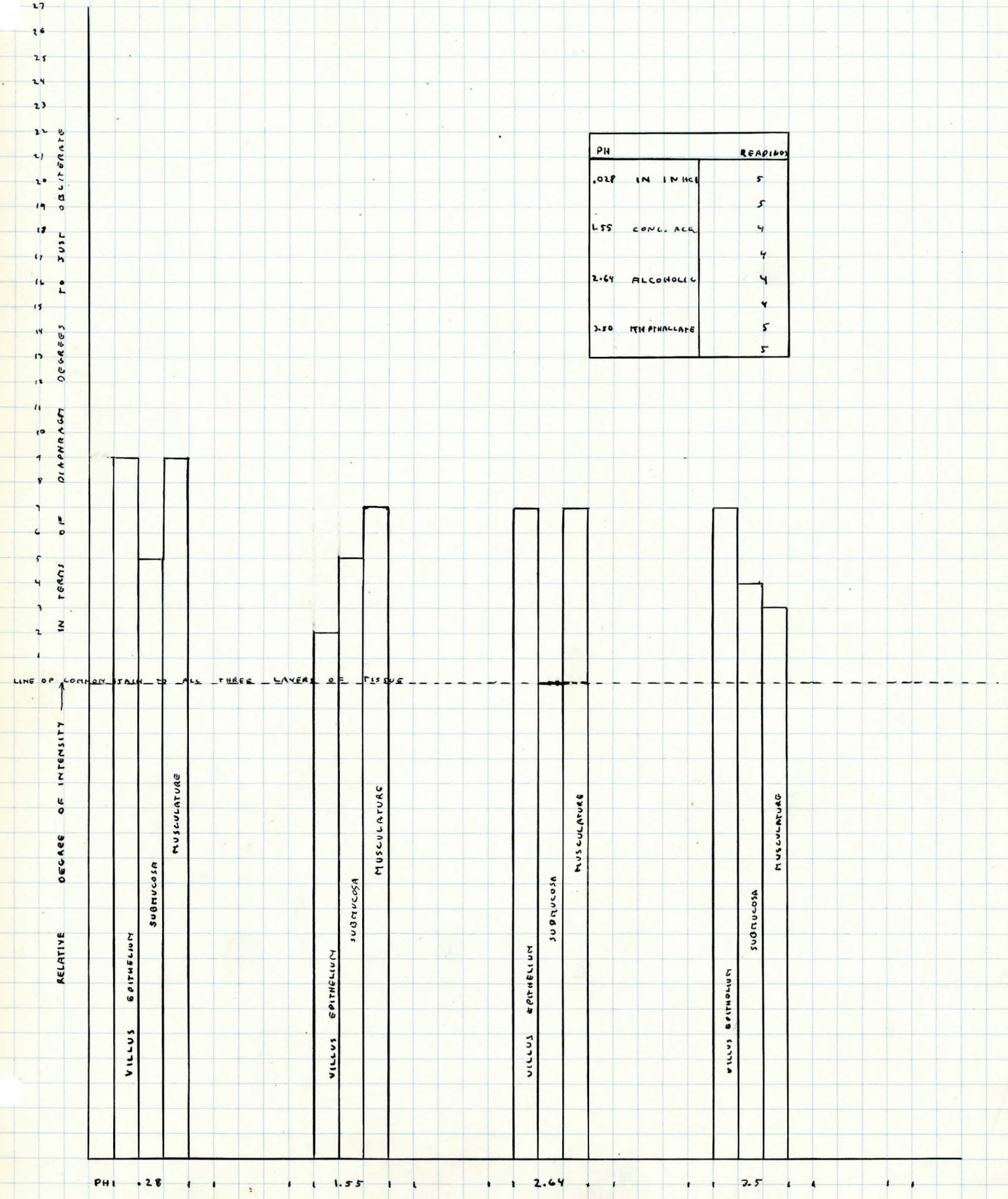
c) size and weight of particle.

d) hydration of ions.

CHART # 1

RELATIONSHIP BETWEEN DEGREE OF STAIN AND PH FOR PICRIC ACID.

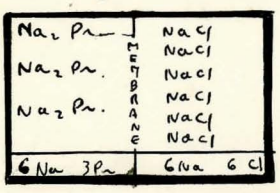
PH		READINGS
.028	IN 1 N HCl	5
		5
1.55	CONC. ACID	4
		4
2.64	ALCOHOLIC	4
		4
2.50	METHYL ORANGE	5
		5



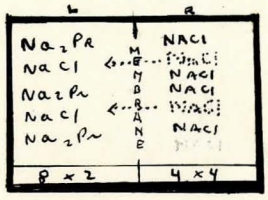
III. Colloid swelling

IV. Sodium and Potassium concentration in fluid. (Na renders membranes more permeable, while K renders membrane less permeable.)

V. Donnan equilibrium. - Potential



ONLY THE Na & Cl IS IN EQUILIBRIUM
6 x 1 = 06
6 x 6 = 36
THE PROTEIN "PR" DOES NOT PENETRATE THE MEMBRANE.



L R
Na Cl = Na Cl
8 x 2 = 4 x 4
16 = 16
AN EQUILIBRIUM IS EST.

VI. pH.

Since the influence of the pH is my investigation, I will recount the membrane potential from data based on the bibliography.

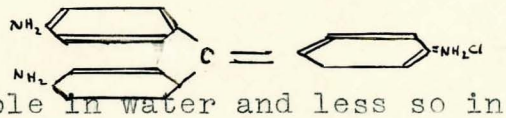
When two aqueous solutions are seperated by a membrane which is impermeable for one ionic variety, namely the cell cytoplasm, a potential arises between the two solutions and also according to osmotic laws an unequal distribution of ions follows. According to W. Ostwald, impermeability to any ionic form gives rise to a membrane charge.

Unfortunately, with acid fuchsin I did not find this variation to be proportional to the pH, but found it to vary directly with the concentration. Optimum penetration was obtained in a 45 per cent acid fuchsin, 50 per cent H_2O to which 5 per cent N/10 HCl had been added. The pH here was found to be 3.49 optimum. As non-corollatory as this is, it aids no end in simplifying the counterstaining problem by having but one variable; the acid fuchsin remaining constant. The pH of concentrated acid fuchsin is 3.5, and since I wanted to thin the solution without changing the pH, I found the above combination of water, acid and dye to give this pH.

Ex. - 1'. In the solution of pH 3.49, muscle stains is greater than villus epithelium is greater than villus connective tissue is greater than submucosa. This is not always readily noticeable, but

if one introduces the tissue into the fuchsin immediately after having been in a solution of 60 c.c. absolute C_2H_5OH, 30 c.c. chloroform, 10 c.c. glacial acetic acid, this can be easily seen. V.

Composition: Fuchsin or magenta is a triphenylmethane dye prepared by oxidizing a mixture of aniline and isomeric toluenes. The chief constituent prepared this way is parasaniline which must be sulfanated:



This dye is highly soluble in water and less so in alcohol. This is in exact reverse to picric acid, but like picric acid, acid fuchsin is very sensative to alkalies, so that overstains can easily be removed in tap water of normal pH 7.2 to 7.4. Acid fuchsin is one of the most widely used cytoplasmic dyes and can be used in many combinations. One of the most frequently employed uses is the Van Gieson counter staining with picric acid as will later be utilized. 1.

Ex. - 2' If the tissue is treated in an alcoholic solution of 60 per cent C_2H_5OH (95%), and 30 per cent glacial acetic acid, the tissue stains, muscle = epithelium > submucosa. This is useful only in single staining since both of these are removed by picric acid in the counter. See slide # VI.

Ex. - 3' In the case that the dehydrating alcohols have been neutral, the tissue is evenly stained with a more intense red than any of the previous tests. I selected this tissue in my Van Gieson counterstain because of its submucosal vividness. If care is taken, even mitotic figures of the cytoplasm can be defined.

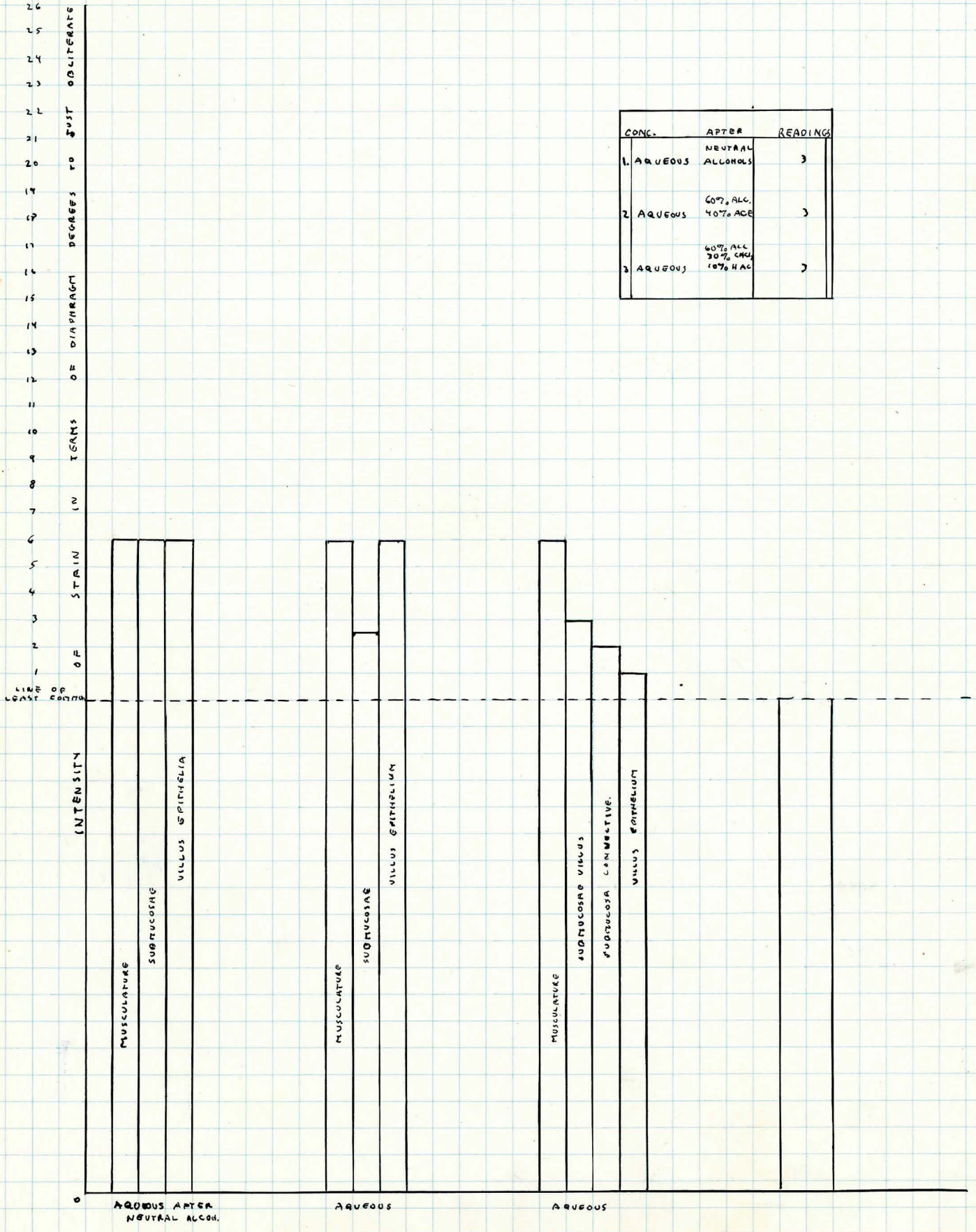
Having thus worked out efficiently the effect of pH on the individual components of Van Gieson's counterstain, I will now proceed with the system itself.

The prescribed formula by Van Gieson is a saturated solution of picric acid to which has been added enough saturated aqueous

CHART NO 2

RELATIONSHIP BETWEEN DEGREE OF STAIN AND CONCENTRATION FOR ACID FUCHSIN

	CONC.	AFTER	READINGS
1.	AQUEOUS	NEUTRAL ALCOHOLS	)
2	AQUEOUS	60% ALC. 40% ACE	)
3	AQUEOUS	60% ALC 30% $CaCl_2$ 10% HAC	)



acid fuchsin to render the solution garnet red. Another formula[2] is to add five parts of 1% solution of acid fuchsin to 100 parts of picric acid solution. On the face of these systems one can see the flimsy limits for error. For one thing everybody's conception of "garnet red" may be different. Secondly the concentrations are neglected in the later case. Furthermore the acidity basicity or neutrality of the dehydrating alcohols are grossly neglected. No mention is made of the effects of applying these stains seperately. It is these weaknesses that I have relied upon to strengthen my own arguments. Going back to my original experiments on the individual components (acid fuchsin and picric acid) , it would appear that a combination of the most successful of each stain would be the most advantageous. That would be, picric acid of pH .28, and acid fuchsin of pH 3.49 in aqueous solution after neutral alcohols. A fact not to be neglected is the striking proximity of the pH of the picric buffered solution at pH 3.50 and that of concentrated fuchsin acid. Let us see how closely my theory can be applied.

Ex. - 1". With a solution brought to a pH of .28 using N HCl with picric acid, and adding this with acid fuchsin of pH 3.49 in a proportion 50-50 by volume, a resulting pH of 1.55 is gotten. This stains muscle and villus epithelium with especial vigor. The submucosa, both superficial and deep stains comparatively vividly, being a pink to a red, depending whether it is subvillus submucosa or deep interstitial connective tissue. We now have a cross section

1. Van Gieson - New York Med. Journal 1889 p. 57.
2. American Microscopic Society XIX 1898 p. 105.

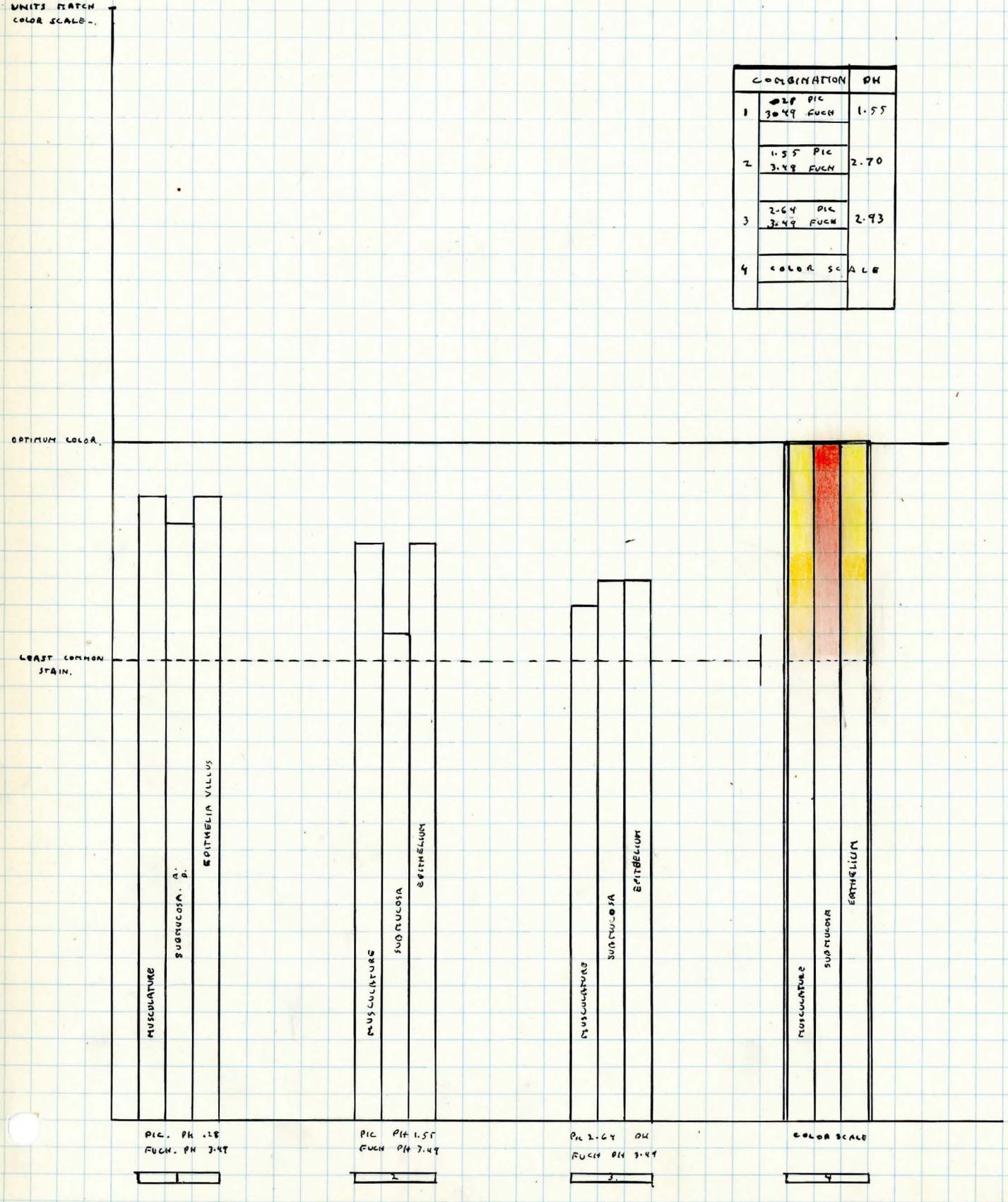
which exhibits an outer ring of yellow (muscle), a middle ring of pink (connective tissue), and an inner ring of yellow (epithelial lining). The nuclei are darkened in places, but for best differentiation Delafield's hematoxylin is added after being mordanted in 2 % ferric alum. See chart # 3 and slide # VI.

Ex.-2." When the combination fluid is a solution of picric acid of pH 1.55 (concentrated aqueous solution) and the standard acid fuchsin, the same general reaction and relation as above take place, but all are less intense and the difference between the epithelium and submucosa is more marked. Time of staining will not be found to be a factor. Both may be overstained. See slide # VII

Note 1. If care is not exercised in the regulation of acidity, very often the yellow picric staining cytoplasm will exhibit a brownish coloration which is permanent and the tissue irretrievable. This coloration comes primarily in the epithelial layer of .28 picric staining. Mitochondria are destroyed in this reaction.

Ex. - 3." In alcohol, the pH of picric concentrated acid changes from 1.55 to 2.64. Consequently the new pH is different from the combination fluid. This new hydrogen ion concentration is 2.93, a little less than half way between the pH of the acid fuchsin and picric acid. One may wonder why it is not exactly half way, especially since it has been mixed in 50-50 by volume/ molarity solution. The difference is caused by the variabilities in solubilities with alcohol as previously discussed. However, to get back to the staining reaction, it is found that the villus epithelium does not stain very heavily. Why the contrast exists between the picric staining staining epithelium, I cannot really justify; but I might suggest that perhaps the muscle sarcostyles may be little more affinitive

COMBINED GRAPH OF ACID FUCHSIN PICRIC ACID COUNTERSTAIN SHOWING — VARYATION WITH THE PH —



	COMBINATION	PH
1	.28 PIC 3.49 FUCH	1.55
2	1.55 PIC 3.49 FUCH	2.70
3	2.64 PIC 3.49 FUCH	2.93
4	COLOR SCALE	

to picric acid, and although this may be uniform throughout all types of pH combinations, it may be because the alcohol is more noticeable at this point. If I had time, I would investigate further, for I believe this is a salient fact frequently overlooked. (The effect of an organic solvent such as C_2H_5OH.) On the whole however, the staining of tissue at this pH is not very intense. Relatively speaking, the submucosa and epithelium are equally stained, but the difference in color is marked enough to distinguish. I do not recommend this solution for staining. See slides # VII, also chart # 3.

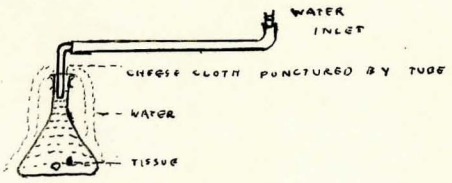
Ex. - 4." Buffered picric acid at pH of 3.5 plus the acid fuchsin at pH of 3.49 gives a fairly standardized combination pH of 3.49. Of course the submucosa will be satisfactory at this pH , but the musculature is very poor. Slide # IX shows this very well. The relationship is epithelia is greater than submucosa is greater than muscular.

DIRECTIONS OF GENERAL PROCEDURE.

In order to assure good results, certain precautions must be observed. I will proceed in order.

Firstly to insure against distortion or desiccation of tissue, immediately after the frog has been pithed, inflate the stomach with Ringer's frog solution (isotonic solution of saline and essential anuran salts)by inserting a pipette into the esophagus and syringing with pressure. Now inject the coelom with Ringer's immediately upon incision of the body wall. You now have the stomach bathed from within and without by an isotonic medium. Remove the section of the stomach desired, and plunge immediately into Zenker's fixative as described above. Allow to remain in the fixative from eight to fifteen hours, with frequent agitations, and at least four

changes. At no time allow the tissue to dry. The tissue must be washed thoroughly in H_2O before carrying on the process. The following piece of apparatus is most satisfactory.

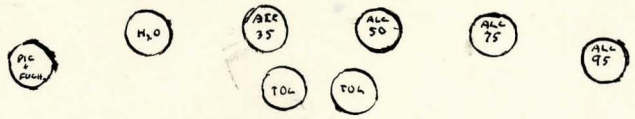


After twenty-four hours of this washing, proceed to dehydrate in neutral alcohol. A preliminary clearing in toluol serves to test dehydration. Proceed now in terpinol mixtures as prescribed by Dr. Brownback.

After imbedding in paraffin, sectioning with microtome will be found most favorable at 12 mu. If possible, a cool day with considerable moisture should be chosen, because warm dry days cause an excess of charges to be thrown upon the knife blade and difficulty in sectioning is encountered as well as in floatation on the glass slides, whereas, on a moist day, the charges are immediatley taken up by the vapor. Further instructions on this can be had by procuring a set of laboratory directions from Biology 15-16. The usual dehydration methods are used with percentages of alcohol ranging 10 %, 35 %, 50 %, 60% , 70-75 %, 95 %, (or absolute if possible.) The pH of all dilutions of alcohol must be taken to assure neutrality. Every day before using the solutions, a sample of some of the alcohols should be taken to check the acidity. Not all of the alcohols need be tested, but the 50 %, 75 %, and 95 % are vital. You will find in your preliminary tests that the distilled water of the laboratory is about 5.7 in pH. In order to cahnge this, you will find that simple distillation only relieves part of this situation (the CI^-). There are organic radicals that also must be removed. When $KMnO_4$ is added, it oxidizes such impurities as may be present and neutral water ensues.

All stains are introduced in aqueous solution, but because of the destaining effect of the alcohols on picric acid, the picrate

used in the combination fluid is used by itself first because of the need for picric overstaining. No alternation in pH is encountered this way.



In final clearing before mounting, two toluols are employed. One to test for possible hydration, the other to definitely clear the tissue. The time of staining is about three minutes in the straight picric, and five minutes in the combination fluid.

PREPARATION OF STANDARD SOLUTIONS.

In order to keep the staining regular, all solutions must be standardized and put in sealed test tubes. These are standardized by titration and then the pH is taken at least ten times in seperate clean containers. The total is divided by ten or the number of tests applied for a mean pH.

Alcohol:- In order to get an absolute alcohol solution from the 95 % given in the laboratory, one needs only to shake some 95 % C_2H_5OH with CaO and allow to settle. Then the supernatent alcohol is withdrawn and together with fresh lime ($CaCO_3$) is carefully distilled. Another method used by McClung * is to add anhydrous copper sulfate to 95 % alcohol. This maintains the dehydrated state of the alcohol, but at the same time introduces a little copper sulfate to the solution.

Paraffin:- Paraffin of various melting points may be used depending on the tissue. The ones used in the laboratory are approximately M.P. 38° C. Strangely enough the older the paraffin, the better the penetration, providing no foreign materials have been introduced.

This concludes my actual experimentation, so I will here summarize my conclusions.

* McClung, Microscopical Technique. 1929. p. 474.

1.pH is definitely a determining factor in histological staining of fixed tissues.

2. This influence extends to intensities of color and differentiation.(To a reasonable degree however, pH cannot be so changed as to obliterate the color except if it be changed from its acidity to basicity or visa versa.)

3. The Van Gieson counterstaining method employing picric-fuchsin mixture is greatly enhanced when the hydrogen ion concentrations are .28 for picric, and 3.49 for the acid fuchsin. This gives a new pH of 1.55 when mixed in 50-50 by volume proportion.

LIST OF APPARATUS

1. Beckman pH determiner (dry cell compensator)
2. Frog stomach (Rana pipiens m.)
3. Processing material
   a) Ethyl alcohol dilutions (see page 15 for preparation)
   b) Neutral distilled H_2O
   c) Pure paraffin
   d) Toluol
   e) Terpinol
   f) Chloroform C.P. Merk
   g) Glacial acetic acid Baker
4. Staining materials
   a) Picric acid C.P. concentrated.
   b) Acid fuchsin A.P.
5. Buffer - K H Phthallate
6. A.H.T. Co. Incubator
7. No. 1 thin cover glasses
8. Mayer's albumin fixative
9. Solutions standardized and titrated with $AgNO_3$ and NaOH.

EXPERIMENTAL TIME

Laboratory analysis:- February 15 - April 20, 1940, four hours per day, five days per week.

Research time:- Approximately three hundred hours.

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