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# Fluorescein As A Reagent In A Spectrophotometric Determination Of Micro-Amounts Of Iodine: Application To Glucose Determination

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# FLUORESCEIN AS A REAGENT IN A SPECTROPHOTOMETRIC DETERMINATION OF MICRO-AMOUNTS OF IODINE: APPLICATION TO GLUCOSE DETERMINATION

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# I. INTRODUCTION

The increasing awareness of the close inter-relationships between biological activity and chemical reactions has resulted in a rapidly growing pursuit of biochemical knowledge. Research in the related fields of medicine, virology, pharmacy, and bio-physics is expanding at a rapid rate. Problems related to food production for an exploding population, space explorations, world health issues, the utilization of the resources of the oceans, and the proper employment of energy sources have spurred man to delve deeper into the secrets of creation. The mysteries of life itself are slowly being revealed through findings in genetic studies.

With this ever expanding biochemical and related research comes the concomitant requirement for new and better analytical techniques. Already quantitative determinations are on the nanogram level. Many procedures are semi- or fully automated.

In this framework of recent advances, the desire to develop an analytical procedure useful to mankind was kindled. In particular, the importance of iodine in certain essential biological systems and biochemical mechanisms led to the problem of this research. The purpose of this study was to determine the possibilities of utilizing fluorescein as an "indicator" in the quantitative determination of

sub-micro amounts of iodine by an existing colorimetric technique. Furthermore, the application of this procedure to the determination of micro-amounts of simple carbohydrates, as glucose, was considered a very real possibility.

The execution of the above stated goals centered around the three chemical reactions: the iodination of fluorescein, production of iodine by the action of iodide with periodate, and the periodate oxidation of glucose. The remainder of this thesis, then, will deal with historical and theoretical aspects taken from the literature; the experimental findings; a discussion of the findings and theory; and conclusions in relation to the above stated chemical reactions and their adaptation to an analytical procedure.

## II. LITERATURE RESEARCH

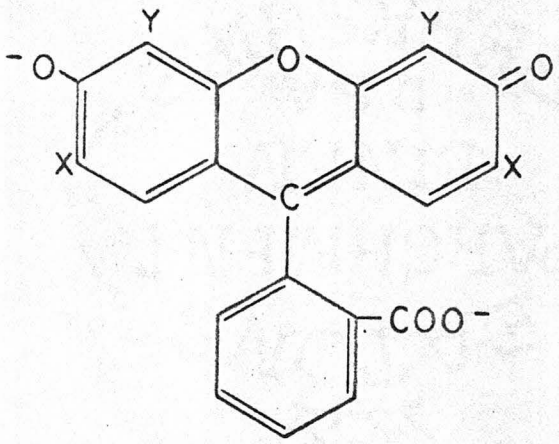
### Fluorescein

Fluorescein, along with phenolphthalein, is probably the most studied of the phthalein dyes. Fluorescein is prepared by reacting phthalic anhydride with resorcinol. Its properties depend on the molecular forms it can assume. The lactone form is colorless but is not common. Most researchers find the quinoid form more probable because of its orange-red color (1,2). Also, absorption spectral analysis shows that fluorescein, in solution is in the quinoid form (2).

In the quinoid form, fluorescein reacts with one mole of base to form a red salt with absorption spectra similar to fluorescein in neutral solution. With two moles of base, a deep orange compound, uranin, is produced (3).

The absorption peaks of fluorescein are reported to lie at 278, 437, 455-457, 485, and 490-491 mu. depending on concentration and pH (4,5,6). Glowacki states that the absorption maxima for an aqueous solution ($4 \times 10^{-6}$ g./cc.) are 4835 and 4560 A. (7).

More detailed studies of the various forms present in solution have shown evidences for a univalent and bivalent anion in addition to the neutral molecular form (4,5,8). The quinoid, dianion form is shown in Figure 1. The pH's at which the various forms are reported to exist are dianion,




| Compound | X | Y |
|---|---|---|
| Fluorescein | H | H |
| Erythrosin | I | I |
| Diiodo-fluoresein | H | I |

FIGURE 1

FLUORESCEIN, ERYTHROSIN, AND DIIODO-FLUORESCEIN STRUCTURE

10.3; monoanion, 5.4; and neutral, 3.2 (9). The pK's are found to be at 6.7, 4.4, and 2.2.

In organic solvents, dyes related to fluorescein obey Beer's law as though they were molecularily dispersed. However, Beer's law is not followed in aqueous solutions, evidently because of molecular aggregation and solute molecular interaction (10). This anomaly in water is not too great, probably because of the unlikelihood of the attached phenyl groups (with COOH) being in the same resonance plane of the main resonance system. Solutions of the sodium salt of erythrosin (tetra-iodo-fluorescein) do follow Beer's law if the pH is sufficiently high (11). It is also reported that the maximum absorbances in the visible decrease as the polarity of the solvent decreases (4).

Halogenation of Fluorescein

Quite a number of colored halogenated fluoresceins are known. Some of the halides are colorless and it is assumed that for these the lactone form is favored (12). Pratt and Coleman report that tetra-iodo-fluorescein (iodines on phenyl ring) exists only in the lactone form (13).

Halogenation of fluorescein has an effect upon its properties. As more halogen is substituted into the fluorescein molecule, the visible and ultraviolet absorption maxima shift to longer wavelengths (6,12). For instance,

the absorbance peak for di-iodo-fluorescein is at 510 mu. (9) and those of erythrosin are reported to be at 263, 351, 356, 524 to 528, and 548 mu. depending on the pH and concentration (6,14). (See Figure 1, page 4, for structures.) Substitution of negative groups, as halides, also usually decreases the fluorescence (15). In the case of iodination, the greenish-yellow fluorescent coloration progressively decreases and a pink color predominates. This decrease in fluorescence is the basis for an analytical procedure in the micro-determination of iodine (15).

Iodination of the non-phenyl portion of fluorescein proceeds rather easily. The di- and tetra- iodo-fluoresceins are most common. Erythrosin (2,4,5,7 tetra-iodo-fluorescein) is used extensively as a red food-coloring agent and photographic sensitizer. It is prepared by treating fluorescein with iodine in an alkaline solution. In an acid medium, there seems to be no reaction. In a neutral solution, a orange-red product results (15). (The composition of this product is discussed in Section V, page 55.)

Halogenated fluorescein dyes are reported to be photochemically converted in the presence of mild reducing agents (9). The two stable products resulting are the dehalogenated form and a colorless, reduced form (leuco). Conversion to the colorless form occurs more readily than

dehalogenation since the latter does not occur at low pH's. The heavier the halogen atom, the easier it is to remove. Also, it is reported that dehalogenation of eosin (tetra-bromo-fluorescein) does not proceed in pure water or ethanol. Similar studies on other halogenated forms evidently have not been reported.

Salt effects upon the iodination of phenolic rings are said to be secondary (16).

The halogenation of fluorescein, yielding colored products, is utilized in the quantitative determination of halogens. In 1912, Labat described a colorimetric procedure for the determination of bromine in the formation of eosin (17). Already mentioned is the use of the decrease of fluorescence upon iodination by Harlay for iodine determination (15). Associated with this fluorometric application, Harlay describes a colorimetric procedure for the determination of micro-amounts of iodine.

Harlay found that the formation of the colored iodinated fluorescein was proportionate to the quantity of iodine present in the reaction medium. The pH was found to play an essential role in the formation of the product. The reaction mixture was acidified slightly with acetic and phosphoric acids, then buffered with di-sodium hydrogen phosphate and borax to keep the pH between 6.5 and 7.5. Using visual colorimetry, quantities of iodine ranging from

30 to 150 ug./ml. were determined. In comparison, the fluorometric method allowed one to estimate values from 5 to 25 ug./ml. (See page 22 for abbreviation explanations.)

In an attempt to determine the exact nature of the iodinated fluorescein product, Harlay titrated the excess, unreacted iodine with sodium thiosulfate and calculated that 3.85 atoms of iodine reacted per molecule of fluorescein. The solutions employed were 0.00125 M fluorescein (purified), 0.1 N iodine, and 0.1 N sodium thiosulfate in a reaction mixture of about 60 ml. He, then, concluded that a di-iodide of fluorescein is being produced. The other two iodines are converted to iodide ions. A further indication that the tetra-iodide derivative was not formed is that the color is orange-red, not red. Also, fluorometric studies showed that a tetra-iodide derivative could only be formed in a definitely alkaline solution.

Iodide-Periodate Reaction

The reactions between the halide ion and the anions of the halogen oxyacids have been thoroughly studied. The determination and separation of the various species in a solution has been a challenge. The ease of reaction depends largely on the oxidation potentials and pH.

With respect to solutions of iodide, iodate, and periodate ions, workers have suceeded in determining one in the

presence of the others. The use of periodate in the structural study and analytical determination of polyhydroxy organic compounds has increased knowledge of iodine chemstry. The most important reactions involved in periodate studies in acid or neutral solutions are as follows:

(A) $IO_4^- + 7\ I^- + 8\ H^+ = 4\ I_2 + 4\ H_2O$

(B) $IO_3^- + 5\ I^- + 6\ H^+ = 3\ I_2 + 3\ H_2O$

(C) $IO_4^- + 2\ I^- + 2\ H^+ = I_2 + H_2O + IO_3^-$

(D) $O_2 + 4\ I^- + 4\ H^+ = 2\ I_2 + 2\ H_2O$

(It is understood that where an excess of iodide ion is present in aqueous solutions, the iodine will be present as $I_3^-$.)

Reactions A and B are rapid in acidic solution. Reaction B does not proceed in bicarbonate or boric acid - bicarbonate solutions (18,19). On the other hand, reaction C proceeds in a borax-borate buffer (18), bicarbonate solution (19), acetic acid-sodium acetate solution (20,21,22), or phosphate buffer (22). Thus, it is possible to determine periodate and iodate ions in each other's presence by a proper adjustment of the pH. Further, reaction C is very slow at high pH's (18,19). Reaction D proceeds in acid solutions but not in neutral solutions (18).

As expected, the reaction rates are increased with increasing iodide ion concentration (21). Also, the reaction time is decreased at higher temperatures for the

iodide-periodate reaction in unbuffered solutions (23). However, the yield of iodine is found to decrease at higher temperatures (19). Most likely, this is due to the vaporization of iodine from solution. This can be controlled by the addition of an appropriate reducing agent.

For the determination of iodine liberated in the periodate oxidation of carbohydrates, reaction A is more favorable than C from the standpoint of the amount of iodine produced per mole of periodate. Disadvantages of reaction A include the possibilities of error due to reactions related to reaction D and to anomalies in carbohydrate oxidations in acidic solutions (24,25,26).

Periodate Oxidation of Glucose

The periodate ion is a very useful and popular reagent in the study of polyhydroxy organic compounds. Malaprade, a pioneer in this field, published an article on the oxidation of several polyalcohols by periodic acid in 1928 (26). In 1932, Fleury and Lange showed that the periodate reaction was specific for alpha-glycols (27). Malaprade published a more general relationship in 1934 as follows:

$$CH_2OH(CHOH)_nCH_2OH + (n+1)\ HIO_4 =$$
$$(n+1)\ HIO_3 + 2\ HCHO + n\ HCOOH + H_2O\ ,$$

and concludes that the reaction is very simple and quantitatively predictable (28).

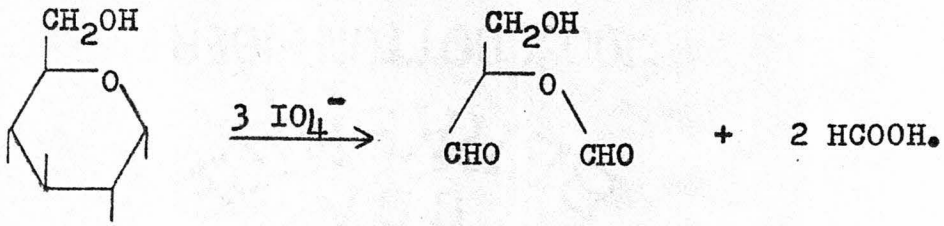
Khouvine and Arragon showed that the reaction is general for sugars (29).

The overall reaction for the periodate oxidation of glucose is:

$$C_6H_{12}O_6 + 5\ IO_4^- = 5\ IO_3^- + 5\ HCOOH + HCHO.$$

The mechanism of this reaction has received considerable study. The effect of pH and temperature on the rate of reaction is very marked. Reports of the time necessary for the theoretical consumption of five moles of periodate per mole of glucose vary from more than one week (30,31), to several days (24), to one day (28), to 12 to 15 hours (32), and, to one hour (28,33,34). Generally, an acid solution reacts faster than a neutral solution and increased temperature reduces the time of reaction. A non-Malaprade oxidation of glucose above pH of 7 is reported as being complete in 100 minutes (35).

The rate of reaction may be studied by the uptake of periodate or by the production of formaldehyde and formic acid. Both methods indicate that the reaction occurs with at least two steps. Hough and associates report that three moles of periodate react rapidly and the last two more slowly (25). They have treated glucose with three moles periodate and obtained two moles formic acid and alpha-o-formyl-D-glyceraldehyde. The following intermediary step is proposed:




The work of Head substantiates this proposal (36). Warsi and Whelan, from their findings for aldohexoses at 0° C., refer to a four-stage process. The first two moles of periodate react in 5 to 10 minutes, the third in 30 to 70 minutes, the fourth in over 7 hours, and the fifth after a week (30). They conclude that glucose is oxidized by stepwise oxidation of hemiacetal groups down the molecule. The rates of the third and fourth steps are lowered because of the accumulation of formyl esters of glyceraldehyde and glycolic aldehydes which lack alpha-glycol groups. Also, evidence showed that in the intermediary stages, more periodate was taken up than free acid formed by as much as 1.2 moles, although the overall reaction conformed to the theoretical consumption of periodate and production of formic acid (24,30).

For the periodate oxidation of carbohydrates, most workers report that best results are obtained in acidic solutions. Evidently, this is not necessarily true for glucose and fructose (32). Anomalies are reported for oxidations in alkaline or strong acid solutions (25). On the other hand, the intermediate products of glucose oxidation

are said to be more stable in alkaline than acidic solutions (24). The rate of oxidation of aldohexoses at a more neutral pH is said to be faster because the linear molecule is supposedly present (37). However, differences of opinion occur on whether the pyranose ring (25) or linear form (29,37) is actually present in glucose oxidations.

Preferences concerning a buffer system, when used, also vary. Lindstedt reports that oxidations are faster in phosphate than acetate buffers at the same pH but that overoxidation occurs in neutral phosphate buffers (32). This is substantiated by Bell, Palmer, and Johns (23). On the other hand, Fleury, Courtois, and Bieder report that results in acidic buffers including phosphate buffers are the same (37).

The use of heat to hasten the oxidation of galactose was described by Khouvine and Arragon as "too brutal" since overoxidation occurred (29). Head reports that with an excess of periodate and in light, the oxidation occurs more rapidly and in excess (36). A solution of sodium periodate (0.03M) decomposed 10 per cent in light in 15 days but was stable in the dark.

Periodate oxidations of other simple sugars are quite similar to that of glucose. With galactose, an acidic solution is not necessary (22). The fructose oxidation is more complex in that four moles of periodate are consumed and 1.25 moles formaldehyde produced (38). But when three moles

of periodate are reacted with fructose, one mole of formaldehyde and 1.5 moles formic acid are produced (25). Reports of quantitative reactions for many other polyhydroxy compounds are reported in the literature.

The quantitative determination of the iodine produced is generally determined by the thiosulfate, Fleury and Lange, and ceric-arsenite titration methods (24). The Fleury and Lange method consists of adding arsenite and iodide solutions to a bicarbonate solution of the sample and titrating the excess arsenite with iodine after 15 minutes. In a modified Fleury-Lange method, the back titration is done immediately. Hughes and Newell report that the thiosulfate method measures the transformation of glucose into its products while the Fleury-Lange method is a close approximation to the rate of reduction of periodate. The modified Fleury-Lange method gives results intermediate to the other two (24). More recently, the iodide catalyzed ceric-arsenite reaction time method is used because of its adaptability to automatic procedures. The refinement of the above procedures has resulted in common sub-microgram determinations of sugars.

## III. EXPERIMENTAL PROCEDURES

### Solutions

Most of the stock solutions were prepared with deionized water. Unless otherwise stated, reagents are of standard analytical grades.

1. Iodine
   a. $10^{-4}$ M (25.2 ug./ml.); prepared with a few ml. ethanol or sublimed. (See below for sublimation method.)
   b. $10^{-1}$ M (25.2 mg./ml.); prepared with 125 mg./ml. KI.
2. Uranin (di-sodium salt of fluorescein)
   0.00133 M (500 ug./ml.); obtained from Eastman Organic Chemicals.
3. Potassium iodide
   0.0033 M (5 mg./ml.)
4. Potassium periodate
   a. 0.00123 M (282 ug./ml.); used for most iodide-periodate studies.
   b. 0.00652 M (1.5 mg./ml.); used for most studies of periodate oxidation of glucose.
5. Glucose
   0.00694 M (1.25 mg./ml.)
6. Erythrosin B (di-sodium salt of erythrosin)

0.0014 M (1.25 mg./ml.); obtained from Allied Chemical Corporation.

7. Buffers

a. Boric acid - borate buffer

6.66 g. $H_3BO_4$ and 0.32 g. $Na_2B_4O_7 \cdot 10\ H_2O$ dissolved in 200 ml. water.

b. Phosphate-borate buffer

Prepared from 0.1 M $H_3PO_4$, 5 g. $Na_2B_4O_7 \cdot 10\ H_2O$ dissolved in 95 ml. water, and 7.5 g. $Na_2HPO_4 \cdot 7\ H_2O$ in 92.5 ml. water.

All glassware was cleaned with detergent and rinsed thoroughly with deionized water. All stock solutions except KI and the buffers were kept in glass-stoppered containers. The solutions were stable for one month, except possibly for bacterial contamination of glucose. The iodine, fluorescein, and periodate solutions were stored in the dark. Because of the undesirability of using ethanol for aiding the dissolution of iodine, a sublimation procedure was used for the $10^{-1}$ M solution. Solid iodine was heated very gently in a glass stoppered volumetric flask until it had sublimed. It was then chilled rapidly in a refrigerator to condense the iodine on the walls of the flask. Water was added and solution occurred overnight.

Laboratory Procedures

Iodination of fluorescein. Generally, this reaction

was studied by adding iodine solution to a buffered fluorescein solution and adding water to make a total solution of 10 or 25 ml. in a stoppered test tube. The reaction was allowed to proceed at constant temperature for a period of time, after which absorption readings were made.

Appropriate dilutions of the iodine solutions were made so that the iodine concentrations were in the range of 0.625 to 10.0 ug./ml. of reaction mixture. The fluorescein concentration was 5 to 10 ug./ml. of reaction mixture. Appropriate amounts of either buffer were used to keep the pH at the desired level. A blank is prepared identically except that iodine was omitted. Most determinations were made at room temperatures and allowed to react at least 5 to 10 minutes before reading their absorptions. A thermostated water bath was used for studies at higher temperatures.

Iodide-periodate reaction. This reaction was studied using periodate concentrations of 1 to 10 ug./ml. reaction mixture with an excess of iodide in buffered solutions in stoppered tubes. The 50 ug./ml. excess iodide that was added was increased by a maximum of 5 ug./ml. iodide generated in the iodine-fluorescein reaction. The stock solution of periodate was diluted daily to give the above concentrations in one ml. or less of solution. The fluorescein concentration was the same as above. To minimize any possible vaporization of iodine, fluorescein was added before the iodine

was added to the periodate. A blank was prepared identically except that the periodate was omitted.

An iodide-fluorescein-buffer solution of the above stated concentrations was found to be convenient in that several steps of adding reagents were eliminated. Most studies were done at 56° C. for a period of 10 minutes. The test tubes were then cooled and the absorption read.

Periodate oxidation of glucose. A working solution of glucose was prepared daily so that 0.5 to 3 ml. would give glucose concentrations of 0.04 to 2 ug./ml. of reaction mixture. The periodate solution was prepared as described above with 0.7 ml. containing 84 ug. The glucose and periodate solutions were pipetted into glass stoppered test tubes containing 1.5 ml. of boric acid - borate buffer to keep the pH near 7. Water was added to wash down the sides of the tubes, bringing the total volume to 5.2 ml. A reference was prepared identically except that glucose was omitted. The reaction mixtures were mixed by careful shaking and placed in a water bath, generally at 56° C. The glass stoppers must be seated properly to avoid their popping off as the tube is heated. After 20 minutes, the tubes were removed from the water bath and cooled for one minute in running water.

Immediately, the glass stoppers were removed and the stoppers and sides washed down with 2.5 ml. water. Next,

one ml. of iodide-fluorescein mixture (50 ug. iodide and 100 ug. fluorescein per ml.) and 1.3 ml. of water was added to bring the total volume to 10 ml. A blank is prepared with 1.5 ml. buffer, one ml. iodide-fluorescein mixture, and 7.5 ml. water and stoppered. All tubes were shaken and heated for 10 minutes at 56° C. The tubes were then cooled, and their absorption read.

Absorption measurements. The Beckman Model DB spectrophotometer, with recorder, was used to determine most of the absorptions of the reaction mixtures. A medium slit width was used. Glass cuvettes, 1 by 1 cc., matched as closely as possible, were used. The same cuvette was always used for the blank. The cuvettes were thoroughly rinsed with water after use. The sample cuvette was occasionally washed in ethanol to remove the reaction product absorbed by the glass.

After a warmup time of at least 15 minutes, the instrument and recorder were set at 100 per cent transmission with blank solution in both cuvettes. With a sample in the sample cuvettes, a plot was begun at 520 mu. and stopped at about 495 mu. An absorption peak was recorded at about 503 to 508 mu. Then, the cuvettes are reversed, and the run continued to 480 mu. with another peak recorded at about 482 to 484 mu. The maximum absorbances for each sample were read since there is a slight increase of peak wave-

length with increasing concentration.

The sample was then discarded, the cuvettes rinsed twice with the next sample, and another reading was taken.

The procedure for use of the Spectronic 20 was similar, except that the wavelength was set at 505 mu. and the blank set at 100 per cent transmission. No studies were made at lower wavelengths.

The Beckman DB manual lists the accuracy between 335 and 574 mu. as better than 5 mu. and the precision as better than 2.5 mu. The linearity is better than ± one per cent transmission or ± 0.01 absorption units at 0.4 absorbance. The 100 per cent line variation is less than 2 per cent for 220 to 700 mu. The specifications for the attached recorder gives the limit of error as ± one per cent of the span with an accuracy of ± 0.35 per cent.

Personal reading accuracy from the instrument scale is ± 0.2 mu. and ± 0.2 per cent transmission and ± 0.5 mu. and ± 0.1 per cent transmission from the recording chart.

Concentration determinations. For the iodination of fluorescein and iodide-periodate reactions, the iodine concentration was plotted against per cent transmission on semi-log paper for each of the absorption peaks. A line of best fit was drawn through the points including the zero concentration - 100 per cent transmission point.

For glucose determinations, the differences between

the maximum absorbancies of the reference and the glucose solutions were plotted against glucose concentration on retangular graph paper for each of the peaks. A line of best fit was drawn through the points including the zero absorption difference - zero concentration point.

Buffers and pH determination. Typically, 1.5 ml. of boric acid - borate buffer per 10 ml. of reaction mixture maintained a pH of 6.8 before glucose oxidation and 7.03 to 7.08 after the iodination reaction.

The phosphate-borate buffer was used mainly for the iodination of fluorescein. Typically, a mixture of 5 ml. of 0.1M $H_3PO_4$, 1.5 ml. $Na_2B_4O_7$ and 2 ml. $Na_2HPO_4$ in a total of 25 ml. kept the pH at 6.8.

The pH's were determined by a Beckman Zeromatic pH meter, using micro electrodes.

Paper chromatography. Ascending paper chromatographic technics were employed in a cylindrical glass jar. Whatman number one strip filter paper was used. Samples were spotted on the paper with a glass capillary tube and air dried. The solvent system used was 0.1 N borax adjusted to a pH of about 10 with dilute sodium hydroxide. The spots were located with the aid of an ultraviolet lamp.

# IV. EXPERIMENTAL RESULTS

## Reaction Conditions and Abbreviations

Unless otherwise stated, the following conditions prevail: the pH is 6.8; the buffer used is boric acid-borate buffer; the temperature is room temperature; fluorescein refers to uranin in solution; and absorption readings are done on the Beckman Model DB spectrophotometer.

The absorption maximum of 505 mu. will be used to refer to the range of 503 to 508 mu. and 484 mu. to the range of 482 to 484 mu.

Besides the accepted measurement abbreviations, mu. will refer to millimicrons, and ug. to micrograms.

## Iodination of Fluorescein

Absorption spectra. Upon completion of the iodination reaction, a reddish orange coloration occurs. A typical absorption curve of this product together with the spectra of fluorescein and erythrosin is shown in Figure 2. The absorption peak at 505 mu. is produced by the colored product while that at 484 mu. corresponds to fluorescein. The break in the curve for the iodinated fluorescein occurs when the cuvettes are reversed. The absorbance at 484 mu. is a measure of the excess fluorescein not reacting. But, the actual absorbance is due to the difference between the amount of fluorescein in the blank and the unreacted fluo-

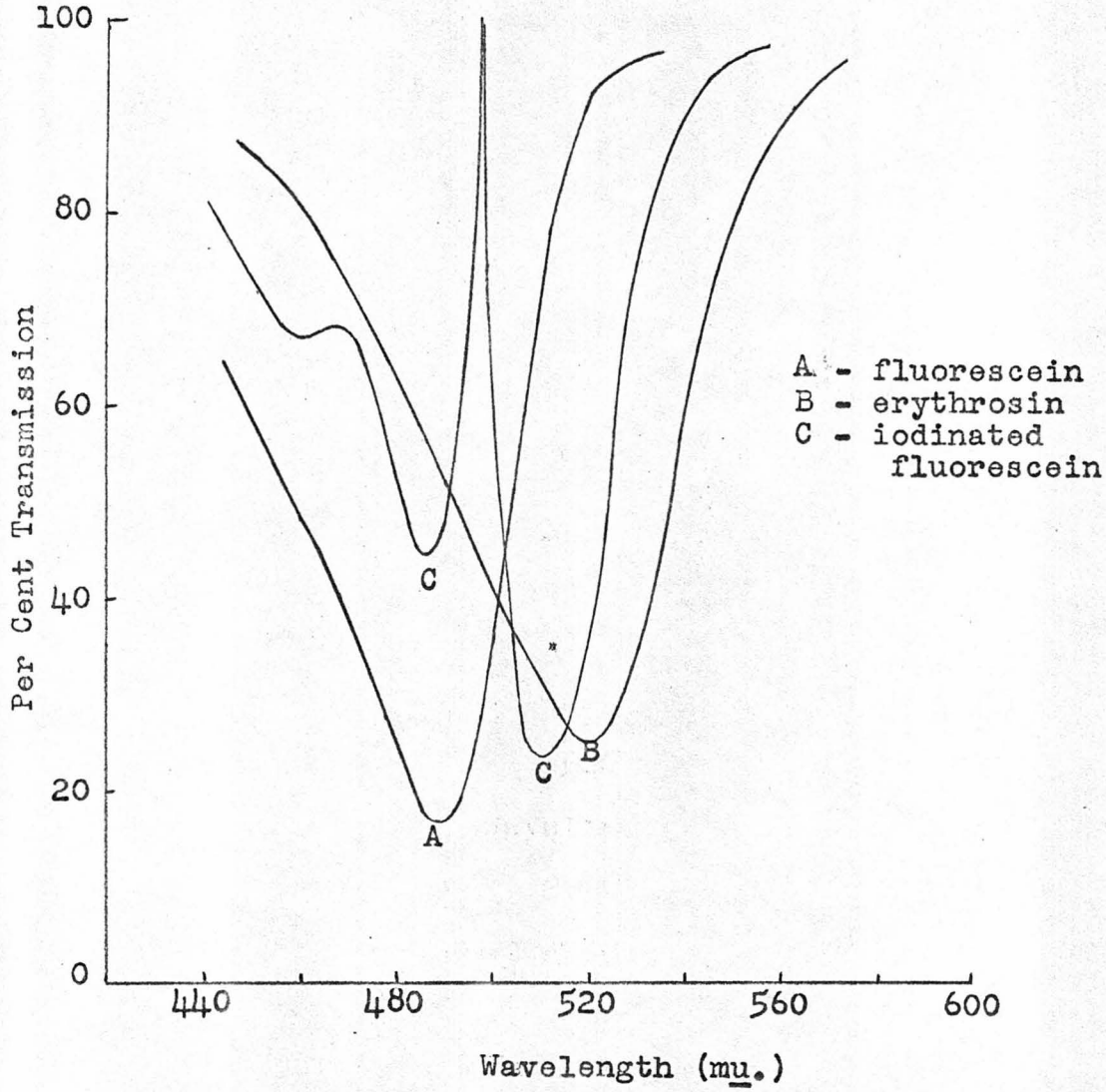



FIGURE 2

ABSORPTION SPECTRA

rescein in the iodinated sample. In Figure 2, the concentrations of the iodine, fluorescein, and erythrosin are 5, 5, and 12.5 ug./ml., respectively.

The iodide ion was found to absorb less than one per cent in the range of 480 to 520 mu.

Absorption-concentration studies. A typical plot of a concentration series study is shown in Figure 3, page 25. The iodine solution contained small amounts of ethanol in the original preparation, and the phosphate-borate buffer was used. The fluorescein concentration was constant at 10 ug./ml.

A plot of the maximum absorbances (as per cent transmission) for each concentration at its absorbance peak against concentration is shown in Figure 4, page 26.

A similar series using iodine-iodide solutions is shown in Figure 5, page 27. The concentration of fluorescein is constant at 10 ug./ml. Another study with lower concentrations and using the Spectronic 20 at the 505 mu. peak only is shown in Figure 6, page 28. The concentration of fluorescein is 5 ug./ml. and the pH is 6.9

A typical precision study is shown in Table X, page 62.

Time, pH, and lighting effects. A study of the effects of time upon this reaction is shown in Table I, page 29. Studies were done at pH's of 7.3 and 6.4 utilizing

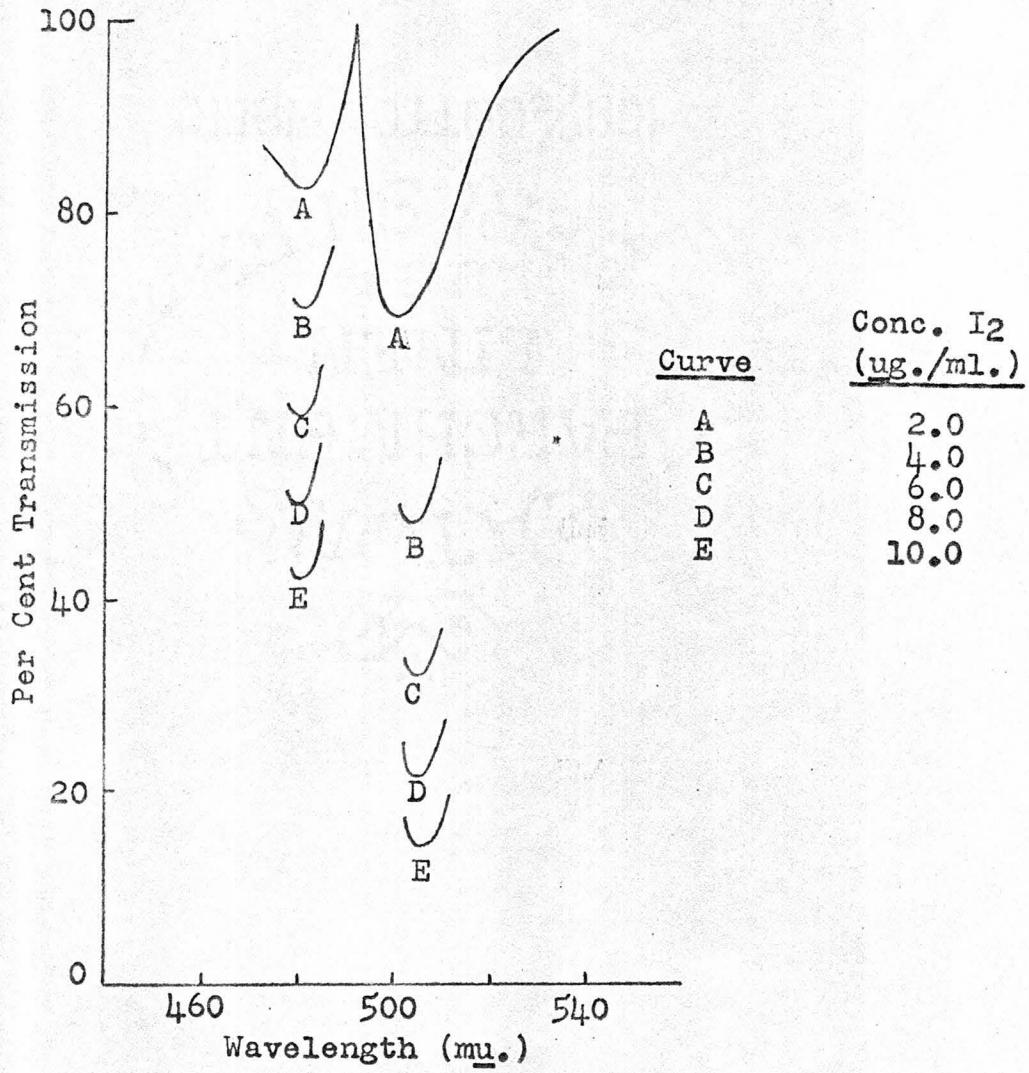



FIGURE 3

ABSORPTION SPECTRA OF IODINATED FLUORESCEIN

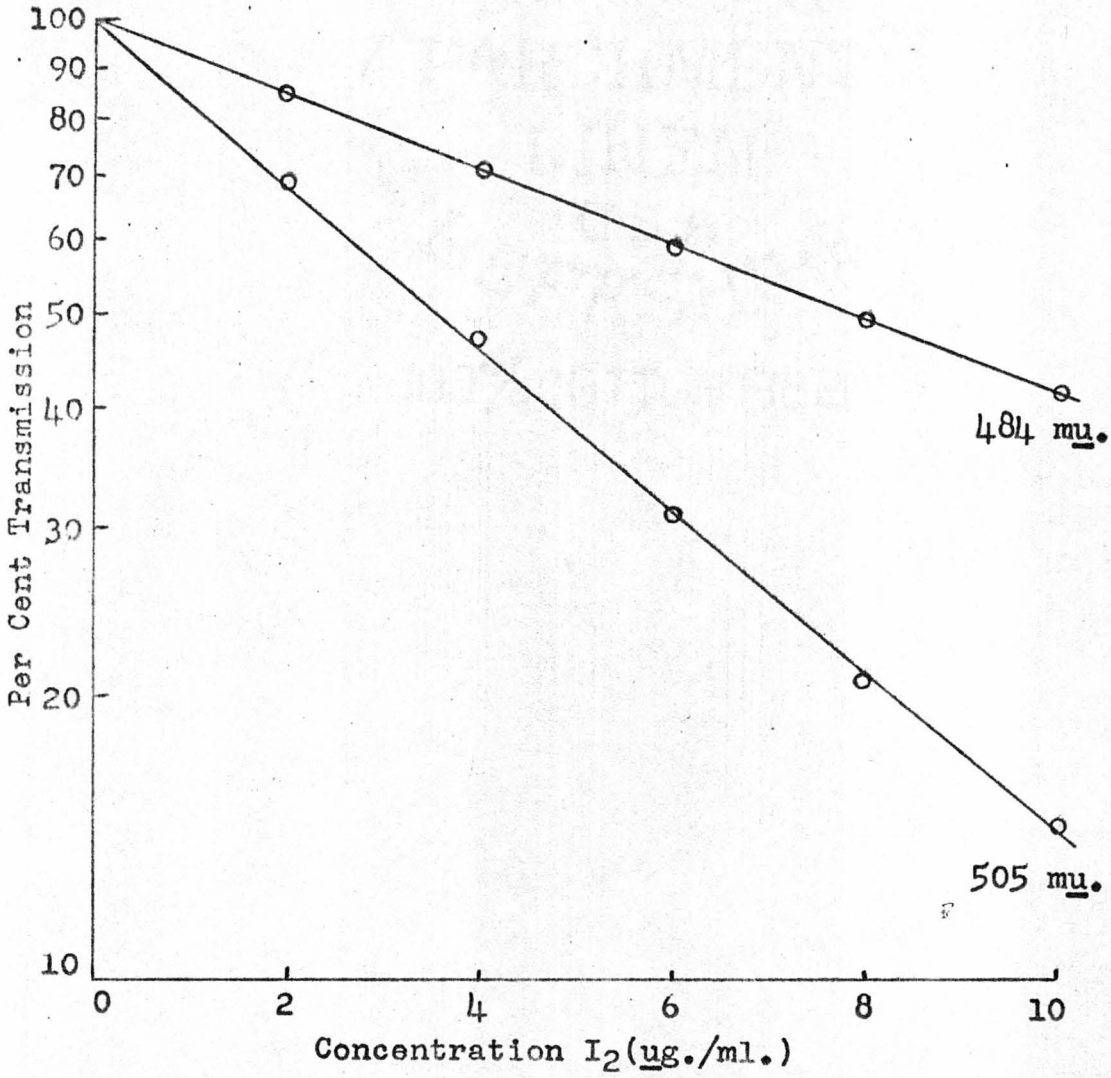



FIGURE 4

PER CENT TRANSMISSION-CONCENTRATION PLOT
(IODINATED FLUORESCEIN)
(IODINE SOLUTION)

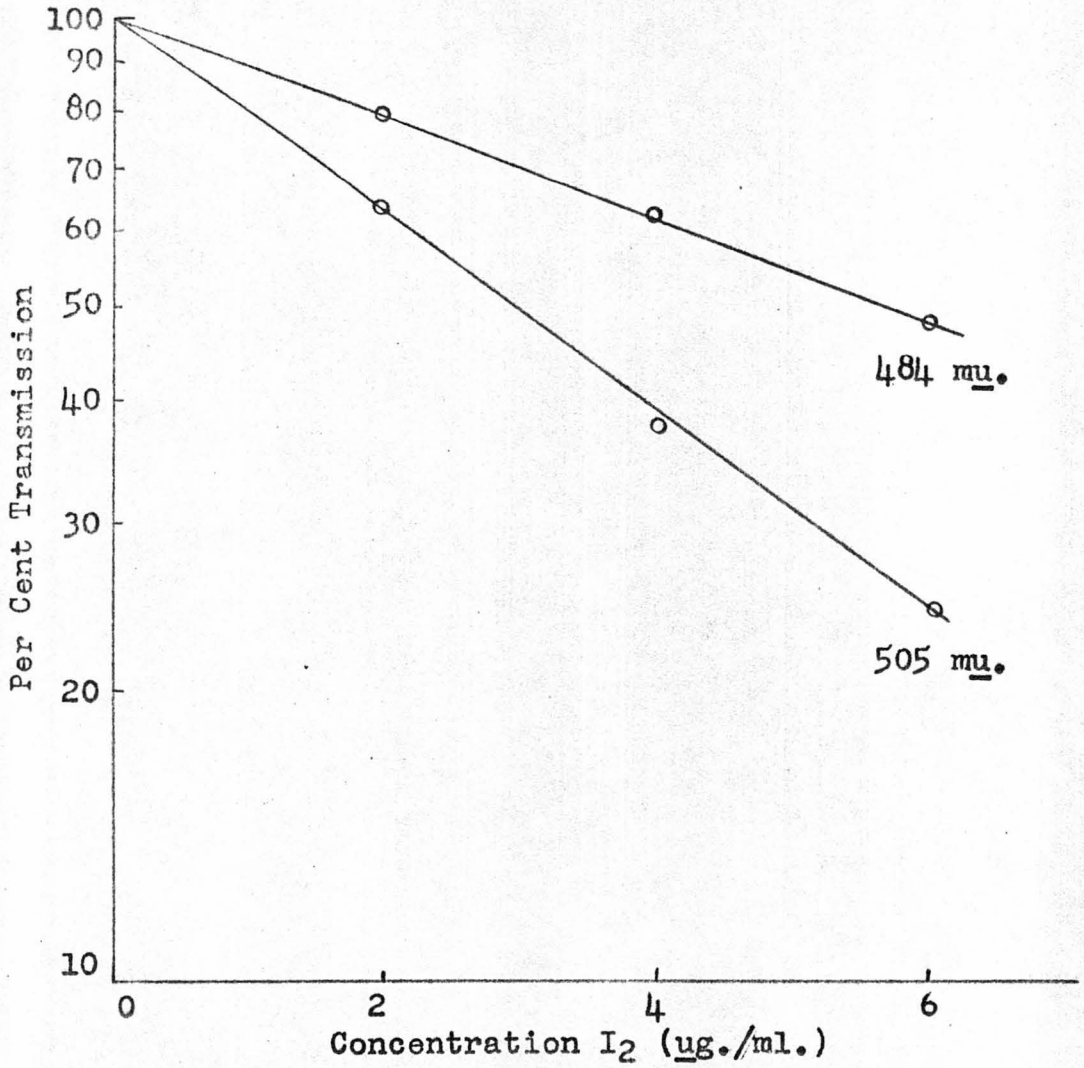



FIGURE 5

PER CENT TRANSMISSION-CONCENTRATION PLOT
(IODINATED FLUORESCEIN)
(IODINE-IODIDE SOLUTION)

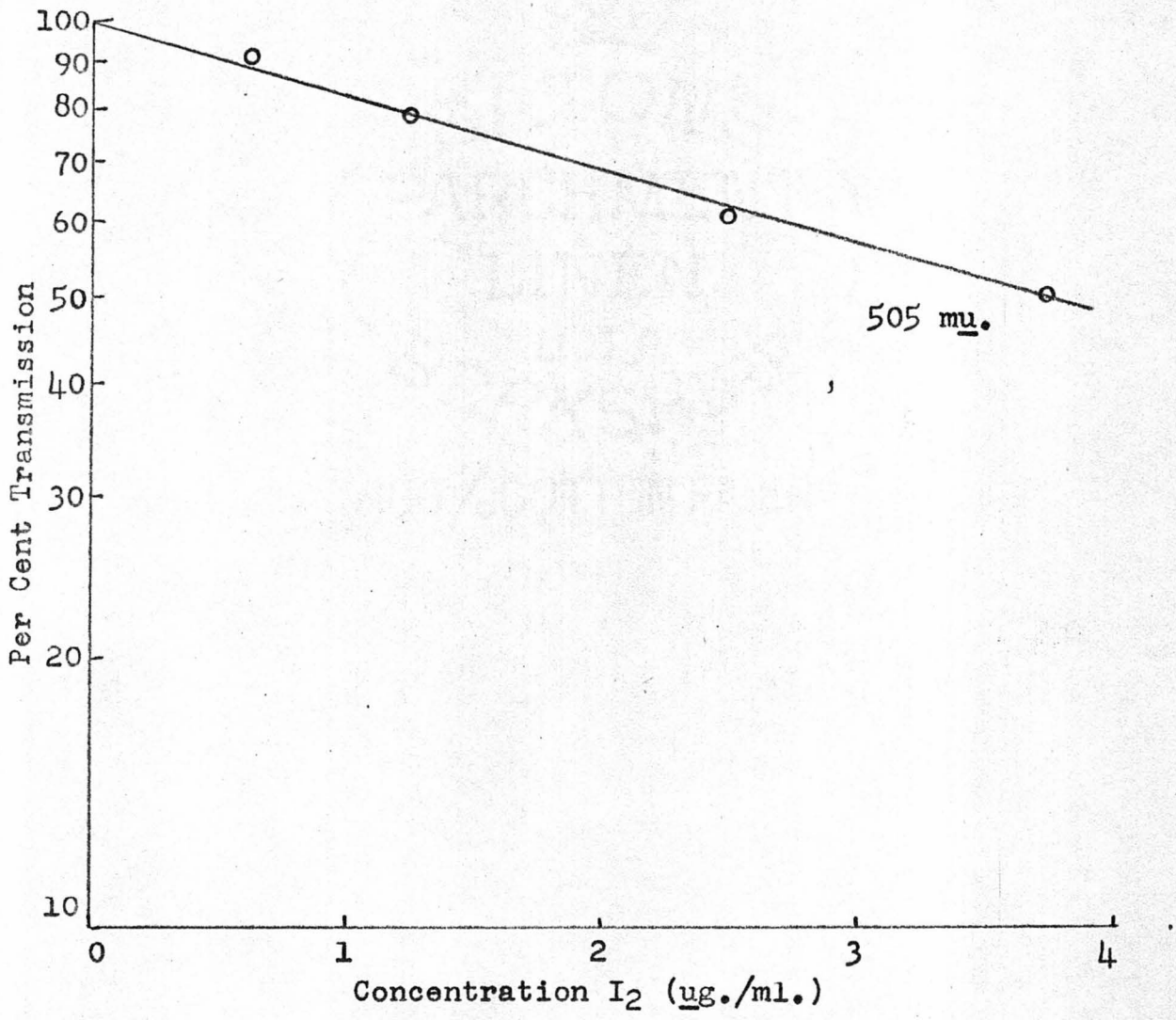



FIGURE 6

PER CENT TRANSMISSION-CONCENTRATION PLOT
(IODINATED FLUORESCEIN)
(SPECTRONIC 20)

TABLE I

TIME EFFECTS (IODINATED FLUORESCEIN REACTIONS, IODINE SOLUTION)

| | Reaction Time Minutes | Maximum % Transmission 505 mμ. | 484 mμ. |
|---|---|---|---|
| A. pH = 7.3 | 1 | 46.0 | |
| | 1.5 | | 53.0 |
| | 3 | 46.2 | |
| | 5 | 46.0 | |
| | 6 | | 52.5 |
| | 7 | 45.9 | |
| | 8 | | 52.7 |
| | 9 | 46.0 | |
| | 10 | | 52.7 |
| | 11 | 46.0 | |
| | 12 | | 52.8 |
| | 14 | | 52.8 |
| | 15 | 46.0 | |
| | 20 | 46.0 | 52.8 |
| | 45 | 46.2 | 52.8 |
| | 90 | 46.2 | 53.0 |
| | 120 | 46.2 | 53.0 |
| | 210 | 46.2 | 53.2 |
| B. pH = 6.4 | 2 | 45.3 | |
| | 3 | | 80.0 |
| | 4 | 44.8 | |
| | 5 | | 80.0 |
| | 6 | 44.8 | |
| | 8 | 44.8 | |
| | 9 | | 80.5 |
| | 10 | 45.0 | |
| | 14 | 44.9 | |
| | 15 | | 80.9 |
| | 30 | 44.9 | 80.2 |
| | 90 | 45.0 | 81.0 |

phosphate-borate buffers. The iodine and fluorescein concentrations were kept constant at 4 and 10 ug./ml., respectively. The solutions were kept in capped cuvettes inside the darkened cuvette chamber of the spectrophotometer between readings.

Table II, shows results obtained using iodine produced in the iodide-periodate reaction in the phosphate-borate buffer at a pH of 7.4. The iodide-periodate reaction was allowed to proceed for 30 minutes, then the fluorescein was added and absorbances were read, beginning after 5 minutes. The iodine and fluorescein concentrations were constant at 7.2 and 6 ug./ml., respectively.

All studies similarily show that the iodination reaction reaches a maximum within 5 minutes.

The effect of pH was studied in a phosphate buffer with time held constant to 10 minutes of reaction time. The iodine and fluorescein concentrations were constant at 4 and 10 ug./ml. of reaction mixture. Generally, the color of the product became redder with increasing pH. Graphical results are shown in Figure 7, page 32.

The effect of normal laboratory lighting (fluorescent fixture) upon the iodination of fluorescein is shown in Table III. The time was constant at 15 minutes reaction time. The pH was kept at 7.4 with the phosphate-borate buffer. The fluorescein concentration was 10 ug./ml.

TABLE II

TIME EFFECTS (IODINATED FLUORESCEIN, IODIDE-PERIODATE REACTION)

| Reaction Time Minutes | Maximum % Transmission 505 mu. | 484 mu. |
|---|---|---|
| 5 | 34.0 | 48.5 |
| 10 | 35.0 | 46.0 |
| 20 | 37.5 | 43.5 |
| 30 | 38.0 | 43.0 |
| 58 | 39.5 | 42.5 |
| 80 | 40.5 | 42.0 |

TABLE III

EFFECTS OF LIGHT (IODINATED FLUORESCEIN REACTION)

| Concentration $I_2$ (ug./ml.) | 2 | 4 | 6 | 8 | 10 |
|---|---|---|---|---|---|
| % Transmission | | | | | |
| 1. 505 mu. | | | | | |
| Dark | 72.1 | 49.0 | 33.0 | 22.8 | 15.0 |
| Room Light | 72.7 | 48.2 | 33.0 | 22.8 | 14.5 |
| 2. 484 mu. | | | | | |
| Dark | 81.4 | 63.0 | 48.0 | 37.8 | 27.5 |
| Room Light | 76.0 | 60.0 | 44.4 | 35.3 | 25.9 |

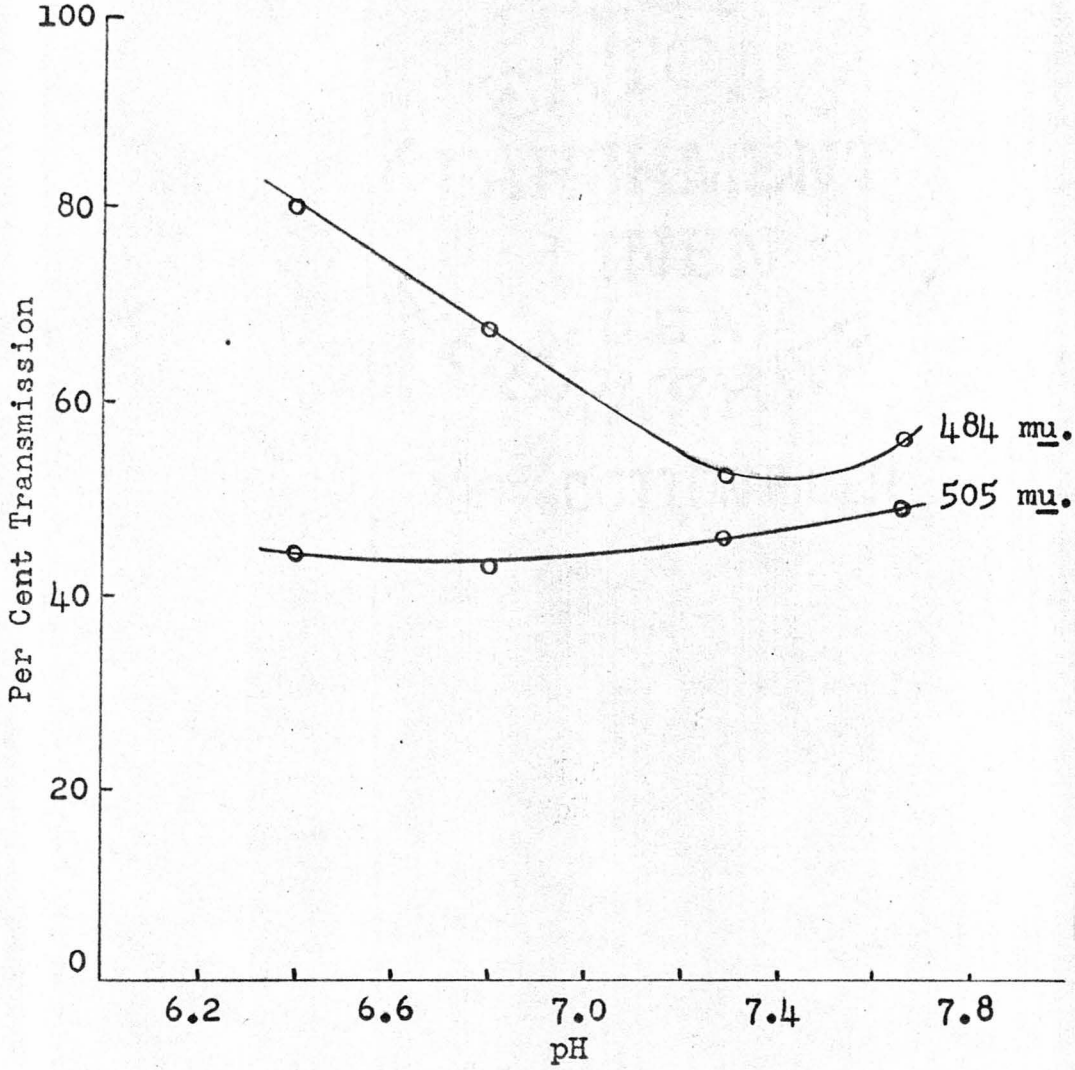



FIGURE 7

PER CENT TRANSMISSION-pH PLOT
(IODINATED FLUORESCEIN)

The series run in the dark was prepared in a non-lighted laboratory and placed in the dark as soon as possible.

The effect of increased fluorescent irradiation and time is shown in Table IV. A reaction mixture, containing 4 ug./ml. reaction mixture was prepared as usual. One half of this was kept as a control in normal room lighting; the other half was placed approximately one foot from a common fluorescent lighting fixture, in stoppered tubes. The temperature of the control was 24.0° C.; that of the strongly irradiated mixture, 24.3° C. Other conditions were the same as described in the preceding paragraph.

Effect of chloride ion. Table V, shows the results of adding chloride ion to a typical reaction mixture. The micro-molar ratio of iodine to chloride ion was 0.0099 to 1.9. The iodine concentration was 5 ug./ml. reaction mixture and was diluted from an iodine-iodide solution preparation. The micro-molar ratio of iodine to iodide was 0.0099 to 0.075.

Stoichiometry. A graphical analysis is shown in Figure 8. Iodine was added to a constant fluorescein concentration of 5 ug./ml. The break in the curve indicates that the iodination is complete when 3 to 4 iodine atoms are used.

In the spectral studies, various mixtures of reagents were combined and compared to the fluorescein and erythrosin

TABLE IV

EFFECT OF LIGHT AND TIME (IODINATED FLUORESCEIN REACTION)

| Time Minutes | % Transmission-505 mu. Room Light | % Transmission-505 mu. Strong Light | % Transmission-484 mu. Room Light | % Transmission-484 mu. Strong Light |
|---|---|---|---|---|
| 10 | 46.7 | | 69.4 | |
| 18 | | 48.8 | | 67.9 |
| 30 | 47.0 | | 69.4 | |
| 36 | | 52.0 | | 65.4 |
| 60 | 47.5 | 59.0 | | 59.8 |

TABLE V

EFFECT OF CHLORIDE ION (IODINATED FLUORESCEIN REACTION

| Conc. $Cl^-$ in Blank (u moles/ml.) | Concentration $I_2$ (ug./ml.) | (u moles/ml.) | Conc. $Cl^-$ (u moles/ml.) | % Trans. 505 mu. |
|---|---|---|---|---|
| 0 | 5 | 0.0099 | 0 | 44.3 |
| 0 | 5 | 0.0099 | 1.9 | 44.2 |
| 1.9 | 5 | 0.0099 | 0 | 44.2 |
| 1.9 | 5 | 0.0099 | 1.9 | 44.2 |

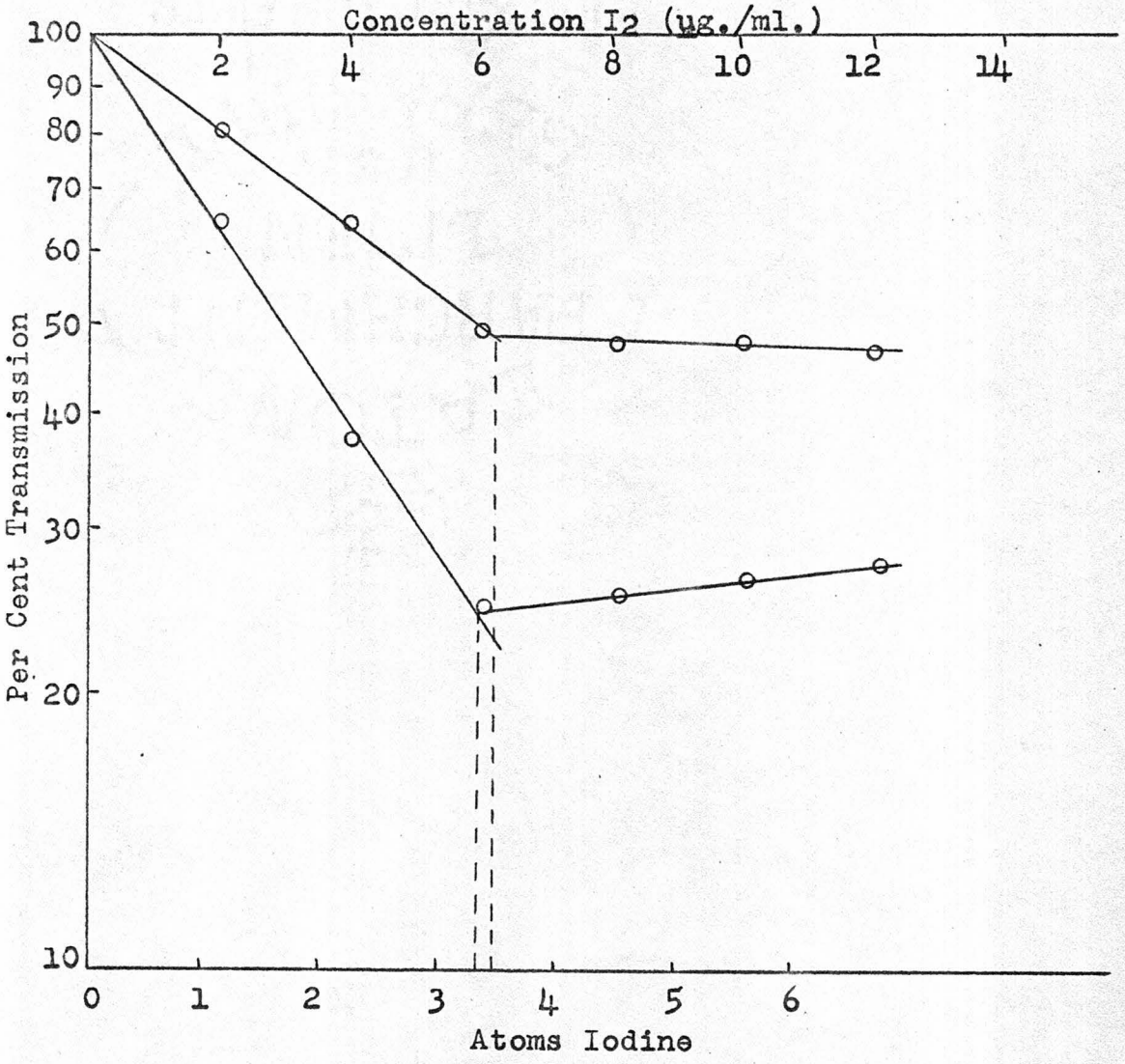



FIGURE 8

STOICHIOMETRY-GRAPHICAL ANALYSIS

spectra. The results are shown in Figure 9, page 37, and Table VI, page 38. The di-sodium salt of erythrosin was prepared and diluted to the same molar concentration as fluorescein. The buffer concentration was held constant in all tests including the blank. Literature values of absorption peaks for fluorescein and erythrosin are 491 and 524 to 526 mu. (6), respectively.

The paper chromatographic studies resulted in $R_f$ values of 0.04 and 0.16 for erythrosin and the iodinated fluorescein product, respectively. For comparison, the $R_f$ value of fluorescein was 0.66. The results are reproduced in Figure 10, page 39. The concentrations of the fluorescein and erythrosin were 5 and 10 ug./ml., respectively. The iodinated fluorescein product was prepared with 5 ug. fluorescein and 10 ug. iodine (a slight stoichiometric excess) per ml. The spots remained very nearly the same as they moved indicating the probability that mixtures were not present.

## Iodide-Periodate Reaction

Absorption-concentration studies. A typical concentration series with excess iodide concentration (50 ug./ml.) and varying periodate concentration is given in Figure 11, page 40. In all of these reactions, the iodide ion concentration is increased slightly by the iodide formed in the

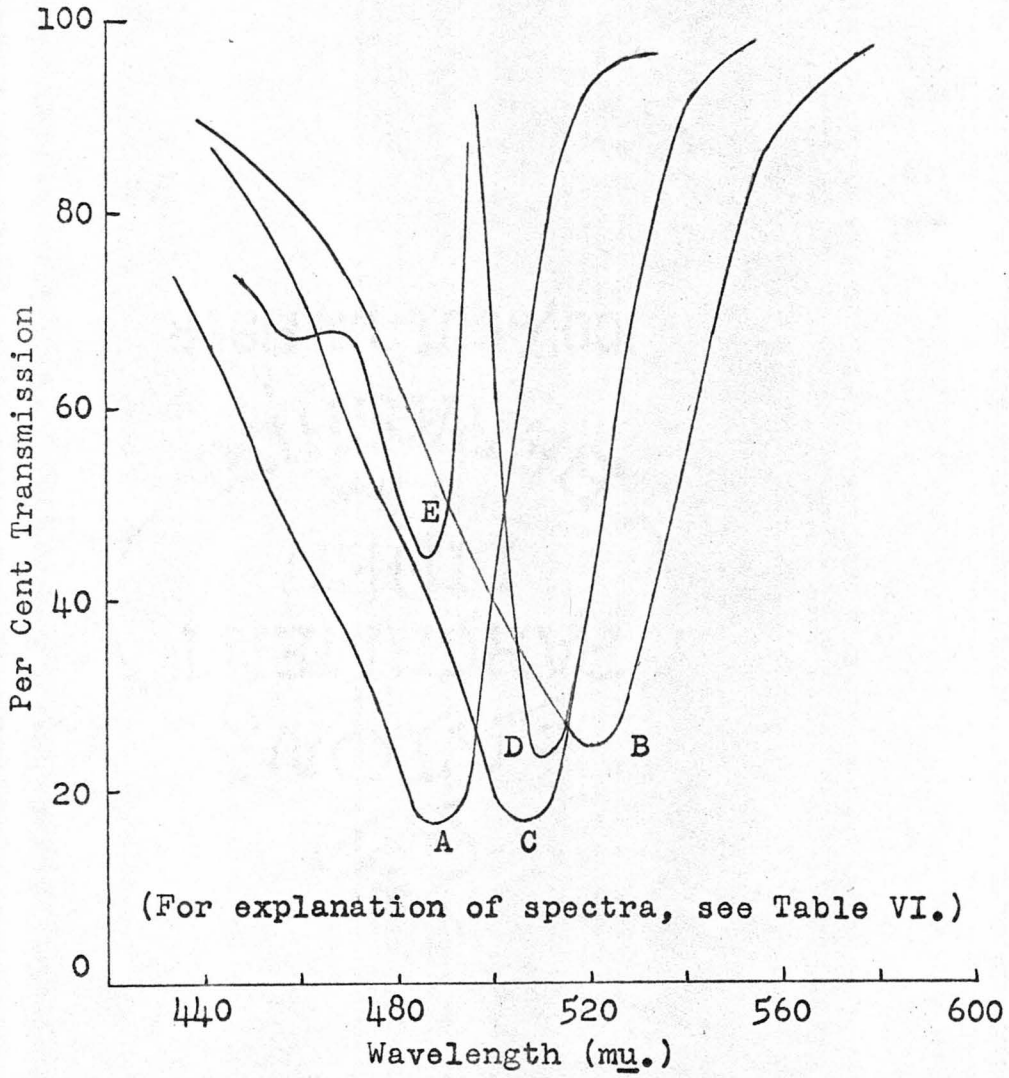



FIGURE 9

STOICHIOMETRY-SPECTRAL ANALYSIS

TABLE VI

SPECTRAL ANALYSIS OF IODINATED FLUORESCEIN REACTION

| Reagent | | Control | | | |
|---|---|---|---|---|---|
| Composition | Conc. (ug./ml.) | Composition | Conc. (ug./ml.) | Spectrum (Figure 9) | Absorption (mu.) |
| 1. Fluorescein | 5.0 | Blank | - | A | 488 |
| 2. Erythrosin | 12.5 | Blank | - | B | 518 |
| 3. Iodinated fluorescein and fluorescein | 8.1 ($I_2$)<br>5.0 | Blank | - | C | 506 |
| 4. Iodinated fluorescein and fluorescein | 8.1 ($I_2$)<br>5.0 | Blank and fluorescein | 5.0 | D | 509 |
| 5. Blank and fluorescein | 5.0 | Iodinated fluorescein and fluorescein | 8.1 ($I_2$) | E | 487 |

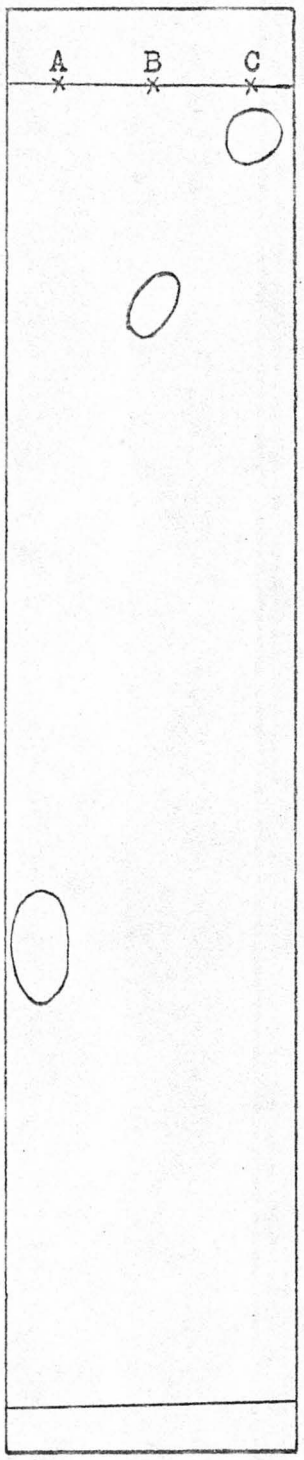



FIGURE 10

STOICHIOMETRY-CHROMATOGRAPHIC ANALYSIS

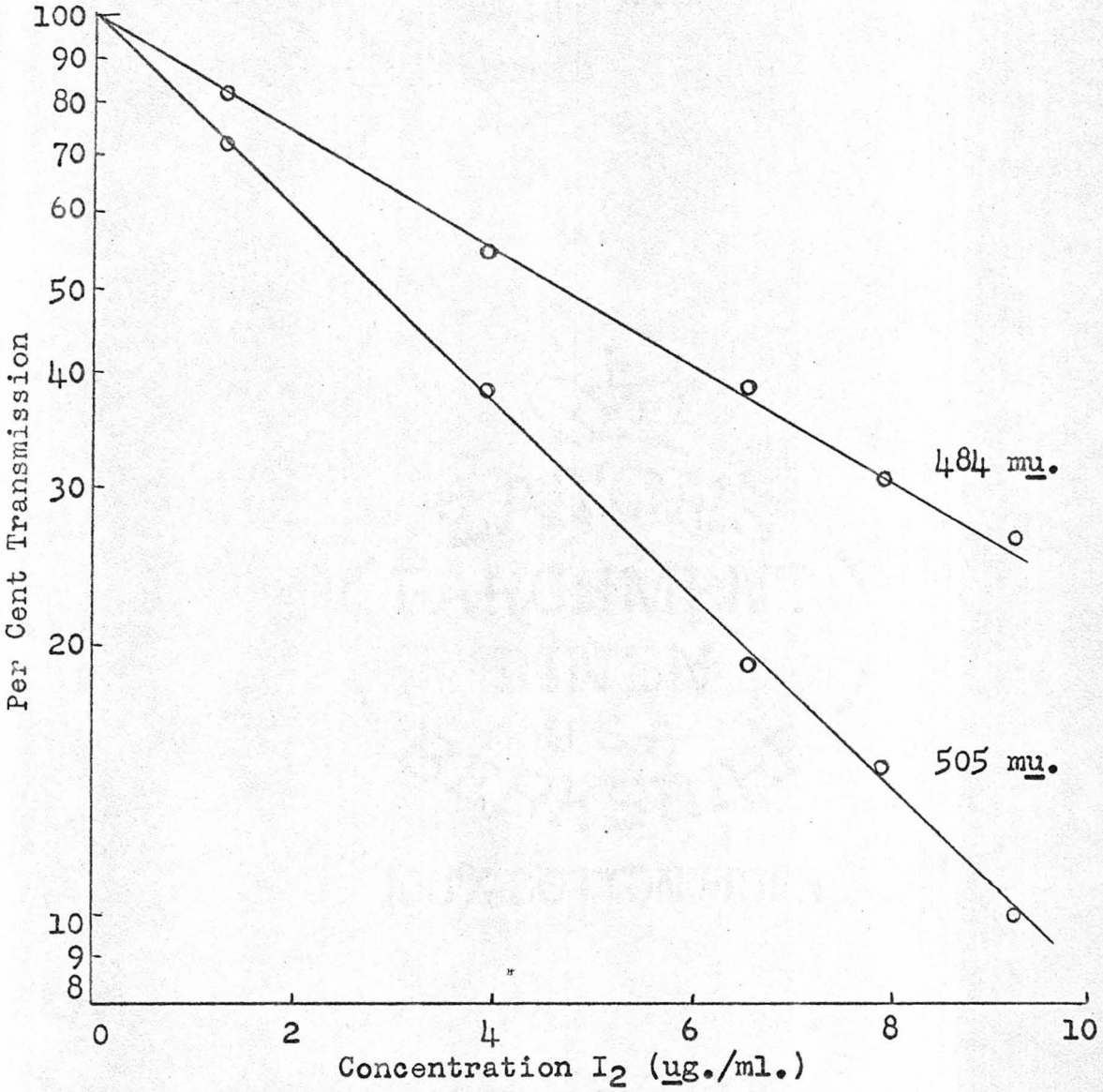



FIGURE 11

PER CENT TRANSMISSION-CONCENTRATION PLOT
(IODIDE-PERIODATE REACTION)

fluorescein-iodine reaction. The spectral plot is shown in Figure 13, page 45. The reactants were heated for 10 minutes at 56° C., cooled, and the absorptions read.

A typical precision study is summarized in Table X, page 62.

Time, temperature, and pH effects. A rate study with a reaction temperature of 56 to 57° C. is shown in Figure 12. The periodate concentration is constant at 5.6 ug./ml. reaction mixture (11.4 ug. KI per ug. periodate), and the fluorescein is added before allowing the periodate and iodide to react. A similar study, but with fluorescein being added after heating the reaction mixture (2.8 ug. KI per ug. periodate), is also shown in Figure 12.

Another study at room temperature and with the iodide-periodate reaction occurring before the fluorescein is added is summarized in Figure 12. The periodate concentration is 6.8 ug./ml. (2.65 ug. KI per ug. periodate). The absorption readings were taken approximately 10 minutes after addition of the fluorescein.

At a lower pH (approximately 0.8) and room temperature, the reaction is essentially complete within one minute.

Effect of iodide ion concentration. The effect of iodide ion concentration on the completion of the reaction after heating 20 minutes at 56 to 57° C. in the presence of

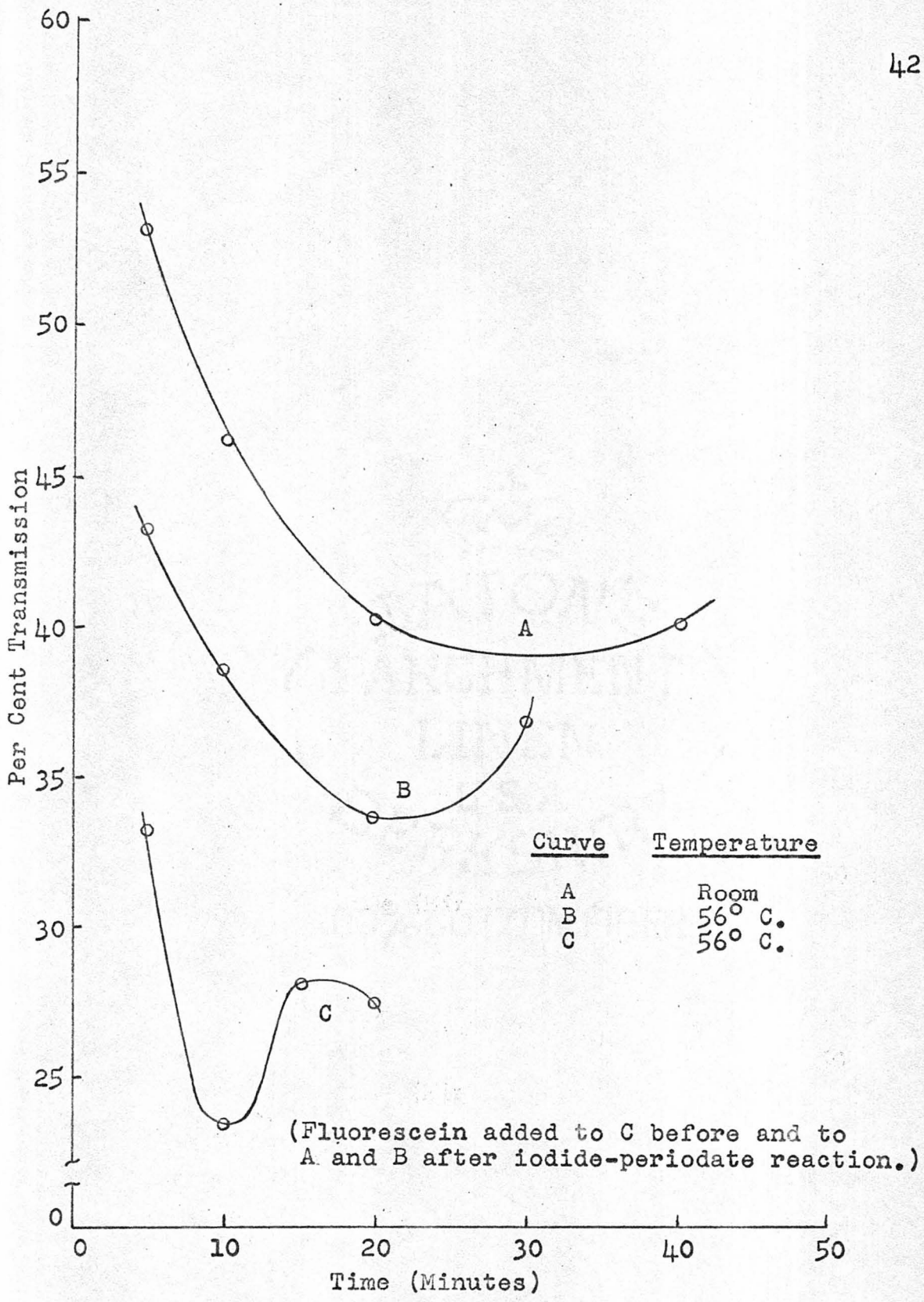



FIGURE 12

TIME AND TEMPERATURE EFFECTS
(IODIDE-PERIODATE REACTION)

fluorescein is shown in Table VII. The periodate concentration is held constant at 5.6 ug./ml. reaction mixture.

Stoichiometry. To determine the stoichimetric relationships in this reaction, a study was completed in which equivalent amounts of iodine were compared with the iodine produced by reaction C (see Section II, page 9). The reaction conditions are the same as employed in the absorption-concentration studies described above. The study was done in two parts and completed in approximately 1.5 hours. The absorption curves are shown in Figure 13, page 45, and a summary of the results given in Figure 14, page 46.

Periodate Oxidation of Glucose

Absorption-concentration studies. A typical glucose concentration series study is shown in Figure 15, page 47, and Table VIII, page 48. The glucose oxidation reaction proceeded for 20 minutes at 56° C.; fluorescein and iodide were added; and, the mixture was again heated for 10 minutes at the same temperature.

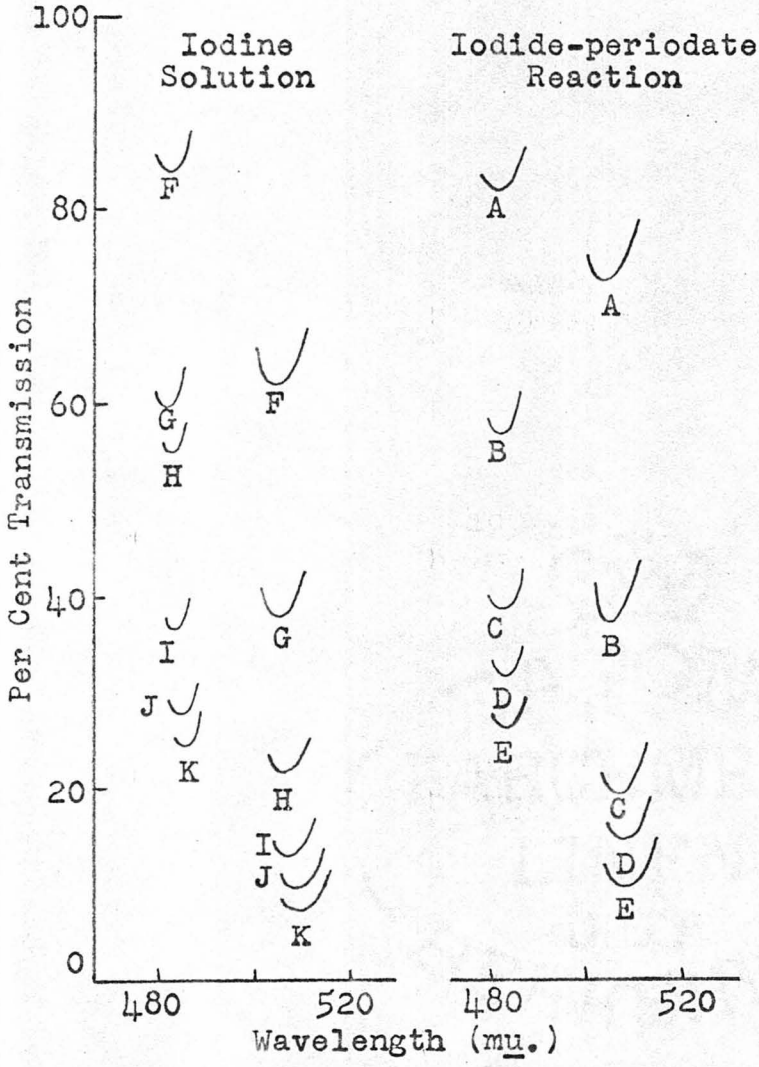
Figure 16, page 49, and Table IX, page 50, show a similar study for lower glucose concentrations. The conditions were the same as above except that an oxidation reaction time of 10 minutes at 98° C. was used.

A typical precision study is shown in Table X, page 62.

TABLE VII

EFFECT OF IODIDE CONCENTRATION
ON IODIDE-PERIODATE REACTION

| Conc. KI (ug./ml.) | Iodide/Periodate molar ratio | % Transmission 505 mu. | 484 mu. |
|---|---|---|---|
| 15.7 | 3.9 | 27.0 | 51.5 |
| 31.4 | 7.8 | 27.3 | 38.8 |
| 47.1 | 11.7 | 25.3 | 49.0 |
| 62.8 | 15.6 | 25.5 | 43.0 |




| Curve | Conc. $I_2$ (ug./ml.) |
|---|---|
| A | 1.3 |
| B | 4.0 |
| C | 6.6 |
| D | 7.9 |
| E | 9.3 |
| F | 2.0 |
| G | 4.0 |
| H | 6.1 |
| I | 8.1 |
| J | 9.3 |
| K | 10.1 |

FIGURE 13

STOICHIOMETRY (IODIDE-PERIODATE REACTION)

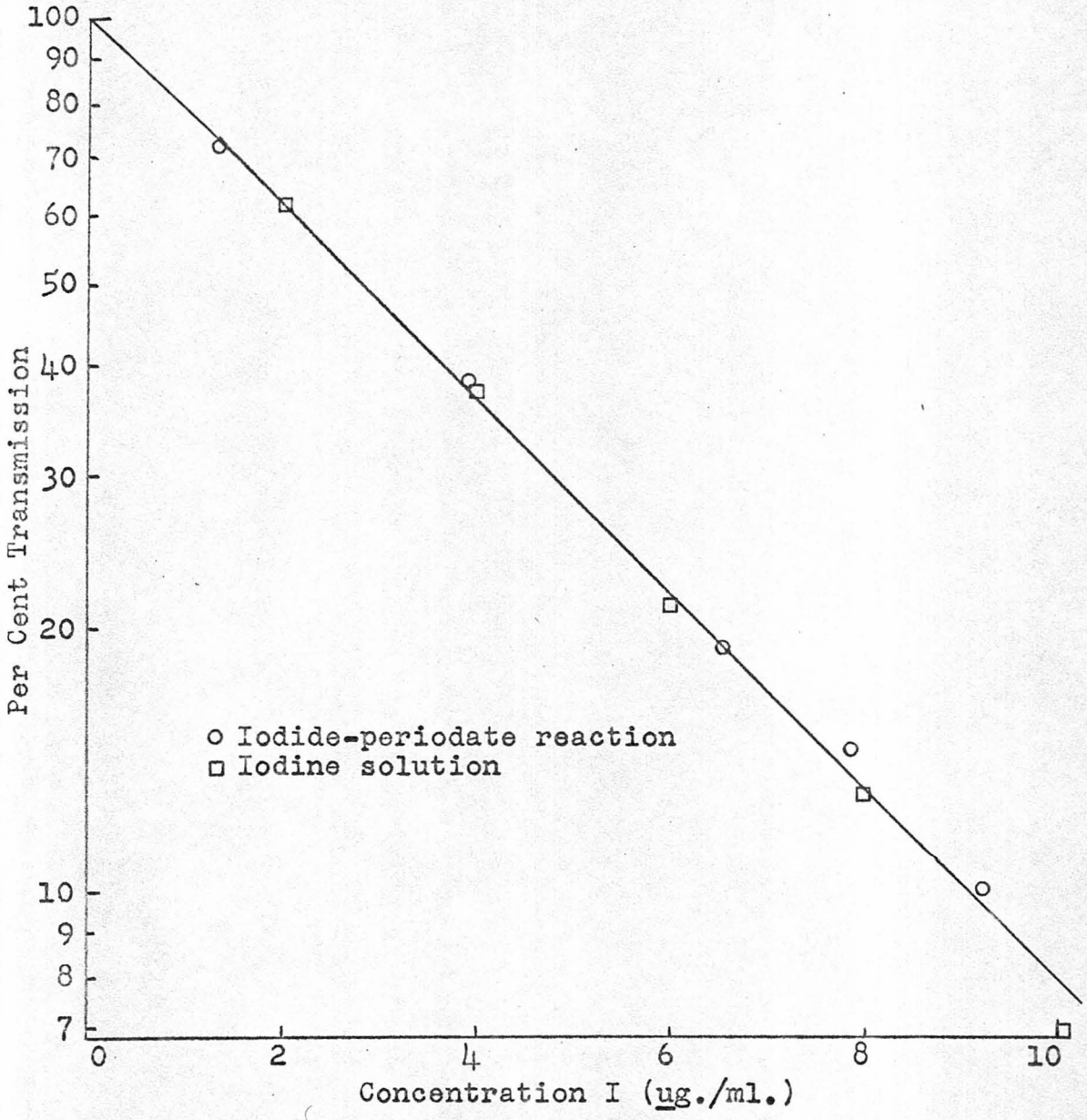



FIGURE 14

STOICHIOMETRY (IODIDE-PERIODATE REACTION)

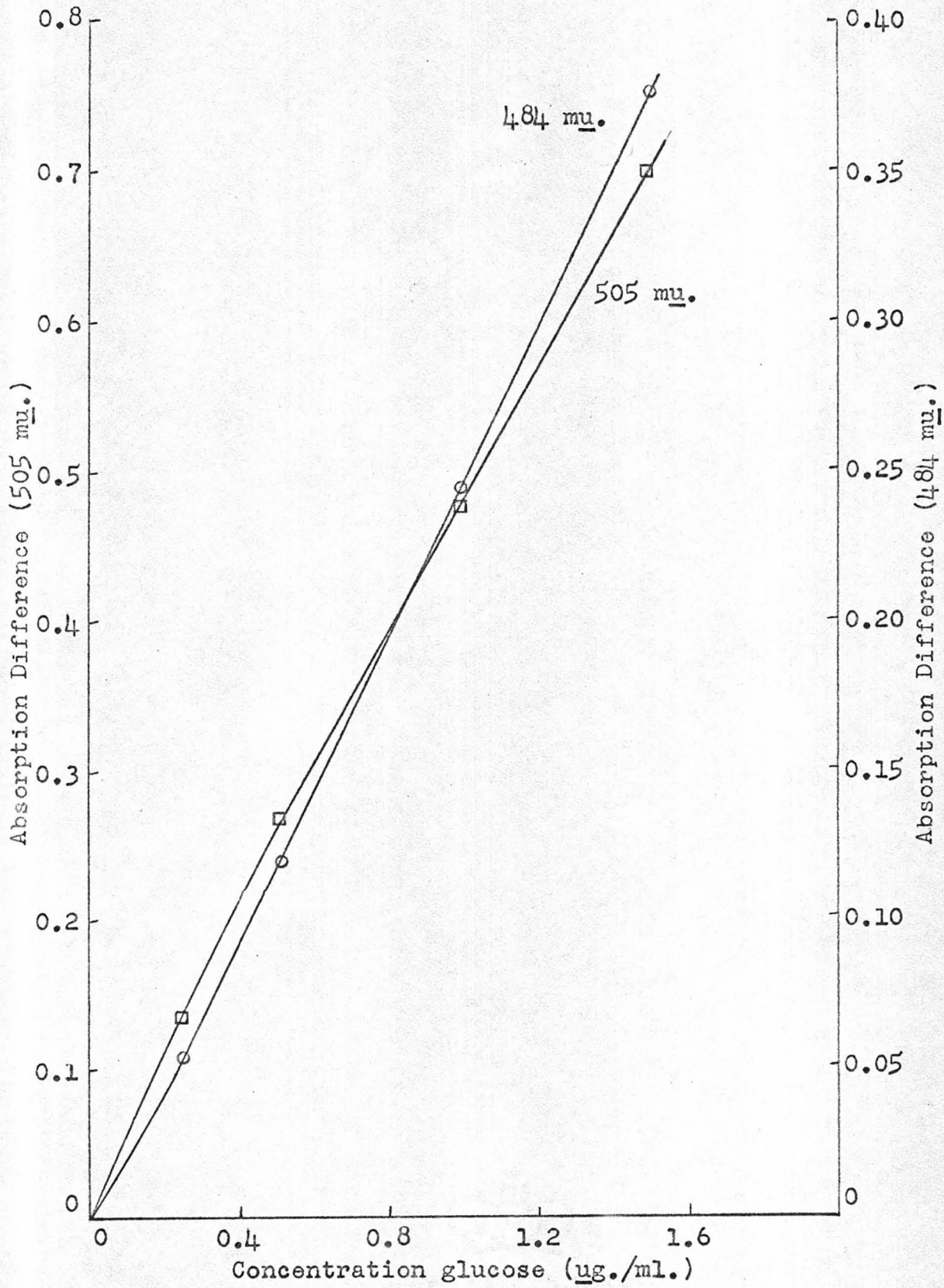



FIGURE 15

ABSORPTION DIFFERENCE-GLUCOSE CONCENTRATION PLOTS
(HIGHER CONCENTRATIONS)

TABLE VIII

TYPICAL TRANSMISSION-CONCENTRATION STUDY
(PERIODATE-GLUCOSE REACTION)
(HIGHER CONSENTRATIONS)

| Conc. Glucose (ug./ml.) | Wavelength (mu) | % Transmission | Absorption | Absorption* Difference |
|---|---|---|---|---|
| 0 | 505 | 9.6 | 1.018 | - |
| 0.25 | 505 | 13.0 | .885 | 0.133 |
| 0.5 | 505 | 17.7 | .752 | .266 |
| 1.0 | 505 | 28.6 | .544 | .474 |
| 1.5 | 505 | 48.0 | .319 | .699 |
| 0 | 484 | 27.7 | .557 | - |
| 0.25 | 484 | 31.3 | .504 | .053 |
| 0.5 | 484 | 36.5 | .438 | .119 |
| 1.0 | 484 | 48.6 | .313 | .244 |
| 1.5 | 484 | 65.8 | .182 | .375 |

*Difference in absorptions of blank (no glucose) and glucose sample.

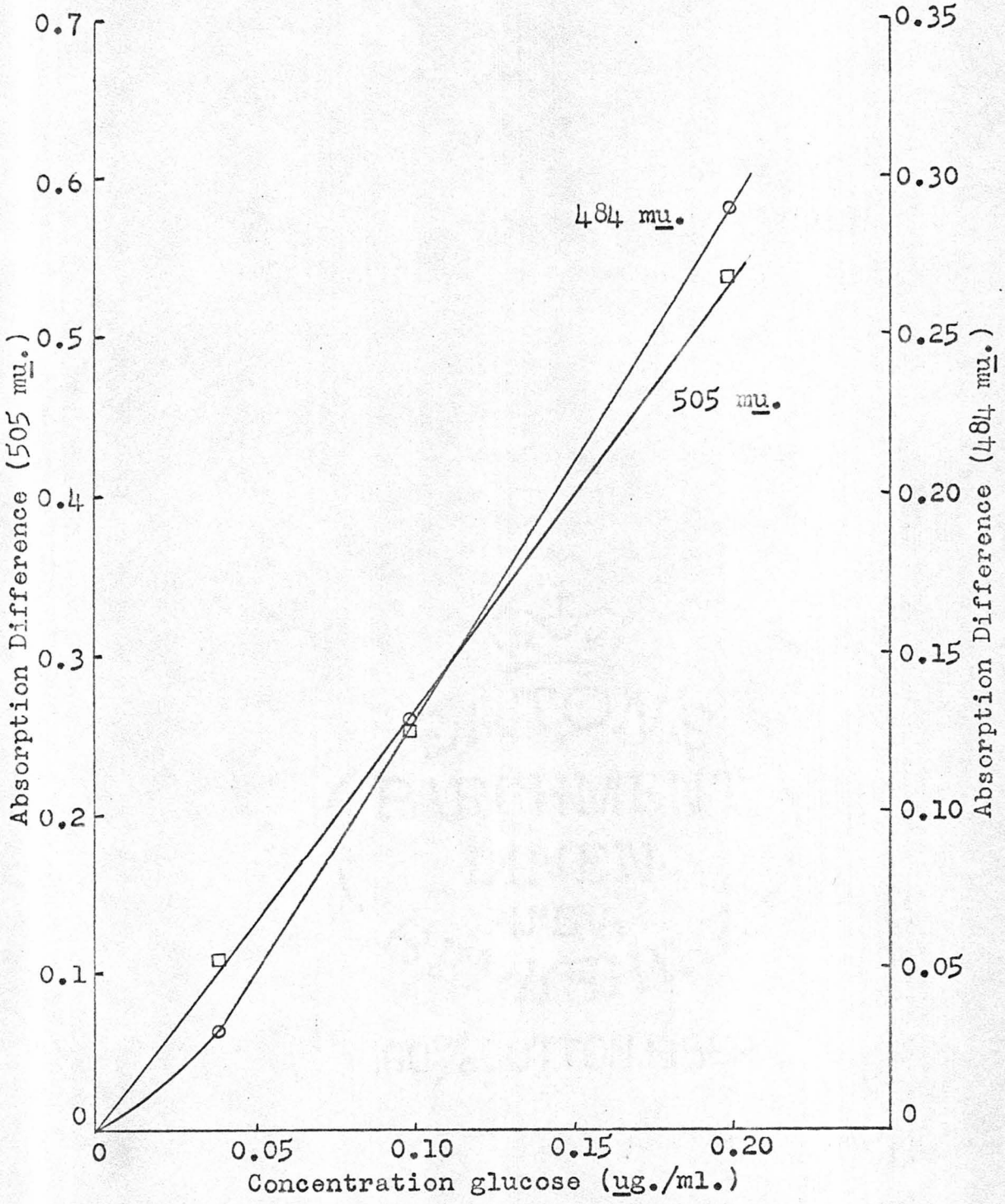



FIGURE 16

ABSORPTION DIFFERENCE-GLUCOSE CONCENTRATION PLOTS
(LOWER CONCENTRATIONS)

TABLE IX

TYPICAL TRANSMISSION-CONCENTRATION STUDY
(PERIODATE-GLUCOSE REACTION)
(LOWER CONCENTRATION)

| Conc. Glucose (ug./ml.) | Wavelength (mu.) | % Transmission | Absorption | Absorption* Difference |
|---|---|---|---|---|
| 0 | 505 | 12.5 | 0.902 | - |
| 0.04 | 505 | 16.0 | .796 | 0.106 |
| 0.1 | 505 | 21.4 | .650 | .252 |
| 0.2 | 505 | 43.3 | .363 | .539 |
| 0 | 484 | 34.1 | .466 | - |
| 0.04 | 484 | 36.6 | .436 | .030 |
| 0.1 | 484 | 46.0 | .337 | .129 |
| 0.2 | 484 | 66.8 | .175 | .291 |

*Difference in absorptions of blank (no glucose) and glucose sample.

Effect of temperature, time, and pH. Results of the effect of temperature and pH with respect to time are shown in Figure 17. In all cases, the glucose concentration was constant at 0.25 ug./ml. of reaction mixture. Also, the iodination of fluorescein reaction time was 10 minutes at 57° C.

A study at a higher temperature is described above. It also appears that higher pH decreases the rate of oxidation.

Stoichiometry. No quantitative stoichiometric studies were conducted for reasons indicated below. (see Section V, page 58.) In the studies summarized in Tables VIII and IX, (pages 48 and 50, respectively), the periodate concentration was kept constant at 8.4 ug./ml. This amount is theoretically equivalent to 6.55 ug./ml. glucose if glucose undergoes complete oxidation. However, the actual highest glucose concentration used in Table VIII, page 48, is only 1.5 ug./ml. and 0.2 ug./ml. in Table IX, page 50.

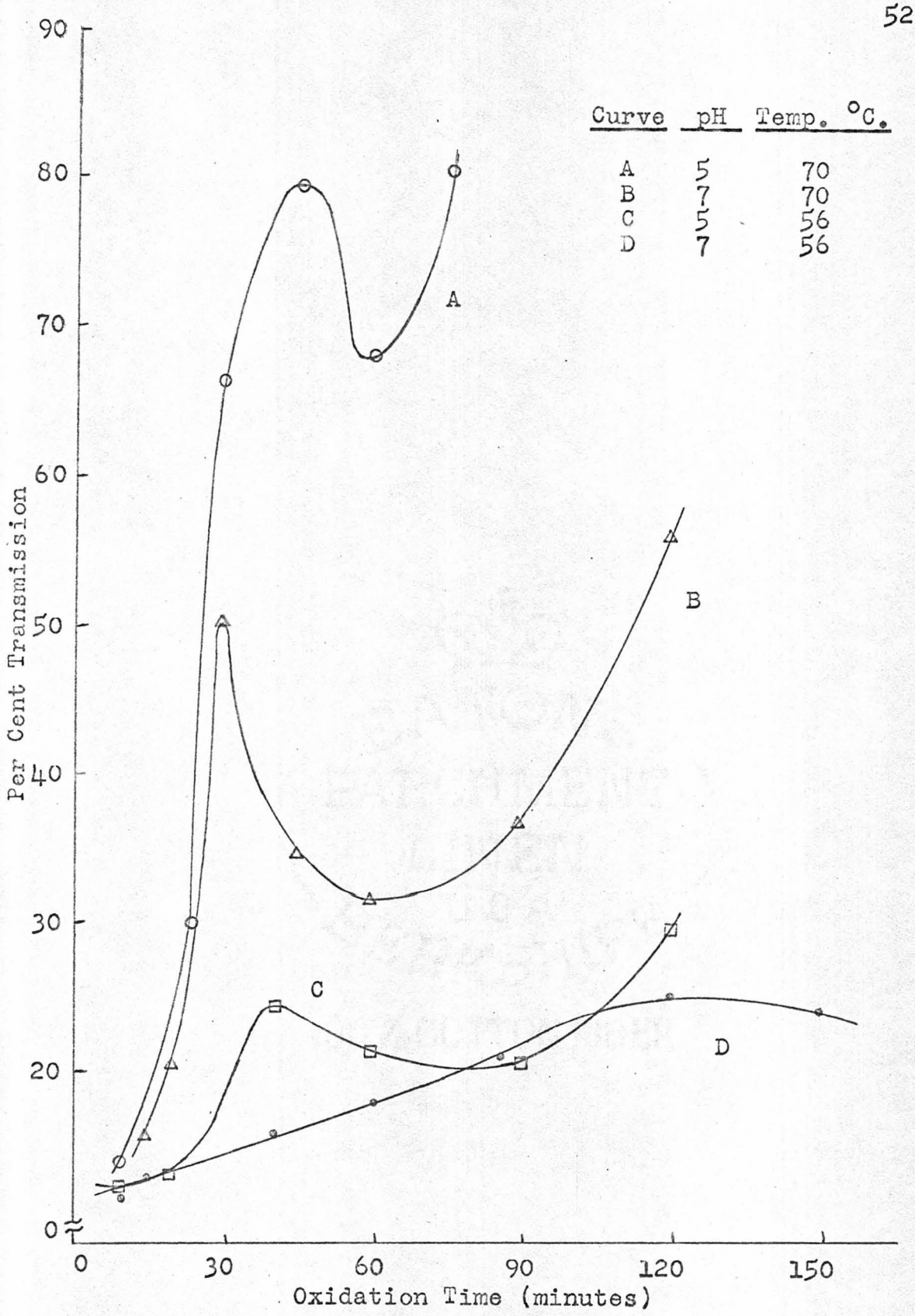



FIGURE 17

EFFECT OF TEMPERATURE, TIME, AND pH
(PERIODATE-GLUCOSE REACTION)

## V. DISCUSSION AND CONCLUSIONS

### Deviations From Beer's Law and Spectral Shifts

All studies indicate a very slight deviation from Beer's law for the iodinated fluorescein product and unreacting excess fluorescein at low concentrations (Figures 4,5,6,10). However, except for very low concentrations, the points do fall on a straight line. Small positive deviations from Beer's law for fluorescein in aqueous solutions are in accord with the literature (10).

Greater deviations are found to occur with the glucose determinations (Figures 15 and 16). But, except for the lowest concentrations, the plots are straight lines. Overoxidation may be a factor resulting in these larger deviations. An increased consumption of periodate decreases the amount of iodine produced, resulting in a lower absorbance than theoretically expected (see Section V, page 59.)

From the various spectral plots using the Beckman Model DB spectrophotometer, it is observed that the absorption maxima shift to slightly higher wavelengths with higher concentrations of the iodinated fluorescein product. The reverse is true for the unreacted fluorescein. In both instances the wavelength shift is in the direction toward the absorption maximum of the pure iodinated fluorescein and fluorescein, respectively, in solution. These shifts would be expected because of the overlap of the absorption

curves (see Figure 2.) Furthermore, the shift is insufficient to imply that additional iodination had occurred.

Iodination of Fluorescein

From Figure 7, page 32, it is evident that pH has very little effect upon the maximum absorbance of the iodinated fluorescein product between pH's of 6.4 and 7.8 in a phosphate-borate buffer. All studies indicate similar results utilizing the boric acid - borate buffer in the same pH range. In this pH range, the dianion of fluorescein would be the predominant species. Visual observation confirms the literature that no reaction occurs in acidic solutions and that the product becomes redder in color for pH's above 7 (15).

The iodination reaction is complete in 5 minutes in neutral solutions at room temperature. Although intense light does effect the product markedly (Table IV, page 34) normal laboratory lighting has virtually no effect up to 15 minutes (Table III, page 31.)

It is evident that the iodinated product does undergo photochemical conversion, especially under intense illumination. Since the wavelength does not shift to lower wavelengths as the intensity of the color diminishes with time, deiodination does not seem to occur.

It is possible that equilibrium conditions exist and

that the equilibrium is shifting slightly towards the unreacted fluorescein and iodine. This may be contradicted by graphical stoichiometric analysis shown in Figure 8, page 35. The curves seem to break sharply indicating a completed reaction (39). Another possibility is the partial reduction of the colored product to its colorless form. However, no species with sufficient reducing power are present in the reaction mixture. This observed behavior substantiates the findings of Karg (9). No further attempt was made to determine the specific cause for this behavior.

The effect of the chloride ion is negligible as would be expected (Table V, page 34) and concurs with the observed minor salt effect of the iodination of phenolic rings.

The spectral, graphical, and chromatographic stoichiometric studies all indicate that the iodinated fluorescein product is probably not erythrosin in the pH range employed. The spectral studies indicate the product may be diiodofluorescein, while the graphical studies seem to show that between 3 and 4 atoms iodine react per fluorescein molecule. A higher $R_f$ value for the iodinated product than that for erythrosin indicates fewer iodine atoms than 4. The possibility of the existence of a mixture of different iodinated fluoresceins is not indicated by the chromatographic studies. Also, the possibility that the graphical results are in error because of impure fluorescein is eliminated by the

chromatographic findings. Visually, the color of the iodinated product is orange-red while that of erythrosin is red. However, at higher pH's the color of the iodinated product becomes redder. Thus, pH appears to be very important in the iodination reaction. These findings are similar to those of Harley. (See Section II, page 7.)

Thus, the determination of the exact composition of the iodinated fluorescein requires further study. Of great importance for an analytical procedure, however, is the fact that the end product is reproducible under the same reaction conditions. The stability of the end product is likewise sufficient for analytical determinations.

Iodide-Periodate Reactions

Results of rate studies were as expected. Increased temperature hastens the completion of the reaction (Figure 11, page 40). An indication of a slight increase of the extent of reaction with increased iodide concentration is shown in Table VII, page 44. And, it would appear that the addition of fluorescein before the iodide-periodate reaction, rather than after, has a greater effect on the rate than increased iodide ion concentration.

Conclusions concerning the rate of reaction are complicated by the possible loss by vaporization of the iodine produced, especially at higher temperatures. To

decrease this loss, fluorescein was added to the reaction mixture before the iodide-periodate reaction was allowed to proceed. Thus, the iodine would react with the fluorescein as it was formed. Finally, it is apparent that after the maximum absorption has been attained, it decreases quite rapidly. Again, the possible shifting of the equilibrium to iodine and fluorescein would explain this behavior.

In acidic solutions, the iodide-periodate reaction is almost instantaneous at room temperatures. But, the use of acidic conditions would not have allowed the iodination of fluorescein as the iodine was formed. (See Section II, page 6.)

The determination of the stoichiometry of the iodide-periodate reaction in a manner similar to the graphical analysis of the iodination of fluorescein reaction was unsuitable. Another approach was successful (Figure 14, page 46.) The plot of the absorbances of the iodine produced in accordance with reaction C (Section II, page 9) compares very favorably with the absorption plot of equal amounts of iodine from an iodine solution. The slight differences may well be due to the fact the experiment was completed in two parts and conditions may not have been exactly similar. Thus, the reaction is shown to be reaction C as the literature states.

Periodate Oxidation of Glucose

The effect of temperature and pH on the extent of reaction is very marked (Figure 17, page 52). The increase of temperature causes the expected increase in the rate of oxidation. A decrease of pH also causes the rate to increase. The rate of oxidation is quite low at room temperatures.

Much experimentation resulted in the choice of a moderate temperature (about 56° C.) for the oxidation step. From Figure 17 it is evident that the rate of increase of percentage transmission with time appears to be quite low at 57° C. compared to 70° C. For the same reason, a pH of about 7 was chosen. In other words, the reaction is more controllable and predictable under these moderate conditions. This is substantiated by the literature (24,25, 29,32).

Since the subsequent iodide-periodate reaction required a more neutral solution, acidic solutions were avoided. (It would be possible to perform an acidic oxidation and then neutralize, but this would require another reagent addition.) An alkaline oxidation was avoided because the oxidation rate seemed slow and the subsequent iodide-periodate reaction proceeded more favorably under neutral conditions.

It is evident from Figure 17 that stoichiometric

studies would be complicated. Under conditions of higher concentrations of glucose and/or higher temperature, over-oxidation (the consumption of more periodate by glucose oxidation than theoretically possible) appeared to be causing considerable errors. No attempts were made to determine the cause of this overoxidation other than to try to decrease its magnitude and by changing from phosphate-borate to boric acid - borate buffer. Overoxidation is mentioned by various authors and various causes have been suggested. Possible causes are the use of phosphate buffers in neutral glucose oxidation (32), and the photodecomposition of periodate (36). As mentioned earlier, Warsi and Whelan have reported an abnormal uptake of the periodate in the intermediate steps of the oxidation.

To compensate for overoxidation at higher concentrations of glucose, an excess of periodate is required. However, the amount of excess periodate is necessarily limited by the lower allowable limit of percentage transmission produced by the reference solution containing no glucose. Stated otherwise, the upper limit of glucose which can be determined is decreased because of overoxidation.

Even with relatively high periodate concentrations, the oxidation reaction apparently is not complete, except possibly for very low concentrations of glucose. This is shown by the near adherence to Beer's law, the straight

line plots (Figures 16,17), and a study of Figure 17, page 52. For, if the lowest concentration of glucose was completely oxidized while the higher concentrations were not, Beer's law would not hold nor would a straight line plot result. For the concentration of glucose used (0.25 ug./ml.) in the studies shown in Figure 17, a stoichiometrically complete reaction would result in the liberation of about 0.5 ug. iodine. This corresponds to a transmission of about 85 to 95 per cent. Thus, it would appear that the most advanced reaction (pH of 5, temperature of 70° C.) is not complete after 45 minutes while the reaction with a pH of 7 and temperature of 56° is far from complete even after 4 hours.

Further study of Figure 17, page 52, tends to corroborate earlier rate and mechanism findings for the periodate oxidation of aldohexoses (25,30,31,36). The maximum peaks correspond to greater periodate uptake (and therefore less iodine produced) and may indicate the rapid, first step. The following decrease in percentage transmission may be explained by equilibrium shifts. The further rise may indicate the slower, second step. Another explanation for the alternate maxima and minima could be the abnormal uptake of periodate in the intermediate steps reported by Warsi and Whelan (30) and Khouvine and Arragon (29).

Precision and Error Studies

Precision studies of the concentration-absorption plots are shown in Table X, while the deviation in amounts of iodine or glucose corresponding to the deviations in percentage transmission are shown in Table XI, page 63. The standard deviations are calculated by summing the squares of the individual deviations from the mean, dividing by the number of measurements minus one, and exacting the square root of the quotient.

It is noted that whereas the various precision studies were done with different systems (each at a different per cent transmission level), the precision at each per cent transmission level for any one of the systems might be expected to be nearly the same. This is substantiated in that the precision for the glucose determination method is the same as the iodide-periodate study and lower than the iodine study rather than higher as might be expected because of the increased complexity of the glucose method.

Generally, the 505 mu. plots have a higher precision that the 484 mu. plots. This is most likely due to the fact that inaccuracies in pipetting equal amounts of fluorescein in each tube results in a greater error for fluorescein determination at 484 mu. than for the iodinated product at 505 mu.

For the determination of iodine and glucose, it is

TABLE X

PRECISION STUDIES

| System | Wavelength (mu.) | % Transmission | Mean % Trans. | Standard Deviation (% T) |
|---|---|---|---|---|
| A. $I_2(I^-)$ and fluorescein (4 ug. $I_2$/ml.) | 505 | 35.4, 35.1, 34.9 | 35.1 | ±0.4 |
| | 484 | 62.0, 60.5, 62.1 | 61.5 | ±1.2 |
| B. Iodide-periodate (equiv. to 8 ug. $I_2$ per ml.) | 505 | 14.5, 14.9, 14.5, 14.6 | 14.6 | ±0.3 |
| | 484 | 35.9, 36.2, 37.1, 36.0 | 36.4 | ±0.8 |
| C. Periodate-glucose (1 ug. glucose/ml. and 8.4 ug. $KIO_4$/ml. | 505 | 25.0, 24.7, 25.3 | 25.0 | ±0.3 |
| | 484 | 49.2, 50.2 50.3 | 49.9 | ±1.0 |

TABLE XI

ERROR ANALYSIS

| Iodine (ug./ml.) | Conc. Glucose (ug./ml.) | Wave-length (mu.) | Standard[a] Deviation (% Trans.) | Corresponding Deviation (ug./ml.) | Per cent Deviation |
|---|---|---|---|---|---|
| 2.0 | | 505 | - | - | (5)[b] |
| 4.0 | | 505 | ±0.4 | ±0.05 | 1.3 |
| 7.94 | | 505 | ±0.3 | ±0.1 | 1.3 |
| 4.0 | | 484 | ±1.2 | ±0.2 | 5.0 |
| 7.94 | | 484 | ±0.8 | ±0.25 | 3.1 |
| | 0.04 | 505 | ±0.3 | ±0.005 | 12.5 |
| | .1 | 505 | ±0.3 | ±0.004 | 4.0 |
| | .2 | 505 | ±0.4 | ±0.004 | 2.0 |
| | .5 | 505 | ±0.3 | ±0.036 | 7.2 |
| | 1.0 | 505 | ±0.3 | ±0.034 | 3.4 |
| | 1.5 | 505 | ±0.4 | ±0.036 | 2.3 |
| | .1 | 484 | ±1.0 | ±0.0075 | 7.5 |
| | .2 | 484 | ±1.2 | ±0.005 | 2.5 |
| | .5 | 484 | ±0.8 | ±0.07 | 14.0 |
| | 1.0 | 484 | ±1.0 | ±0.07 | 7.0 |
| | 1.5 | 484 | ±1.2 | ±0.64 | 4.3 |

[a] Taken from Table X.
[b] Estimated.

necessary to establish a reference straight line using at least two reference samples. For iodine, these points should be between 4 and 10 ug./ml. (Figure 4, page 26); for glucose, between 0.5 and 1.5 ug./ml. (Figure 15, page 47); or between 0.1 and 0.2 ug./ml. (Figure 16, page 49). Below these concentrations, Beer's law deviates. However, fairly good results can be obtained by using a reference point at a low concentration and the zero point to establish a straight reference line.

In the determination of iodine, the precision can be increased by plotting the points on a one-cycle semi-log graph paper of larger dimensions.

Reference line plots for the study employing the Spectronic 20 compare favorably with the others (Figure 6, page 28). While the concentration range is lower, the span of percentage transmission from the lowest to highest concentration of iodine is not as great as in the concentrations studied with the Beckman Model DB spectrophotometer. However, the precisions and accuracies do not appear to be as good as above.

Errors in colormetric results are minimized if transmissions are kept within the range of 10 to 75 per cent (40). The most favorable experimental conditions for photometric instruments is to determine the concentrations at 36.8 per cent transmission. At this point, the ratio of the relative

concentration error to the relative photometric error (provided the galvanometric current is directly proportional to light intensity) is at a minimum. It is noted that all studies done at 505 mu. (except for the Spectronic 20 study) fall around this point. But, the majority of studies done at 484 mu. have higher transmissions and may thus be considered to be less desirable. Also, it is noted that nearly all studies were done in the range of 10 to 75 per cent transmission.

Another method of determining the concentration range over which most accurate work can be done is by constructing a Ringbom plot (41). The plot is useful whether Beer's law is followed or not. This is a plot of absorbances (or percentage transmission) as the ordinate versus the logarithm of the concentration as the abscissa. The accuracy is greatest when the slope is at a maximum. The ratio of the relative concentration error to the relative photometric error, $E_p$, can be shown to equal 2.303 divided by the slope of the Ringbom plots. A Ringbom plot is shown in Figure 18 for the studies given in Figures 4, 6, 15, and 16. The values of $E_p$ are found in Table XII, page 67. The lines for glucose have a negative slope because glucose concentrations (and not iodine) were used.

A study of the plots will substantiate quite well the validity of the concentration ranges chosen over which a

| Curve | Wavelength (mu.) | Conc. Range (ug./ml.) | Figure |
|---|---|---|---|
| 1. Iodine | | | |
| A | 505 | 2-10 | 4 |
| B | 484 | 2-10 | 4 |
| C | 505 | .625-3.75 | 6 |
| 2. Glucose | | | |
| D | 505 | .04-.2 | 16 |
| E | 505 | .25-1.5 | 15 |
| F | 484 | .04-.2 | 16 |
| G | 484 | .25-1.5 | 15 |

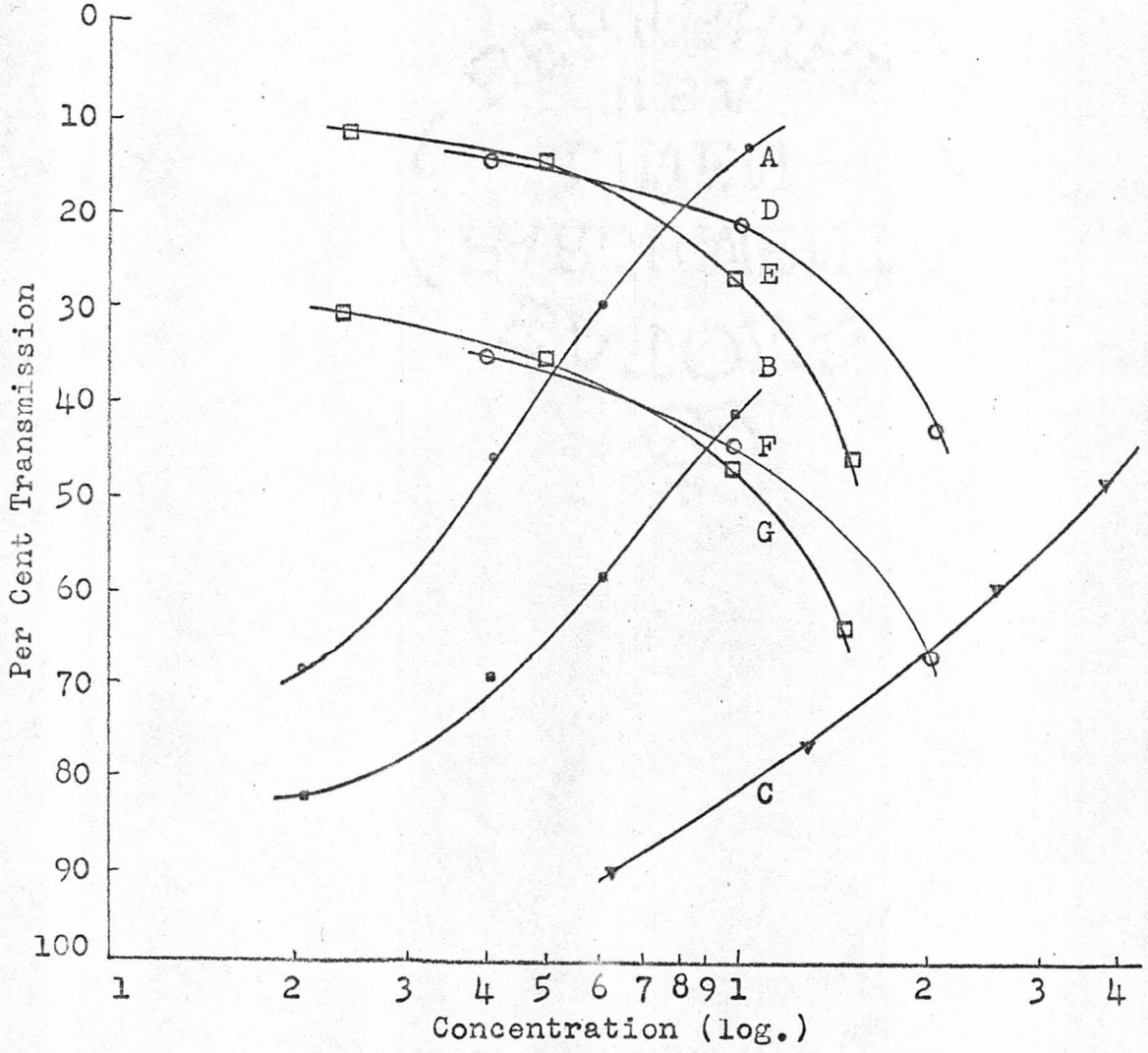



FIGURE 18

RINGBOM ERROR PLOT

TABLE XII

RINGBOM ERROR ANALYSIS

| System | Wavelength (mu.) | Conc. Range (ug./ml.) | Slope* | $E_p$ |
|---|---|---|---|---|
| 1. Iodine | | | | |
| | 505 | 0.625 - 1.25 | 41 | 0.056 |
| | 505 | 1.25 - 3.75 | 80 | .029 |
| | 505 | 2.0 - 4.0 | 75 | .031 |
| | 505 | 4.0 - 10.0 | 86 | .027 |
| | 484 | 2.0 - 4.0 | 42 | .055 |
| | 484 | 4.0 - 10.0 | 70 | .033 |
| 2. Glucose | | | | |
| | 505 | 0.1 - 0.2 | 67 | .034 |
| | 505 | 0.5 - 1.0 | 39 | .059 |
| | 505 | 1.0 - 1.5 | 109 | .021 |
| | 484 | 0.1 - 0.2 | 69 | .033 |
| | 484 | 0.5 - 1.0 | 40 | .058 |
| | 484 | 1.0 - 1.5 | 98 | .024 |

*Approximated from Figure 18.

standard reference line could be constructed. All $E_p$ values, except one, show that errors for the 505 mu. studies were less than those for 484 mu. Furthermore, these errors increased as the concentration of iodine or glucose decreased. Also, the method of determining precisions (as mentioned above) is shown to be quite valid with regard to these types of errors.

Besides the errors inherent in colorimetric instruments, manipulative and procedural errors must be considered. As mentioned, errors at 484 mu. are probably increased by the sensitivity of small amounts of fluorescein inaccurately pipetted. Clean reaction vessels, free from dust and lint, must be used. A working area free of other iodine studies is advisable because of the possibility of the volatilization of iodine, and possible contamination when working with micro-amounts. The contamination or decomposition of standard iodine and glucose solutions must be avoided for accurate work. The accuracy of the concentrations of the other solutions is not as critical. For glucose determinations, the reaction vessel must be well stoppered to prevent the possible loss of iodine by vaporization in the heated iodide-periodate conversion step. Also, critical for glucose determinations is the washing down procedure immediately after the periodate oxidation step. Most of these errors, as well as pipetting errors, practi-

cally compensate for each other when procedures are employed which are easily and exactly repeated during a particular determination. This is one of the advantages of this type of comparative colorimetric methods.

It is concluded from the precision studies and the Ringbom error analysis, that manipulative and procedural errors are not great (with the exception of the 484 mu. studies) and are nearly constant. It would also seem likely that the increased errors at low concentrations may be due to the inherent manipulative errors as well as to the increase in instrumental errors.

Quantitative Determination of Iodine

From Figures 4 and 5, and Table XI, it is evident that iodine in amounts between 4 and 10 ug./ml. of solution can be determined with good accuracy using the Beckman Model DB spectrophotometer. Below 4 ug. iodine per ml., accuracies are not quite as good when the standard reference plot for the higher concentration is used. Better accuracies are obtained for low concentrations by plotting an auxiliary reference line as described above in the error analysis (page 64). With the Spectronic 20, fair accuracies can be attained in the concentration range of 1.25 to 3.75 ug./ml.

The procedure described has extended Harlay's method

from a concentration range of 30 to 150 ug./ml. to 4 to 10 ug./ml. with good accuracy and to 1.25 to 3.75 ug./ml. with fair accuracy. Further, an instrumental procedure with a recorder is clearly advantageous to the visual colorimetric method used by Harlay.

The procedure which was developed through this study is rapid, requiring less than one hour for the entire operation. Only semi-micro quantities are necessary. The solutions required are easily prepared and are fairly stable. The iodinated end product is reproducible and of sufficient stability for an analytical procedure. The method is uncomplicated requiring a minimum of reagent addition. No special temperature control is necessary. The use of two absorption peaks provides two results for each concentration in each determination. The procedure does not require specially trained personnel. The only specialized equipment is a spectrophotometer or good colorimeter. Although other methods enable determinations of small amounts of iodine, these procedures generally require more costly equipment.

This procedure is limited to certain concentration levels. Thus, preliminary experiments are necessary to determine the proper dilution of unknown iodine solutions. Because Beer's law is not followed entirely, two standard concentration determinations are necessary for each series for accurate results.

It is very likely that micro-technics may be utilized if micro-cuvettes are employed. Micro-cuvettes may also provide the means in determining still smaller amounts of iodine by this method. Utilization of a more polar solvent or mixed solvent system may extend the concentration range to lower levels.

In summary, this method provides a means of determining micro-amounts of iodine with reasonably good precision and accuracy using readily available equipment.

Quantitative Determination of Glucose

From Figure 15 and Tables VIII and XI, it can be seen that glucose can be determined with good accuracy in amounts of 0.5 to 1.5 ug./ml. Below 0.5 ug./ml. poorer accuracies prevail. Figure 16, page 49, shows that good accuracies are possible between 0.1 and 0.2 ug./ml. when the periodate oxidation is carried out at 98° C. At the lower concentrations, better accuracies can be obtained by using an auxiliary reference line as described above.

The advantages and limitations described for iodine determination above also pertain to glucose determinations except for the factors of time and temperature. A series determination can be completed within 1.5 hours. A simple, thermostated water bath or boiling water bath is also required.

The described procedure, then is a fairly rapid,

uncomplicated means of determining micro-amounts of glucose in semi-micro reaction solutions with good accuracy. Although the periodate oxidation step is quite critical, good results are obtainable if reaction conditions are held constant for all reaction solutions.

It is very conceivable that other sugars and poly-hydroxy organic compounds, with some modifications, may be determined in like manner.

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