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Chemistry of glycerol dichlorohydrin as a reagent for the determination of vitamin A

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CHEMISTRY OF GLYCEROL DICHLOROHYDRIN AS A REAGENT FOR THE DETERMINATION OF VITAMIN A

by

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INTRODUCTION

The analysis of vitamin A by colorimetric methods has been the topic of many investigations in the past twenty five years. Numerous methods have been proposed, tested and finally discarded with the conclusion that they lacked specificity and/or the required sensitivity to measure small amounts of vitamin A in biological materials.

The method that appears to be the most accepted and most used in biological and investigational work is the so-called Carr-Price or antimony trichloride method. Although this method produces an intense blue color with vitamin A which permits testing of very small quantities of vitamin A, the color builds up to a maximum and then begins to fade within 5 to 10 seconds at room temperature. The color must be developed in the absence of moisture to avoid the formation of a suspension of antimony oxychloride which results in a false instrument reading. The color development and fading rate are dependent upon the intensity of light striking the reaction mixture. Many improvements in technique and apparatus for measuring the blue color have tended to minimize the disadvantages of the antimony trichloride method, but for the average analyst a reliable and sensitive method which can be used without numerous precautions and special techniques would be highly desirable.

Within the last four years a new colorimetric reagent, which has many desirable features for the measurement of vitamin A, has been introduced. The reagent is glycerol dichlorohydrin that is activated by vacuum distillation with a small amount of antimony trichloride. The violet color produced with vitamin A is stable for several minutes but has an extinction coefficient only about one-fourth that of the antimony trichloride color. Traces of moisture apparently do not interfere with the color reaction. The color may be measured with practically any type of colorimeter or spectrophotometer, and Sobel and Snow (211) reported a method of measurement which approximates the sensitivity of the antimony trichloride method. Because of the advantages of the activated glycerol dichlorohydrin method (214), it is being used in some analytical laboratories.

Regarding the use of activated glycerol dichlorohydrin, Dann (43) has stated that "the reagent appears more likely to displace antimony trichloride than any other which has yet appeared, and it deserves to be widely tested". As yet, very little information regarding the chemistry of the activation of glycerol dichlorohydrin or its reaction with vitamin A has been reported.

This investigation was initiated in an attempt (1) to determine the nature of the activation of glycerol dichlorohydrin with certain acids, metallic and nonmetallic halides,

acyl halides and other compounds, (2) to determine the structural requirements necessary in glycerol derivatives to produce good color when mixed with a vitamin A solution, (3) to determine the effect of various solvent-reagent ratios on the absorption spectra of the products of the reactions of vitamin A with activated glycerol dichlorohydrin, (4) to determine the effect of added impurities on the development of color, and (5) to determine the probable mechanism of the reaction of vitamin A with activated glycerol dichlorohydrin.

REVIEW OF THE LITERATURE

Discovery of Vitamin A

Although the medicinal use of cod liver oil was general in Western Europe by 1840 (83), it is only since the recognition of deficiency diseases that progress regarding the cause of avitaminosis has been made. Detailed accounts of early work dealing with this subject are presented in Sherman and Smith's *Monograph* (201) and the Medical Research Council's Survey of the Vitamins (125).

In 1913, McCollum and Davis (117) found that growth ceased prematurely in rats fed on a synthetic diet in which the fat supplied was lard. Upon the addition of butterfat or an ether extract of egg yolk to the diet growth was resumed and it was concluded that the effect might be due to the presence of complex organic lipins or substances associated with the latter. Osborne and Mendel (167, 168) observed that growth of rats was not maintained on a diet of purified foodstuffs and "protein-free milk" but was resumed on the addition of either butter or cod liver oil. Further work by McCollum and Davis (119-122) and Osborne and Mendel (169) indicated that two distinct types of food accessory factors are required for normal growth, one was named "fat-soluble A" and the other "water-soluble B". To simplify the nomenclature of these accessory factors Drummond (45) recom-

mended the use of the terms vitamin A and vitamin B.

Mellanby (126) demonstrated that rickets is a deficiency disease due to a shortage of an accessory substance which was considered to be fat-soluble A. Substances containing and associated with fat-soluble A were thought to be concerned with calcification processes. Cod liver oil possessed factors which would promote growth and would cure rickets. Although both of these properties were originally ascribed to the same factor, evidence that there was only a limited correlation between growth promotion and antirachitic activity soon accumulated. McCollum and coworkers (123) found that the vitamin A activity of cod liver oil could be destroyed by aeration for 20 hours at 100°C. while the antirachitic activity was unaffected. Lesné and Vagliano (110) came to similar conclusions. It was then concluded that cod liver oil contains two distinct food accessory factors which became known as vitamins A and D.

Properties of Vitamin A

Several vitamins A (A_1, A_2, A_3 (?), neovitamin A) have been reported but only vitamin A_1 will be considered here. It is the most abundant and biologically important of this group of vitamins.

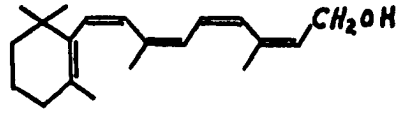
The chemical nature of vitamin A_1 (to be called vitamin A in this report) has been quite thoroughly investigated.

McCollum and Davis (118) in 1914 demonstrated that it could be extracted from saponified butterfat. Numerous investigators have since shown the vitamin to be concentrated in the nonsaponifiable fraction and many attempts to isolate it are based on this principle (see (83) and (187) for further discussion of this point).

The following properties of vitamin A have been established:

Crystalline form	pale yellow crystals
Empirical formula	$C_{20}H_{30}O$
Molecular weight	286
Boiling point	120-125° C. at 5 x 10^{-3}mm (85)
Melting point	63-64°C. (8)
Solubility	Soluble in most fats and organic solvents
Optical activity	none
Absorption maximum	325-328 mμ (172)
Absorption maximum of $SbCl_3$ reaction product	620 mμ
Absorption maximum of activated glycerol dichlorohydrin reaction product	555 mμ (213)
Fluorescence	green in ultraviolet light (215-217)

The chemical constitution of vitamin A has been established mainly by the work of Karrer and coworkers (98, 99). The formula they suggested has been confirmed by Heilbron, Morton and Webster (84) and is as follows:



Milas (141), Gridgeman (71), Heilbron (80) and Johnson (92) have reviewed the research on the synthesis of vitamin A and closely related compounds. Several more recent publications include the synthesis of vitamin A (198), vitamin A esters (90), and vitamin A ethers (78, 90, 142).

The vitamin occurs in nature in both the ester and free alcohol forms. The alcoholic form predominates in eggs (159) and normal blood serum (87, 177), while the ester is the principal form in colostrum and milk (176), fish, fowl and mammalian livers (68, 69, 177, 184) and fish liver oils (85, 183). The esters are thought to be more stable than the free alcohol (5, 9, 50, 85, 187). The decomposition of vitamin A is an oxidative reaction catalyzed by heat and light (15, 51).

The dietary deficiency of vitamin A may result in night blindness, poor growth, atrophy of epithelial tissues and tissues of the eye, increased lability to general infection and other difficulties. The physiology of this vitamin is adequately covered by several reviews (13, 83, 185, 187).

Development of Assay Methods

Biological Assay

Early studies have shown that the growth of rats is related to the vitamin A content of the diet. The prevention and cure of certain pathologic conditions has also been

attributed to vitamin A.

The growth method. As early as 1920 the growth method was introduced as a technic for assaying vitamin A in test materials (46, 170). Since then considerable experimentation has resulted in many refinements in the method. Nelson and DeWitt (160) have reviewed much of the literature leading to the development of the presently accepted biological assay.

The essential features of the method are as follows: Young rats weighing between 40 and 50 grams are fed a basal diet which supplies all essential nutrients needed for growth except vitamin A. When the animals cease to grow the vitamin A stores have been depleted. The animals are then placed in separate cages and fed a specified daily allotment of the test material as a supplement to the basal diet. During this test period individual weights are recorded at regular intervals. The increase in weight during the test period may be used as a measure of the vitamin A content in the material being tested. Several levels of the vitamin A-containing material are generally fed to matched groups of animals and the resulting growth is graded to the dose. The relation between dose and growth response is not linear but may be represented by a curve. Coward and her coworkers (37, 40, 41) have studied this relationship quite extensively.

Munsell (153) has reviewed much of the literature concerning the important variables affecting the accuracy of the

results obtained by the biological assay. The variables which have been investigated include: selection of a basal diet free of vitamin A and yet complete in all other factors; age, weight and dietary history of experimental animals; acceptable animal weights at the end of the depletion period; determination of suitable depletion criteria; length of the test period; use of females and males; method of administering test doses; number of animals required for an assay group; number of groups receiving graded doses of test material required to establish the relation between gain in weight and the vitamin A intake.

Coward (38) has described in some detail most of the important details of biological assay methods. The method adopted by the United States Pharmacopoeia is the one most extensively used at the present time. The procedure is described in considerable detail (180) and requires careful study for a full understanding of all the details. The description of the method is mainly for the purpose of law enforcement but for most routine assay laboratories much of the detail is not important.

In 1931, the Health Organization of the League of Nations (108) adopted a vitamin A standard. This consisted of a preparation of carotene made from carrots and the International Unit was defined as the activity of one microgram of the standard. The same organization in 1934 (218) changed

the standard to pure β-carotene and the unit was defined as the vitamin A activity of 0.6 microgram of the standard. Gridgeman (70) has reviewed the pertinent literature regarding the calibration of cod liver oils in the preparation of U.S.P. Reference Cod Liver Oils.

Guerrant et al. (74) demonstrated that vitamin A acetate offered definite advantages, as a vitamin A standard, over U.S.P. Reference Cod Liver oil and over commercially available crystalline vitamin A alcohol and β-carotene. Chilcote, Guerrant and Ellenberger (30) have recently investigated the stability of vitamin A acetate when stored under various laboratory conditions. They reported that vitamin A acetate in the crystalline state and when dissolved in a refined cottonseed oil is sufficiently stable to warrant its use as a vitamin A standard. The U.S.P. Vitamin A Reference Standard (232) now in use is a solution of crystalline vitamin A acetate in cottonseed oil contained in a special gelatin capsule. This oil solution has a biological potency of 10,000 U.S.P. units of vitamin A in each gram. Ellenberger, Guerrant and Chilcote (49) have made comparisons of the extinction coefficients and the relative biological potencies of the U.S.P. Reference Cod Liver Oil no. 3, the new Vitamin A Reference Standard, crystalline vitamin A acetate and the International Standard.

Gridgeman (70) and Heilbron et al. (83) have reviewed the subject of errors that may be encountered in the bio-

logical assay procedure.

Single-dose growth method. The growth method involves feeding vitamin A supplements at periodic intervals for several weeks and the estimation of vitamin A potency is based on weight gain over the test period. However, this technic may be modified by administration of a single dose of test material to vitamin A-depleted animals. The survival period of the animals may be taken as a measure of the vitamin A content in the test material (161, 200). Sherman and Todhunter (202) applied this assay technic by using a curve relating weight to survival time.

Vaginal smear method. One of the earliest vitamin A deficiency symptoms to appear is abnormal estrus and keratinization of vaginal epithelial cells in the female rat. Evans and Bishop (59) showed that this condition could be corrected by feeding vitamin A. Several investigators (7, 39, 143, 182) have attempted to apply this phenomenon as the basis of a quantitative method for assaying vitamin A potency of test materials. Although the method has several advantages, it does not lend itself for general adoption, because of the rather tedious procedure and because the response to a small dose is difficult to evaluate. Coward (38) presents a good review of the research concerning this method.

Liver storage method. Guggenheim and Kock (75) have recently proposed a biological assay based upon the fact that

the amount of vitamin A stored in the liver varies directly with the dose fed. Foy and Morgareide (64) made a study of this method and report several advantages over the U.S.P. curative-growth method: a short depletion period of six days; a very short dosing period of two successive days with 0.1 ml. of oil containing the vitamin A; maintenance of experimental animals in good health since they grow normally throughout the test; good specificity and precision; and economy. The principal disadvantage is in the high sample potency required. Further work on liver storage of vitamin A fed to depleted rats has been reported by Guerrant (73).

Physico-chemical Methods

As more information regarding the physical and chemical properties of vitamin A accumulated the application of some of this information in the development of new quantitative assay methods was forthcoming. The methods which have been most extensively studied are the selective ultraviolet absorption and the colorimetric reactions of vitamin A and compounds closely related to vitamin A.

The ultraviolet absorption method. As early as 1925 Takahashi and associates (225) made a study of an active vitamin A preparation (Biosterin) and stated that this material showed a selective absorption at 320 mμ. Morton and Heilbron (147) showed that vitamin A in fish liver oils has a selective absorption band at 328 mμ. Crystalline vitamin A has

one symmetrical absorption band with a high extinction coefficient (E 1%,1cm) at a peak which occurs between 320 and 330 mμ (10, 88, 246). Baxter and Robeson (10) report a mean extinction coefficient of 1750 ± 21 for nine different crystalline vitamin A preparations in ethanol solution. The extinction coefficients (E 1%,1cm) of vitamin A esters are lower than that of vitamin A alcohol (9).

Gridgeman (70) has summarized the available data on the effect of the various solvents on the extinction coefficient at 325 mμ. Pure ethanol, isopropanol and cyclohexane appear to be satisfactory solvents while chloroform invariably gives a lower extinction coefficient.

That there is good correlation between absorption at 328 mμ and the biological activity of oils and concentrates has been demonstrated by several groups of investigators (47, 60, 115, 147, 174). Certain oils, however, display at 328 mμ extraneous absorption which may be eliminated for the most part by determining the absorption on the nonsaponifiable fraction (40, 124, 150).

It is quite generally agreed that the principal source of error in the estimation of vitamin A in various materials by this method is extraneous absorption. Oser and his coworkers (174) suggested that there is more likely to be an agreement between the biological assay and the spectrophotometric assay of a fish liver oil when its extinction ratio

$E_{300/328}$ is less than 0.72. This is based on a quantitative estimate of the departure of the sample curve from that of pure vitamin A. Morton and Stubbs (149, 150) have recently devised a mathematical method for correcting absorption curves for the extent of deviation from that of true vitamin A. Corrections can be made for distortion of the shape of the curve and displacement in its height by irrelevant substances. Oser (171) has constructed a nomogram which facilitates correction of vitamin A absorption curves.

Recently Servigne, Pinta and Montgareuil (199) have advocated a new technic for the removal of vitamin A in oils for spectrophotometric analysis. The oil is saponified and the unsaponified material is extracted with petroleum ether. The extract is passed through a column containing iron filings which adsorb the vitamin A. After elution with 60% ethanol the vitamin A is measured by its ultraviolet absorption over the range of 300 to 350 mμ.

Little (111) suggested using the method of destructive irradiation for the determination of vitamin A in fish liver oils and concentrates which have extraneous absorption at 328 mμ. The validity of destructive irradiation technics is dependent upon (1) complete destruction of vitamin A at 328 mμ and lack of absorption by its decomposition products and (2) absence of any change in absorption of substances other than vitamin A. These conditions were met when filtered ir-

radiation which did not contain wavelengths coincident with absorption maxima of substances other than vitamin A were used. The method was used for the determination of vitamin A in oils, liver and muscle tissue. This principle has been applied in the analysis of butter (157), margarine (158) and blood serum (12, 113).

Vitamin A in the presence of carotenoids may be determined spectrophotometrically by using other technics. Gillam (67) has assayed butter by making a correction for the contribution of carotenoids to the total absorption at 325 mμ. Awarapa, Mattson, Mehl and Deuel (4) have applied a chromatographic method for the removal of vitamin A from an aliquot of a sample. The absorption of light is measured at 300, 322, and 350 mμ before and after the chromatographic treatment. The vitamin A content is calculated from the difference in readings at 322 mμ. The method is reported to be applicable to the determination of vitamin A in animal tissues and vegetable oils low in vitamin A and containing appreciable amounts of nonsaponifiable materials. Müller (151) has reported a chromatographic separation of vitamin A alcohol, vitamin A ester and β-carotene and their spectrophotometric determination. Wilkie (238) has reviewed the results of collaborative chromatographic spectrophotometric estimation of vitamin A in margarine.

Munsell (153) and Dann (43) have reviewed the types of instruments used in the spectrophotometric method. These include various types of spectrophotometers, spectrographs and the Hilger vitameter which is an instrument developed for the express purpose of determining vitamin A in fish liver oils. The instrumental error in the ultraviolet absorption method has been adequately summarized (43).

The factor for conversion of the observed extinction coefficient of a sample to its biological potency has been discussed in numerous articles and the subject is too confusing to warrant a thorough discussion here. Gridgeman (70) presents a rather complete coverage of this topic. Other authors (38, 43, 146, 153) have reviewed the subject to some extent. Recently Ellenberger, Guerrant and Chilcote (49) calculated conversion factors of twenty-seven representative fish oils (nonsaponifiable fraction) in terms of the present U.S.P. vitamin A reference standard. These ranged from 840 to 2240 with a logarithmic mean of 1500. The variability of conversion factors indicates that no single factor would be readily applicable to all fish oils.

A rather complete discussion of the ultraviolet absorption method for determining vitamin A has been published by the Association of Vitamin Chemists, Inc. (3).

Colorimetric methods. Color tests for vitamin A have been studied quite extensively for over twenty five-years,

but actually a test used as long as sixty years ago to indicate the quality of cod liver oils apparently depended upon the vitamin A content of the oil (33). As early as 1920, Rosenheim and Drummond (188) suggested a possible relationship between the color reaction of cod liver oil with concentrated sulfuric acid and the fat-soluble accessory factor. Much investigation since that time has revealed a number of reagents which give colorimetric reactions with vitamin A. Rosenheim and Drummond (189) reported color reactions of arsenic trichloride, dimethyl sulfate, trichloracetic acid, acetyl chloride and benzoyl chloride (the last two in the presence of $ZnCl_2$) with cod liver oil and suggested the use of arsenic trichloride in chloroform as a colorimetric reagent for the quantitative estimation of vitamin A.

Fearon (62) claimed that good color developed on treating oils containing vitamin A with a petroleum ether solution of trichloracetic acid in the presence of pyrogallol or other polyphenols. This test was further investigated (239) and later shown to be non-specific for vitamin A (191, 192, 240).

A number of color reagents were systematically studied by Carr and Price (25). These included arsenic trichloride, trichloracetic acid, stannic chloride, ferric chloride, aluminum chloride, silicon tetrachloride, antimony trichloride and phosphorus oxychloride. Antimony trichloride dis-

solved in chloroform was recommended and its advantages listed. Several early investigators (32, 40, 56, 241) made comparisons of the antimony trichloride test with other color tests and the general conclusion was that the former was the most satisfactory. The blue color developed by the reaction of vitamin A with antimony trichloride in chloroform was measured in the early investigations by matching the color against standard tinted glasses in a Lovibond tintometer (190). Further discussion of the antimony trichloride test will be given later.

Several variations of the antimony trichloride (Carr-Price) test have been suggested. Morton (145) proposed the addition of 7-methylindole to the reaction mixture to cause alterations in the absorption spectrum of the pigment which have better correlation between the ultraviolet absorption and the color reaction. Rosenthal and associates (193, 194, 196) added catechol or guaiacol before the antimony trichloride and heated the mixture for about one minute to obtain a rather stable violet-red color. Rosenthal and Szilárd (195), Tompkins and Bolomey (228) and Sycheff (223) have applied the Rosenthal reaction to quantitative measurements of vitamin A. Gutzeit (76) reported the use of hydroxylamine hydrochloride in the antimony trichloride test.

Antimony pentachloride has been used both in qualitative (226) and quantitative analysis (14) of vitamin A in

liver oils. Chloroform solutions of 1% and 2% antimony pentachloride were reported to give weaker but more stable blue colors than a saturated antimony trichloride solution. Troitskiĭ (229) made a spectroscopic study of the color reaction of antimony pentachloride with vitamin A and related compound.

Still other color tests have been reported but not applied in quantitative estimation of vitamin A. Takeda (226), Ueno, Ota and Ueda (231), Schaltegger (197) and Pacini and Taras (175) list a number of tests used strictly on a qualitative basis. A number of investigators (57, 104, 106, 116, 127, 138, 224, 234, 245) have described the formation of color when vitamin A comes in contact with certain acid clays.

Several new colorimetric tests have been suggested. Nassi (156) devised a method which depends on the interaction of vitamin A with a chloroform solution of diphenylamine in the presence of ferric chloride and concentrated hydrochloric acid to form a blue-green color with a maximum intensity after 30 minutes. Gridgeman (72) reported little success with this method. Robin (186) suggested the reaction of vitamin A with the Liebermann-Burchard reagent to give a blue-green color and recommended the use of acetic anhydride to make the reaction mixture homogeneous. The use of glycerol dichlorohydrin as a colorimetric reagent was introduced

in 1945 (63, 212), and full discussion of this reagent will be given later. The most recent color reaction for vitamin A is similar to the Obermayer's test for indican which involves reaction with ferric chloride in concentrated hydrochloric acid solution (26).

The antimony trichloride method. The antimony trichloride method was originally proposed by Carr and Price (25) and is based on the measurement of the unstable blue color formed by the interaction of antimony trichloride and vitamin A.

Some early studies of the antimony trichloride reaction with oils (163, 164, 243) showed that the color was not proportional to the concentration of vitamin A; however, results obtained with the nonsaponifiable fraction gave a more linear relationship (40, 206, 242). These values checked reasonably well with the biological method (40, 107, 206).

Much of the early spectrographic data concerning the color developed by reaction of vitamin A with antimony trichloride has been reviewed by Munsell (153). Under suitable conditions it was found that the absorption at certain wave lengths were altered by changing concentration, temperature or by adding certain chemicals (19, 55, 145, 165). It is now quite generally agreed that vitamin A in any of its forms possesses a single strong absorption band at 620 mμ.

Although the so-called Carr-Price reagent has been widely used, it has several disadvantages which have caused investi-

gators to seek new and better colorimetric reagents. It is corrosive, poisonous and hygroscopic, which make it unpleasant to handle. The color produced is unstable and begins to fade within a few seconds after its formation.

Ender (56) demonstrated that the blue color is extremely evanescent at room temperature but is stable for hours at low temperatures (-40°C.). The stability of the color is influenced even by small temperature changes, an increase of 10°C. above ordinary room temperature makes the color fade more quickly while a corresponding fall in temperature has the opposite effect. Caldwell and Hughes (21) made a study of the effect of temperature on fading of the antimony trichloride colors of vitamin A and the common carotenoid pigments. The rate of fading of the vitamin A-antimony trichloride color is strongly accelerated by increase in temperature, but the carotenoid-antimony trichloride colors develop and fade much more slowly.

The time required for the blue color to reach a maximum is influenced by the type of sample tested, the concentration of vitamin A, the intensity of the incident light beam and also by the degree of damping of the galvanometer used in the instrument employed in the measurement of the color (3). Caldwell and Parrish (22) found that the lowest rate of color loss was obtained with instruments employing a minimal light source. A special cuvette holder has been devised which per-

mits the use of the antimony trichloride method in the Beckman spectrophotometer (94).

Other factors influencing the fading include the age of the reagent (44) and the particular batch of antimony trichloride being used (87).

Although certain carotenoids give a blue color with antimony trichloride, Caldwell and Hughes (20) demonstrated that vitamin A possesses chromogenic powers 10-to 25-fold greater than the common carotenoids. In the measurement of vitamin A in the presence of carotenoids, an appropriate correction may be made by determining the contribution of the carotene blue color to the total blue color measured at 620 mμ (3, 44, 144). The necessity for the calibration of each photometer for the determination of vitamin A, as well as the correction factor for the presence of carotenoids has been adequately discussed by Caldwell, Parrish and Shrenk (23).

Certain substances interfere with the color reaction. Corbet, Geisinger and Holmes (36) have identified and tabulated a number of these and stated that the most significant characteristic associated with substances producing colors by themselves with antimony trichloride is unsaturation. Norris and Church (162) stated that oleic acid and unsaturated oils accelerate the rate of fading of the blue color, while Emmerie (54) indicated that certain unsaturated fatty acids (oleic and linoleic) do not inhibit the color reaction.

The presence in natural products of substances which interfere with the measurement of the antimony trichloride-vitamin A color has been recognized by a number of workers (12, 18, 35, 42, 54, 65, 81, 155, 165, 166, 173, 178).

One method of correcting for the presence of substances which modify the intensity of the blue color is the increment method of Oser, Melnick and Pader (173). By adding a known increment of vitamin A to a chloroform test solution the standard is subjected to the same inhibitory effect as the vitamin A originally present. This method is applicable only when the instrument used for measurement of color gives a linear response over the assay range.

The most generally used method for removing most of the interfering substances is saponification prior to extraction of vitamin A (12, 105, 178).

Under special conditions the removal of certain of the interfering pigments from vitamin A extracts prior to reaction with the antimony trichloride reagent has been successful. Methods employed include chromatography (18, 34, 35) and precipitation (16).

Meunier and Rauol (137) made comparisons of the determinations of vitamin A in fish liver oils by the antimony trichloride reaction and ultraviolet spectrography and found that in general the results were in fairly close agreement and no constant difference was evident. Differences as high as 23% were occasionally observed.

Kinetic studies of the antimony trichloride reaction have been reported by Meunier and Rauol (135, 136).

Several modifications have been suggested to improve the method of measurement of the blue color. Hock (86) devised a micromethod which permits a photographic recording of the Carr-Price reaction so as to eliminate errors due to fading. Urban, Milder and Carruthers (233) have developed a micromethod for simultaneous determination of both vitamin A and carotene on the same sample at 0°C. They claim the method is over one hundred times as sensitive as that of Dann and Evelyn (44) for measuring vitamin A and carotene in blood. Gibson and Taylor (66) have described a dynamic method which involves measuring the color in a flowing mixture so that the time of observations are unaffected by the transient nature of the color reaction. Hock and Kaplan (105) suggested the simultaneous measurement of the blue color on both standard and unknown, cancelling errors due to fading.

The antimony trichloride reagent forms blue colors with other substances related to vitamin A, including subvitamin A_1, anhydrosubvitamin A_1, anhydrovitamin A_1, vitamin A_2 and anhydrovitamin A_2 (52), axerophten (100), isoaxerophten (97), divitamin A ether (129) and kitol (53).

Several publications (3, 11, 102, 227) have devoted considerable space in discussion of the antimony trichloride method which includes the application of the method to an-

alysis of biological materials, reasons for low results, precautions necessary, and other points that should be considered by anyone who contemplates use of the method.

The glycerol dichlorohydrin method. Sobel, Mayer and Kramer (208) first suggested the use of glycerol dichlorohydrin as a new colorimetric reagent for vitamins D_2 and D_3. The reagent gave satisfactory color reactions in the presence of acetyl chloride or other halides of acid nature.

The use of glycerol dichlorohydrin as a colorimetric reagent for vitamin A was reported in 1945 almost simultaneously by Feinstein (63) and Sobel and Werbin (212). The former indicated that glycerol dichlorohydrin reacted with vitamin A in either ethylene dichloride or chloroform. The reaction mixture was 5 ml. of reagent containing 2 drops concentrated hydrochloric acid and 10 ml. of a chloroform solution containing 600 to 3000 U.S.P. units of vitamin A. The mixture was shaken 5 minutes then after 30 minutes the blue-green color was read in a photoelectric colorimeter using a 660 mμ filter. The vitamin A content in the test solution was then determined by reference to a standard curve. The absorption spectrum of the resulting color showed four maxima between 400 and 800 mμ within 10 to 20 minutes after the start of the reaction. These peaks were located at approximately 440, 580, 640 and 740 mμ with a deep minimum density at 500 mμ. Without hydrochloric acid in the reagent an ab-

sorption maxima was observed at 560 mμ and a low density value at 620 mμ.

Sobel and Werbin (212) proposed the use of Eastman, practical grade, glycerol dichlorohydrin with no special precautions. Upon addition of the reagent to a chloroform solution containing vitamin A an immediate but rapidly changing blue color developed which had an absorption maximum at 625 mμ (similar to but not identical with the antimony trichloride-vitamin A color). The blue color was soon replaced by a violet color with an absorption maximum at 555 mμ which was reported to be stable from 2 to 10 minutes after the initial mixing of vitamin A and the reagent. The advantages of this method over the antimony trichloride technic are the greater stability of the color produced and the stability of the reagent. The chief disadvantage is that the extinction coefficient at 555 mμ is about one-fourth that of the antimony trichloride blue color. The interference due to carotene was reported to be similar to that encountered in the Carr-Price reaction. The reaction between carotene and the reagent produces a colored product with absorption maxima at 475 and 625 mμ with minima at 550 and 700 mμ. Agreement between the glycerol dichlorohydrin and antimony trichloride methods was reported.

In 1946 Sobel and Werbin (213) reported that glycerol dichlorohydrin from other firms, and most of that from East-

man Kodak, did not give color when added to vitamin A dissolved in chloroform. When these dichlorohydrins were distilled with 1 to 5% antimony trichloride at 4- to 40-mm. pressure, the activated products reacted with vitamin A. The concentration of antimony calculated as the trichloride in the activated reagents varied from a trace to 0.67%. However, antimony trichloride per se added in levels ranging from 0.1 to 1.0% did not cause activation of glycerol dichlorohydrin. Activated and practical glycerol dichlorohydrin reacted similarly with vitamin A but gave different reactions with carotene. The activated reagent was reported to be relatively stable for two months or more when stored in the absence of light. There was no appreciable difference in the absorption curves obtained over the range of 400 to 750 mμ when the activated reagent reacted with vitamin A alcohol or vitamin A acetate. The color resulting from the reaction of vitamin A and activated reagent has an absorption maximum at 555 mμ. The color obeys Beer's law over a wide range. The interference of vitamin D_2, ergosterol, 7-dehydrocholesterol and cholesterol is negligible, while that of carotene ranges from 7.35% with the Beckman spectrophotometer to 11% with the Coleman spectrophotometer. The absorption curve of the carotene-activated glycerol dichlorohydrin (GDH) color increases quite rapidly from 700 to 900 mμ whereas the vitamin A color no longer absorbs above 630 mμ. By taking readings

at 555 and 800 mμ it is possible to measure both vitamin A and carotene. Traces of moisture present on the most humid days do not affect the vitamin A-GDH color.

Sobel and Werbin (214) made a comparison of the spectrophotometric, antimony trichloride and activated glycerol dichlorohydrin (GDH) methods for estimating the vitamin A in fish liver oils. On the whole, it was concluded that the agreement between the values obtained by the GDH and antimony trichloride methods was close. Most of the values obtained by the ultraviolet absorption measurements were higher than the corresponding results obtained by the other two methods. Taking the values obtained by the GDH method on whole oils as 100, the per cent deviation of the antimony trichloride values were 1.63% lower while the E (1%,1cm) x 2000 values were 17.11% higher on oils not giving atypical colors. On the nonsaponifiable fractions the per cent deviations of the antimony trichloride values were 4.11% lower and the spectrophotometric values were 26.25% higher. Whole oils analyzed by the GDH method tended to give results which were closer to the nonsaponifiable values than the other two methods.

Activated glycerol dichlorohydrin has been used for the estimation of vitamin A in human serum (211). Because of the stability of the GDH-vitamin A color it is possible to measure the light absorption in a horizontal cell with a 5

cm. light path rather than in the regular Coleman cuvette which has a 1.3 cm. light absorption path. The color can be read more accurately than the $SbCl_3$ color, which required a test-tube type cuvette for rapid reading. A modification in instrumentation, the use of a 555 mμ filter in place of the PC-4 filter in the Coleman universal spectrophotometer was suggested. The change increased the sensitivity to that of the Beckman spectrophotometer. The interference of carotene was found to be higher than with the $SbCl_3$ reaction when light absorption measurements were made with a wide band width of light like that given by filter photometers or the Coleman spectrophotometer. In a study of sixteen sera, the vitamin A values resulting from the determination with GDH on whole sera were higher than the $SbCl_3$ results on the same samples. Good agreement, however, was found between saponified serum analyzed with $SbCl_3$ and both whole and saponified sera estimated with GDH. This indicated that saponification is not necessary when GDH is used as the colorimetric reagent for the estimation of vitamin A in human serum. However, for the most valid results, particularly when high values are anticipated, they suggested saponification of serum before extraction. Vitamin A values with and without anhydrous precautions were in good agreement. A further simplification of the analytical procedure which involved measurement of carotene at 800 mμ following its reaction with

GDH was investigated. This method of measuring carotene was only slightly less precise than the measurement at 440 mμ in petroleum ether extract, and the use of long wave lengths (830 mμ) was suggested as a means of improving the precision. The vitamin A and carotene values in the final simplified method (no anhydrous precautions and carotene measurement at 800 mμ) compared favorably with the conventional method. Directions for the analysis of small quantities of serum (1 ml.) were presented along with those for the preparation and use of calibration charts for vitamin A, carotene and carotene interference. The method may be used on the Coleman and Beckman spectrophotometers, filter photometers and visual colorimeters.

Activated glycerol dichlorohydrin has been employed as the colorimetric reagent for the estimation of vitamin A in other biological materials. Sobel, et al. (210) used it in the analysis of rat blood serum. Squibb (219) and Squibb, Cannon and Allen (220) applied it in the measurement of vitamin A in blood plasma from lactating dairy cows; the procedure used was essentially that recommended by Kimble (103) except for the substitution of GDH for antimony trichloride as the colorimetric reagent. Recoveries of 95.9 to 100 per cent of natural vitamin A ester added at four different levels were reported. Murley and associates (154) employed this reagent for measuring vitamin A in blood plasma from

young dairy calves being fed various filled milks. Fresh whole milk has also been analyzed for its vitamin A content by using GDH (221); the method used was essentially that of Boyer, et al. (17) except for the substitution of GDH for antimony trichloride.

In an investigation of the effect of dispersion on the absorption of vitamin A and carotene by young dairy calves, Yang (244) employed GDH for the estimation of both vitamin A and carotene in the blood plasma. Vitamin A was measured at 555 mμ and carotene at 950 mμ in the Beckman model DU spectrophotometer. Part of the analyses were made on whole and part on saponified plasma samples. The latter appeared to give more acceptable results.

Allen, Wise and Jacobson (1) found that certain substances in the blood plasma from some young dairy calves interfered with the development of the GDH-vitamin A and GDH-carotene color reactions. Saponification with freshly prepared 1 N KOH in 95% ethanol prior to extraction with Skellysolve A removed or counteracted most of these inhibitory substances. Recovery of vitamin A added as internal standard was not always good when the plasma was not saponified, but was generally excellent in the cases of saponified plasma samples. Calves receiving a whole milk diet which was supplemented occasionally with vitamin A or carotene concentrates appeared to have more inhibitory substances in

their blood plasma than calves on other diets. The authors recommended that saponification be included in routine analysis for vitamin A and carotene in calf blood plasma. Nonsaponification and saponification procedures were described.

Antoniani and Artom (2) reported the use of glycerol 1,3-dichlorohydrin in the colorimetric measurement of vitamin A in an oil concentrate. Their procedure involved addition of a 30% solution of antimony trichloride in chloroform to a mixture of a chloroform solution of the oil and glycerol dichlorohydrin. The color was measured in a spectrophotometer after 3 minutes. The reagent was used to improve the intensity and performance of the color obtained with antimony trichloride.

Müller (152) made a comparison of the antimony trichloride and GDH methods for estimating vitamin A. The specificity of both color reactions was found to be approximately the same. The advantages of the new reagent are the good stability of the color reaction between 3 and 10 minutes and in the low susceptibility to small temperature variation. Between 15° and 30°C. the extinction coefficient increases about 0.5% per degree rise in temperature. Its disadvantages are that strong illumination and long reading time in the step-photometer result in a distinct fading of the color. Because of the sensitivity of the reaction mix-

ture to light it was suggested that the development of color should be in the dark or diffuse light up to the time of measurement and that the measurement should be made as quickly as possible (within 30 seconds).

Wall and Kelley (237) made a study of the application of the vitamin A-GDH reaction to the determination of vitamin A in fortified poultry mashes. Certain substances present in both unsaponified and saponified extracts suppressed or interfered with the color reaction of vitamin A and GDH. An adsorbent mixture of 3 parts Hyflo Super-cel and 1 part activated magnesia No. 2641 removed most of these substances from the feed extract when the length of the adsorption column, volume of eluant and quantity of the sample adsorbed were carefully regulated. False vitamin A reaction with GDH was observed in the extract of a mash containing high proportions of fish meal and distiller's solubles. The method was found to be applicable for routine analysis of feeds which contain over 1500 I.U. vitamin A per pound. The disadvantages of the procedure were (1) an empirical correction formula was necessary in calculating the vitamin A content of the feed and (2) the method could not be applied to the evaluation of free vitamin A alcohol since the vitamin would be strongly adsorbed through the OH group.

Activated glycerol dichlorohydrin has been employed as a colorimetric reagent for estimating vitamin D_3 (24), er-

gosterol (58) and for distinguishing between 7-hydroxy-cholesterol and 7-dehydrocholesterol (209).

Chemistry of the Vitamin A Determination

This discussion will be limited to a presentation of the known chemistry of the antimony trichloride and glycerol dichlorohydrin reactions with vitamin A.

The Antimony Trichloride Method

As early as 1931, Heilbron, Gillam and Morton (81) proposed the possible formation of the compounds vitamin A.$SbCl_3$ and vitamin A.$2SbCl_3$ since antimony trichloride was known to form certain double compounds, $C_6H_4(CH_3)_2 \cdot SbCl_3$, $C_6H_4(CH_3)_2 \cdot 2SbCl_3$, etc. They postulated that certain inhibitors could remove $SbCl_3$ from the sphere of action with vitamin A and thus modify the equilibria:

$$\text{vitamin A} + SbCl_3 \rightleftharpoons SbCl_3 \cdot \text{vitamin A}$$

$$SbCl_3 \cdot \text{vitamin A} + SbCl_3 \rightleftharpoons 2SbCl_3 \cdot \text{vitamin A}$$

No actual proof of these complexes was given.

In 1932, Ender (56) found that a blue oil formed by the reaction of vitamin A concentrate with antimony trichloride at low temperatures (-30° to -50°C.). The chloroform layer was decanted and an ice-cold mixture of water and 10% KOH was added until the blue color disappeared. The mixture was extracted with benzene, and the benzene removed by vacuum distillation. A highly unsaturated reddish-yellow oil re-

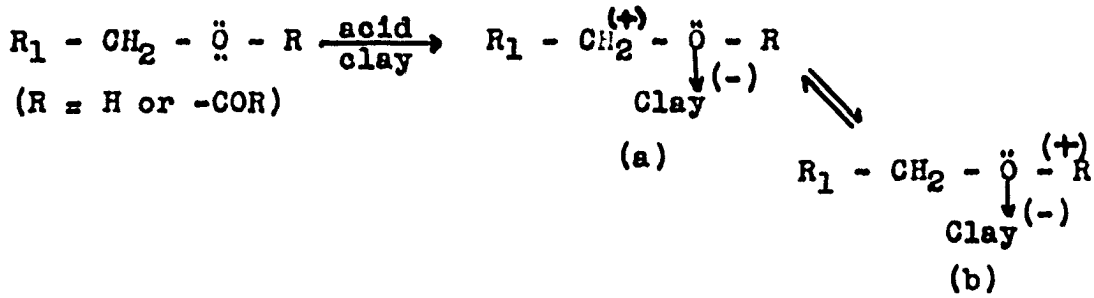
mained which reacted with antimony trichloride to give about one-third the color value of the original vitamin A concentrate. This product was not biologically active and was quite unstable. The reddish yellow oil had absorption bands at 390, 370, 351 and 300-290 mμ.

Edisbury, et al. (48) attempted to recover vitamin A from the blue solution obtained by reaction with antimony trichloride by pouring the mixture into much water. Partial decomposition of the vitamin had occurred and was accompanied by the appearance of narrow absorption maxima at 399, 376, 357 and 340 mμ, and sometimes by additional less definite maxima at 425, 324, 308 and 280 mμ. These data appear to support the view that the initial reaction product is a vitamin A-$SbCl_3$ loose addition product, but secondary processes (perhaps a condensation) cannot be excluded and may account for the narrow bands. Condensation was also suggested as a possible reaction mechanism by Wokes and Willimott (243).

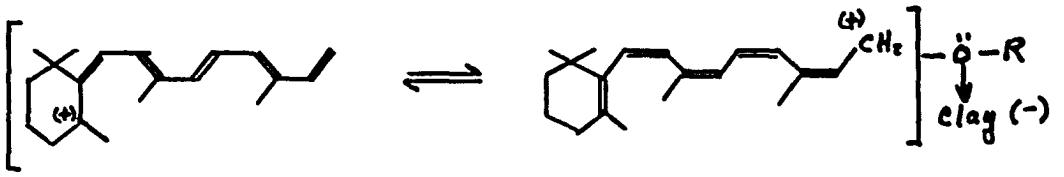
A chromogenic material was also recovered from the antimony trichloride reaction product of vitamin A by Shantz, Cawley and Embree (205). The technique employed was rapid quenching of the blue color with ethanol, addition of aqueous HCl to effect a separation of layers, extraction with petroleum ether and recovery of the extracted product. The product was called anhydrovitamin A and had absorption maxima

at 390, 370, 350 and sometimes 332 mμ. It was concluded that anhydrovitamin A is an intermediate in the development of the antimony trichloride blue color. Several possible structures for anhydrovitamin A were postulated. It is evidently a hydrocarbon with at least five and probably six double bonds.

Meunier (129) has proposed a mechanism of the reaction of vitamin A with antimony trichloride in which the reagent acts as a reaction adsorbent (similar to acid clays), causing ionization and color formation. Earlier studies with acid clays (127, 138) indicated that some clays which possess incomplete electronic octets are able to give intense blue color with vitamin A in a non-polar solvent. By donating unshared electrons to acid clays the vitamin A molecule undergoes polarization and forms positively charged, strongly resonating structures. These electronic changes may be represented as follows:



This phenomenon of mesomerism is made more evident by showing the entire vitamin A molecule in formula (a),



(a_1) (a_2)

The position of the double bonds cannot be precisely stated. The formula limits are probably one or the other and because of this state of electronic instability the intense blue coloration results. While the positive pole position is indeterminant, the negative charge is maintained on the clay by the alcoholic oxygen. The vitamin A could exist in four ionic forms (two cations, two anions). It is impossible to isolate this blue color from the clay. This indicates that the coloration is due to the phenomenon of adsorption. Additional electronic theory of the reaction of vitamin A with acid clay has been published by Javillier and Meunier (91).

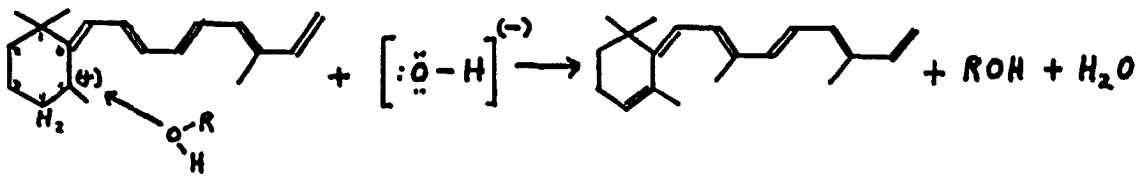
When vitamin A reacts with antimony trichloride, the molecule is ionized at the OH group and is held by the antimony trichloride by sharing of an octet with the oxygen atom (129):

$$R_1 - CH_2 - \underset{\downarrow}{O} : \overset{(+)}{R} \qquad \text{and} \qquad (a_1 = a_2)\ \overset{(+)}{:}\ \underset{\downarrow}{O} - R$$
$$SbCl_3^{(-)} \qquad\qquad SbCl_3^{(-)}$$

The mesomeric state may be responsible for the blue color, with maximum absorption at 620 mμ, which is attributed to

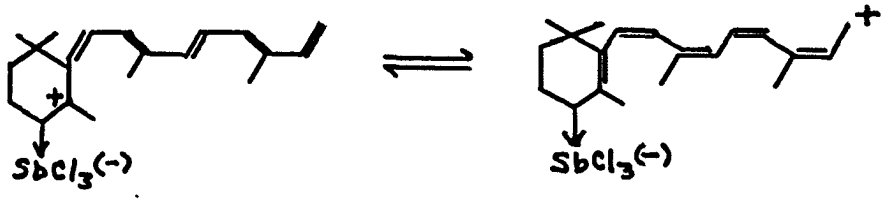
the oscillation of the five conjugated double bonds.

As mentioned above there is quite good evidence for the formation of anhydrovitamin A during the colorimetric reaction of vitamin A with antimony trichloride. Anhydrovitamin A has been called axerophtene (133, 134) and cyclized vitamin A (84) by others. A mechanism for the formation of the anhydrovitamin A during the antimony trichloride reaction has been proposed by Meunier and coworkers (129, 133, 134). Let us consider formula a_1 above in which the position of the double bonds is different than in the original vitamin A. At the moment of treatment of the blue color with a solvent (alcohol) which may donate electrons, the reaction of the ion may be represented as follows:



This reaction involves the loss of a proton from carbon atom 4 and the formation of a double bond between carbon atoms 4 and 5. The resulting carotenoid is a hydrocarbon with six conjugated double bonds. The structure of this compound has been postulated by several workers (84, 133, 205) and is generally accepted in the above form (100, 129,

132, 204). Antimony trichloride reacts with this carotenoid to form a blue color with an absorption spectrum that is virtually identical with the vitamin A-antimony trichloride color (52, 129, 205). Meunier (129) suggested that $SbCl_3$ shares an octet with carbon atom 4 of the ring leaving a mesomeric state among the five double bonds present. This may be represented as follows:



Divitamin A ether is reported to be another product of the reaction of vitamin A with antimony trichloride or an acid clay (129, 139, 140). The ether, after extraction from the reaction mixture, gives a color reaction with $SbCl_3$ (maximum absorption at 589 mμ) which is the same as that of β-carotene. The mechanism suggested for the formation of this ether involves the formation of the anion $(R - CH_2 - O)^{(-)}$ and the cation $(R - CH_2)^{(+)}$. These then combine to give the symmetrical divitamin A ether $R - CH_2 - O - CH_2 - R$. This ether apparently reacts with antimony trichloride by sharing an octet with each of the two electron doublets adjacent to the ether bridge, leaving two oscillating systems of four double bonds each within the molecule.

A mechanism of the reaction of β-carotene and other

carotenoids with antimony trichloride has also been proposed by Meunier (128-131). The colors appear to result from the wandering of electric charges across the system of double bonds and the intensity of the color is proportional to the square of the length of the conjugated system.

The Glycerol Dichlorohydrin Method

The chemistry of the activation of glycerol dichlorohydrin and the reaction of this reagent with vitamin A is quite obscure.

Several methods for the activation of glycerol dichlorohydrin have been described by Sobel and Werbin (213). The addition of concentrated hydrochloric acid, acetyl chloride, phosphorus pentachloride, anhydrous aluminum chloride or benzoyl chloride to inactive glycerol dichlorohydrin gave reagents which reacted with vitamin A to give blue colors changing to violet. Concentrated sulfuric acid, stannic chloride and zinc chloride were reported to produce reagents which gave blue color which did not change to violet.

The preferred method for activating glycerol dichlorohydrin is the distillation of the compound with 1 to 5% antimony trichloride at 4- to 40-mm. pressure. The first suggested method was to add 100 ml. of $CHCl_3$ containing 10 to 50 grams of antimony trichloride to 1000 ml. glycerol dichlorohydrin. Later it was found that the solid could be added directly. The chloroform fraction was discarded then

the activated reagent collected at 4- to 40-mm. pressure. Several kinds of glycerol dichlorohydrin were used in the activation studies. These included Eastman, practical grade; 1,3-dichloro-2-hydroxypropane from the Ohio Chemical Mfg. Co.; and a mixture of 1,3- and 2,3-dichlorohydrins from Shell Chemical Co.

No mechanism for the activation by vacuum distillation with antimony trichloride was proposed. The activity apparently was not due to the antimony trichloride per se, since the addition of this compound in levels ranging from 0.1 to 1.0% failed to cause activation.

Glycerol 1,3-dichlorohydrin, glycerol 2,3-dichlorohydrin and glycerol 1,3-dibromohydrin were reported (213) to be activated with antimony trichloride. Apparently a number of compounds have been tested in an attempt to determine the structural requirements for the preparation of activated reagents. Sobel (207) implied that the compounds listed in another report (208) were tested with negative results. These included ethylene chlorohydrin, trimethylene chlorohydrin, propylene chlorohydrin, propylene glycol, acetylene tetrachloride, trichlorohydrin, 1-chloro-2,3-epoxypropane and glycerol α-monochlorohydrin. It therefore seems evident that a hydroxyl and two halogen groups are required to produce an activated reagent. This is further borne out by the fact that the acetylated derivative of glycerol dichlorohy-

drin could not be activated.

As far as could be ascertained the only other report of the activation of glycerol dichlorohydrin involves the use of concentrated hydrochloric acid. Penketh (179) claims that the activating principle is hydrochloric acid (or perhaps hydrogen ions, since sulfuric acid has some activating effect), small quantities of which are formed during distillation with antimony trichloride. Activation by addition of about 2 per cent of concentrated hydrochloric acid produces a reagent which, if used within a short time, behaves in a similar manner to that activated by the usual manner. On standing the activation increases somewhat but loses the desirable property of stability of the chromophor.

No mechanism for the reaction of activated glycerol dichlorohydrin with vitamin A has yet been proposed. The product or products of the reaction have not been described.

APPARATUS

Most of the glycerol derivatives used in this investigation were vacuum distilled from a 1-liter Claisen flask submerged in an oil bath. A thick-walled capillary tube was sealed into a glass stopper to fit the filling neck of the flask. A piece of rubber tubing was placed on the top section of the capillary tube. By adjusting a screw clamp on the rubber tubing, the rate of bubbling of air through the solution was easily adjusted. All joints in the apparatus were glass. Vacuum was obtained with a water pump and the pressure was indicated by a mercury manometer.

The absorption spectral data were collected by utilizing the Cary recording spectrophotometer (see Figure 1). This instrument was a Model 12 manufactured by the Allied Physics Corporation, Pasadena, California. Prior to running the absorption spectrum of a given substance, the multipot adjustment was made over the spectral range to be used. During this adjustment each cell was filled with the blank solution which was set to read zero optical density. The multipot adjustment corrected for slight differences in the absorption of light by the cells at various wavelengths. The 50 mm. absorption cells were used when making studies in the visible range. When the ultraviolet spectrum was required, the 10 mm. absorption cells were employed. Details

Figure 1. Cary Recording Spectrophotometer

regarding operation and maintenance of the instrument are found in the manual supplied by the concern that manufactures the machine.

The remainder of the spectral absorption data was obtained by using a Beckman quartz spectrophotometer, model DU. Calibrated Corex cells with 1 cm. light path were used throughout this work. The slit width setting was kept at 0.065 mm. unless otherwise indicated.

METHODS

Purification of Glycerol Derivatives

Most of the glycerol derivatives studied were purified by vacuum distillation. Quantities up to about 700 or 800 ml. could be placed in the 1-liter Claisen flask. The temperature of the oil bath which surrounded most of the bulb section of the distilling flask was maintained at 110° to 120°C., except in the case of the distillation of glycerol monochlorohydrin which required higher temperatures. An even boiling in the distilling flask was assured by controlling the rate at which air was bubbled through the liquid. A steady distillation temperature was generally used as the criterion of purity of the fractions collected.

Activation of Glycerol Derivatives

Qualitative Tests

Qualitative activation tests were made by placing 1 ml. of reagent in a small test tube followed by the addition of one drop of activating agent. After mixing, 0.25 ml. of a chloroform solution of vitamin A was added and the tube inverted to mix the reactants. The resulting color formation and change, if any, was noted and recorded.

Antimony Trichloride Method

Two methods of activation with antimony trichloride were used. The first involved vacuum distillation of the

glycerol derivative with approximately 1% Baker's analyzed antimony trichloride according to the method of Sobel and Snow (211). The first fraction was discarded and the reagent distilling at the proper temperature was collected for use in activation studies.

The second method involved the addition of Baker's analyzed antimony trichloride to GDH then subjecting the reagents to dry heat for varying lengths of time. The reagents were prepared by adding weighed quantities of antimony trichloride to inactive GDH and mixing until the trichloride had dissolved. Each reagent was divided into several equal portions in glass-stoppered volumetric flasks. After the flasks were tightly stoppered, each was subjected to a different heating period at a constant temperature. After cooling, the reagents were ready for tests of activation with standard vitamin A solutions.

Acid Methods

When activation was accomplished by the use of concentrated acids, a known volume of acid was pipetted into a glass-stoppered volumetric flask followed by the addition of inactive GDH to make a known volume of solution. After thorough mixing, the reagent was ready to use.

Activation with chlorosulfonic acid was accomplished by weighing small quantities of Eastman practical grade acid in a glass-stoppered volumetric flask, followed by the addition

of inactive GDH to make a known volume of reagent. After mixing, the reagent was ready to use, or other reagents containing lesser quantities of acid were prepared by dilution with inactive GDH.

Activation with anhydrous hydrogen chloride was accomplished by passing a stream of dry HCl into inactive GDH until it was partially or completely saturated with the gas. The dry gas was prepared by adding concentrated sulfuric acid to a mixture of concentrated hydrochloric acid and sodium chloride. To remove final traces of moisture, the gas was bubbled through a concentrated sulfuric acid drying column. The concentration of HCl in GDH was determined either by weighing or by titration with standard NaOH. Reagents containing lesser quantities of dry HCl were prepared by dilution of standardized reagents with inactive GDH.

Preparation of Vitamin A and Carotene Solutions

The chloroform used in the preparation of standard vitamin A and carotene solutions was prepared by drying U.S.P. grade chloroform over anhydrous sodium sulfate followed by distillation in an all-glass apparatus and finally storage over anhydrous sodium sulfate. The reagent was kept in a clear, glass-stoppered bottle in a dark cupboard.

The standard vitamin A solutions were prepared in the following manner: A sample of U.S.P. Vitamin A Reference

Standard Oil (crystalline vitamin A acetate dissolved in cottonseed oil) was weighed in a glass-stoppered volumetric flask. Redistilled, dry chloroform was added to the calibration mark and the contents mixed thoroughly by inversion. Calculation of the vitamin A content was based on the fact that each gram of Reference Standard Oil contains an equivalent to 3000 micrograms of vitamin A. In the preparation of vitamin A reference standard curves, solutions containing lesser quantities of vitamin A were prepared by dilution of the standard solution. Standard solutions were used within a few hours after preparation.

Standard carotene solutions were prepared by dissolving crystalline β-carotene (General Biochemicals, Inc.) in chloroform. From 8 to 10 mg. of carotene were accurately weighed then dissolved in chloroform to give 100 ml. of solution. Solutions containing less than 10 micrograms per ml. of chloroform were prepared by dilution of the standard solution with chloroform.

Absorption Spectra

The absorption spectra of the products of reactions of vitamin A with various glycerol derivatives were obtained with the aid of the Cary recording spectrophotometer. Accurately measured quantities of reactants were mixed in 50-ml. glass-stoppered centrifuge tubes and then poured into

50 mm. absorption tubes. The cells were placed in the instrument within 1 minute and spectral absorption tracings were begun at 1.5 minutes (± 2 seconds) after the reactions were initiated. The scanning speed was 2 mμ per second when the visible spectral range was being used. Tracings were begun at a wavelength of 700 mμ and proceeded in the direction of shorter wavelengths. At the conclusion of an absorption tracing, the scanning and chart motors were stopped and the machine reversed to the original starting position with the tracing needle off the chart. Another curve was then traced over the same spectral range. A series of seven or eight curves was made for most reaction mixtures. The stability of the absorption at each absorption maxima could then be determined from the chart.

Ultraviolet absorption spectra were obtained with the same instrument. The 10 mm. absorption cells were used in all ultraviolet studies. Tracings were begun at 400 mμ and proceeded toward shorter wavelengths. Scanning speeds varied according to the substance being investigated; a speed of 1 mμ per second appeared to be the most satisfactory.

Optical Density Measurements

The optical densities at certain specific wavelengths were measured with a Beckman quartz spectrophotometer, model DU. Accurately measured quantities of reactants were mixed

in glass-stoppered flasks then quickly poured into a Corex cell with a 1 cm. light path. After covering with a glass top, the cell was then placed in the cell carrier. The machine was adjusted to read zero optical density with a solution containing solvent and reagent. Optical density measurements were then made at specified wavelengths at various time intervals. Extinction coefficients were calculated from all optical density values.

Preparation of GDH Containing Added Impurities

The compound or solution to be tested was weighed in a glass-stoppered flask. GDH was then added and mixed with the impurity being studied. The flask was reweighed and the percentage of added impurity calculated. Several concentrations of the impurity were prepared by pipetting 5-, 2-, and 1-ml. portions into 10-ml. glass-stoppered volumetric flasks. GDH was added to each flask to make a final volume of 10 ml. Each flask was stoppered and the contents thoroughly mixed.

EXPERIMENTAL RESULTS

Experiments with Glycerol Dichlorohydrin (GDH)

Several companies sell glycerol dichlorohydrins of varying purity and composition. Generally speaking the products fall into two categories: (1) glycerol 1,3-dichlorohydrin or (2) a mixture of glycerol 1,3- and 2,3-dichlorohydrins. Both types were available for this investigation.

Shell Glycerol Dichlorohydrin

At the time that this work was begun, one gallon of glycerol dichlorohydrin from the Shell Chemical Company was available for use. This product is a mixture of about 75% 2,3- and 25% 1,3-dichlorohydrin. It was a bright yellow color when received. Upon testing with a chloroform solution of vitamin A acetate, this GDH was found to be inactive (gave no color test).

Purification. Before any purification work was begun, activation by vacuum distillation with 1 to 5% antimony trichloride was attempted according to the method described by Sobel and Werbin (211). The product was active when tested with vitamin A, but on standing the reagent darkened considerably. This property was indeed undesirable. Redistillation failed to give a clear product, so purification prior to activation was attempted.

The "as received" GDH was subjected to vacuum distil-

lation. The first fraction to distil boiled at a lower temperature than GDH and, at first, was a two-phase system. Part of this distillate was probably water. When the temperature reached 76°C. at a pressure of 15 mm. Hg, the contents in the Claisen flask began to boil vigorously. At this point the receiver was changed and the GDH collected over the range of 76° to 78°C. at 15 mm. pressure. This distillate was clear and colorless when first collected, but on standing for several days in a clear, glass-stoppered bottle it gradually turned yellow. Activation of this product by codistillation with 1 to 5% antimony trichloride under vacuum again resulted in an active product which was clear but faintly yellow in color. On standing a few hours this reagent turned yellow-brown and thus was not acceptable as a colorimetric reagent. Two redistillations did not give a product which was clear and colorless.

Treatment of any of the above yellow or yellow-brown products with activated charcoal (Nuchar) failed to decolorize them even when the mixture was heated to 100°C. for several minutes.

A clear and colorless product was finally obtained by simply adding about 100 ml. of U.S.P. chloroform to each 500 ml. of GDH and then subjecting the mixture to vacuum distillation. The chloroform fraction was discarded and the clear, colorless GDH collected at 76° to 78°C. at 15 mm.

pressure. This product could then be activated by codistillation with 1 to 5% antimony trichloride to give a clear and colorless reagent which did not change color upon standing for several weeks. The exact function of the chloroform in the purification treatment is not known, but it seems plausible that the chromogenic impurity could be carried over with the chloroform fraction.

Qualitative activation tests. Clear Shell GDH which had been previously activated by codistillation with antimony trichloride was deactivated by treatment with activated charcoal (Nuchar). Approximately 5 grams of Nuchar was mixed with 500 ml. of GDH in a beaker. The mixture was slowly heated, with occasional stirring, to 60°C. and then filtered several times through a Buchner funnel. This product was clear, colorless and inactive when tested with a chloroform solution of vitamin A. Various compounds were then tested for their activating influence. The color formation was observed and recorded (see Table 1).

The following compounds appeared to produce good activity: concentrated hydrochloric acid, 60% perchloric acid, concentrated sulfuric acid, chlorosulfonic acid, methyl sulfate, acetyl chloride, benzoyl chloride, phosphorus oxychloride, arsenic trichloride (liquid), aluminum chloride and p-toluene sulfonic acid. In each case a blue color was first formed which changed to violet. The blue color changed more

Table 1

Activation of Shell Glycerol Dichlorohydrin with Various Agents

Activating Agent	Color Change when Vitamin A was added	Remarks
Aqueous HF (52%)	Faint blue	Color quite unstable
Concentrated HCl	Bright blue changing to violet	Color quite stable
Aqueous HBr (40%)	Blue changing to violet	Color quite unstable
Aqueous HI (47%)	Orange	Reagent turned yellow when acid was added
$HClO_4$ (60%)	Bright blue changing to violet	Color quite stable
Concentrated HNO_3	No color	
Concentrated H_2SO_4	Bright blue changing to violet	Color quite stable
$ClSO_3H$	Bright blue changing to violet	Color quite stable
Methyl Sulfate	Bright blue changing to violet	Color quite stable
Naphthalene-β-sulfonic acid	Light blue changing to violet	Color quite stable
p-Toluene sulfonic acid	Blue slowly changing to violet	Color quite stable
CH_3COOH, glacial	No color	
$CH_2ClCOOH$	No color	

Table 1
(Continued)

Activating Agent	Color Change when Vitamin A was added	Remarks
CCl_3COOH	No color	
Citric acid	No color	
CH_3COCl	Bright blue changing to violet	Color quite stable
Benzoyl chloride	Bright blue changing to violet	Color quite stable
PCl_3	Yellow	
$POCl_3$	Bright blue changing to violet	Color quite stable
$AsCl_3$, liquid	Bright blue changing to violet	Color quite stable
$AlCl_3$, anhydrous	Bright blue changing to violet	Color quite stable
Acetic anhydride	No color	
$BiCl_3$	Light blue	Color quite unstable

slowly in the case of GDH activated with sulfuric acid, p-toluene sulfonic acid and methyl sulfate. It is interesting to note that no color resulted when GDH was treated with nitric, acetic or trichloracetic acid.

HCl activation. Two methods of activation with hydrogen chloride were investigated. The first involved simple addition of concentrated hydrochloric acid to redistilled, Nuchar-deactivated GDH in quantities ranging from 0.1 to 4.0% (volume basis). The resulting reagents were then mixed with chloroform solutions containing either 13.9 or 13.6 micrograms of vitamin A per ml. In each case the reaction mixture was one part of the vitamin A solution and four parts of activated GDH. The absorption spectra of the resulting colors were measured in the Cary recording spectrophotometer over the spectral range of 330 to 700 mμ. Changes in the absorption spectra as the colors aged were determined by scanning the same spectral range every 5 minutes, always starting at the red end of the spectrum.

From the absorption spectrum, which is actually a plot of optical density against the wavelength, the extinction coefficient ($E^{1\%}_{1cm}$) at any wavelength was calculated by using the equation

$$E^{1\%}_{1cm} = \frac{D}{c \times 1}$$

where D is the optical density, c is the concentration of

vitamin A in grams per 100 ml. and l is the length of the light path through the absorption cell. The absorption spectra of the colors produced by the reaction of vitamin A with these HCl-activated reagents are shown in Figure 2.

Table 2

Stability of the Color Developed by the Reaction of Vitamin A with Shell GDH Activated with Several Levels of Concentrated Hydrochloric Acid

Time after mixing (minutes)	Concentration of HCl in GDH					
	0.1%	0.5%	0.5% + $SbCl_3$[a]	1.0%	2.0%	4.0%
			$E^{1\%}_{1cm}$ at 553 mμ			
2.22		1039				
2.72	1110		1010	995	964	894
6.72		822				
7.72	918		803	715	657	504
11.22		594				
12.72	708		610	473	414	275
15.72		424				
17.72	538		461	317	266	173
20.22		306				
22.72	410		349	225	187	130
24.72		229				
27.72	310		273	170	144	108
29.22		184				
32.72	247		217	136	122	102
33.22		155				

[a]added 0.013% antimony trichloride.

These curves represent the first absorption curve taken with each reaction mixture.

Table 2 summarizes part of the data collected with the recording spectrophotometer and shows the effect of aging on

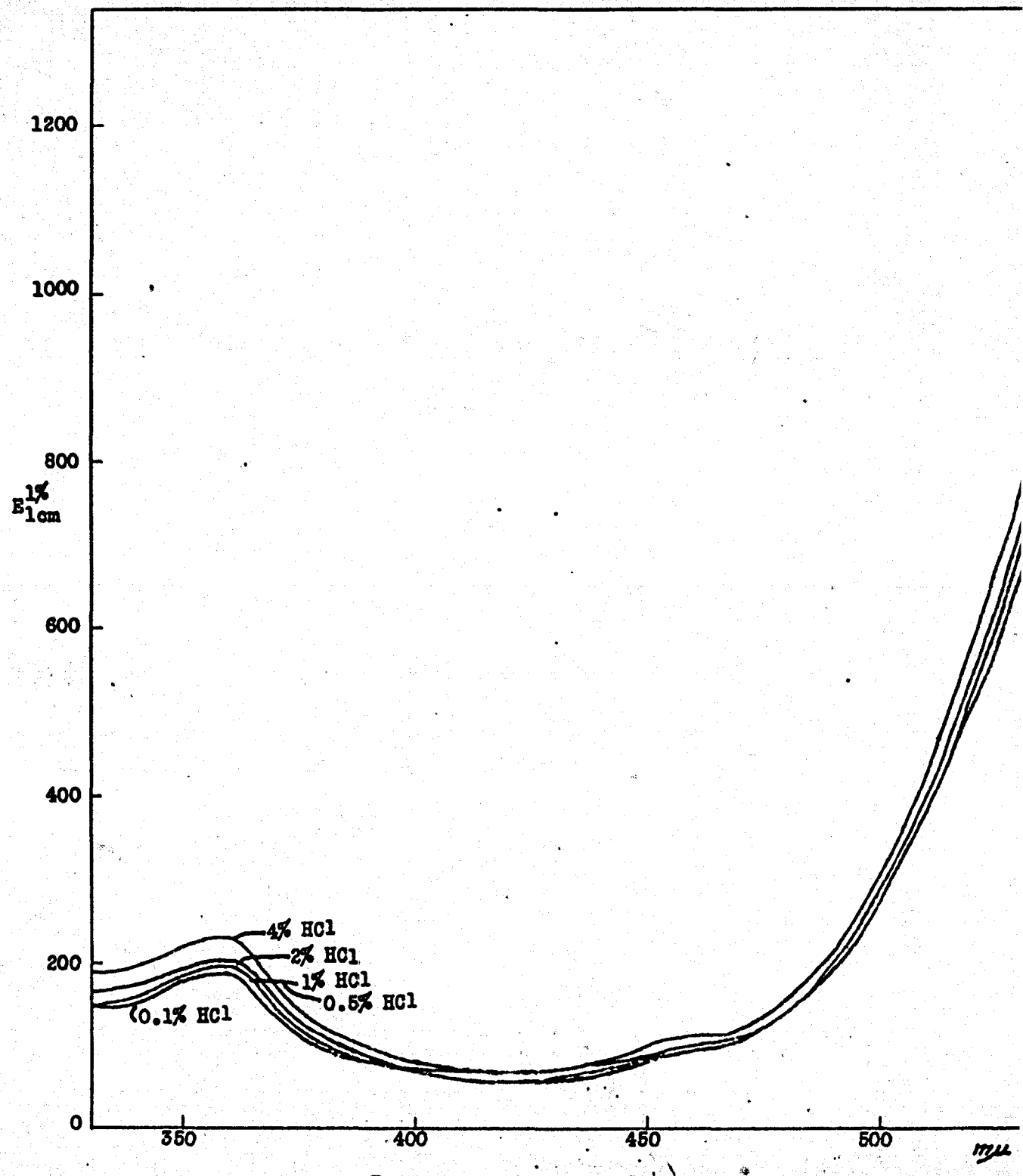
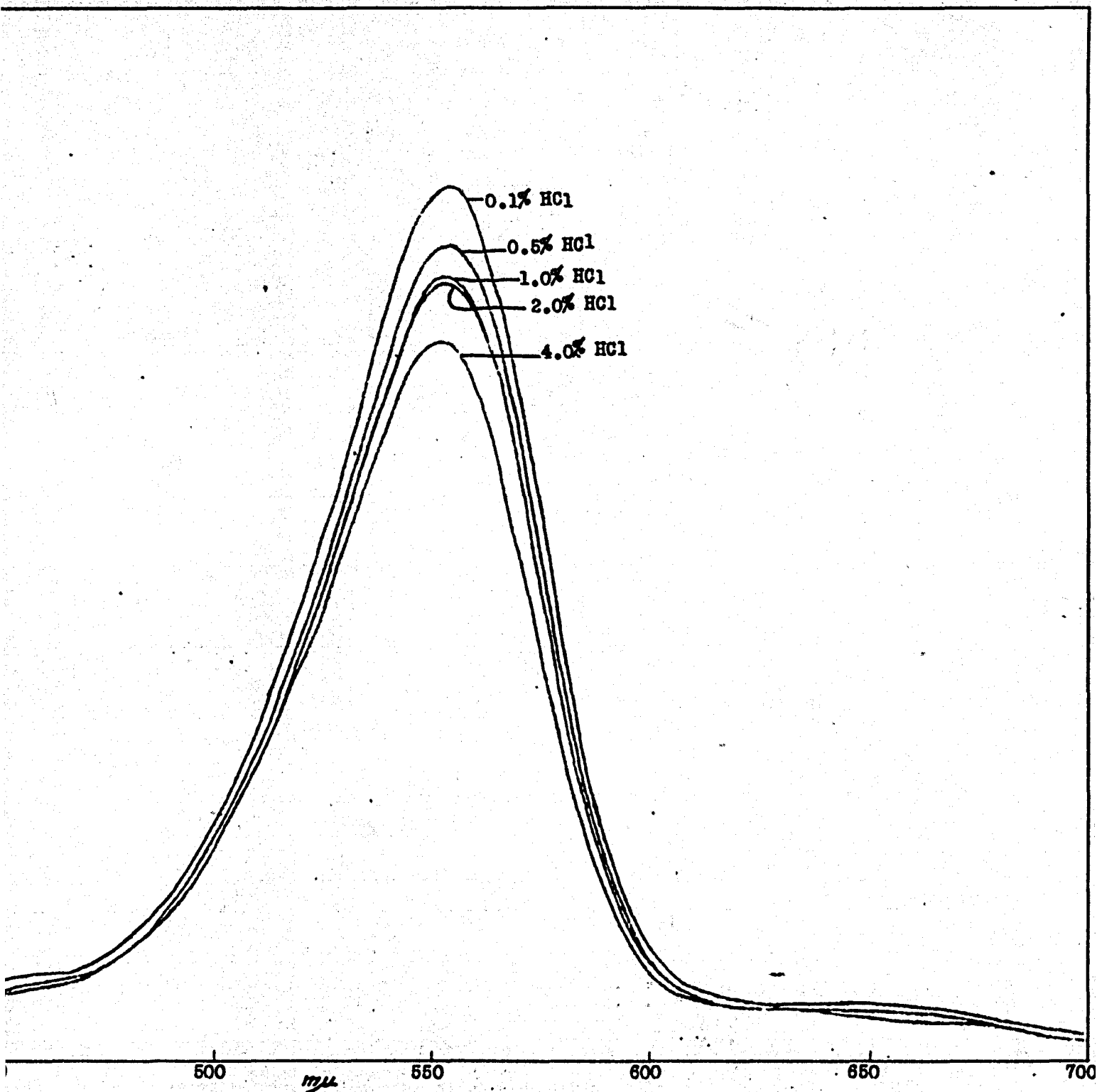


Figure 2. Absorption Spectra of the Colors Produced by the Addition of Several Levels of Concentrate



otra of the Colors Produced by the Reaction of Vitamin A with GDH Activated by
f Several Levels of Concentrated Hydrochloric Acid.

the color at the maximum absorption band (553 mμ). Column 4 of this table indicates the result of adding 0.013% antimony trichloride per se to a reagent activated by the addition of 0.5% concentrated hydrochloric acid. These data were plotted (Figure 3) and it is evident that the reagent containing 0.1% concentrated hydrochloric acid is the most active. It is also of interest to observe that the addition of antimony trichloride per se improved the stability of the color. Both activity of the reagent and stability of the GDH-vitamin A color decreased as the concentration of hydrochloric acid in the reagent increased.

The other method of activating GDH with HCl was by way of addition of various levels of anhydrous HCl. This gas was bubbled into inactive GDH until the reagent became saturated. Reagents containing various levels of anhydrous HCl were prepared by mixing HCl-saturated GDH with inactive GDH. The normality of each solution was determined by titration with standard NaOH using phenolphthalein as indicator. The formation of a pink color which remained for about 5 seconds was designated the end-point of the titration since it was found that GDH would react slowly with dilute NaOH.

Another set of HCl-activated reagents were prepared by bubbling dry HCl into inactive GDH until the reagent was approximately 0.4 N with respect to HCl as indicated by increase in weight. This was diluted with inactive GDH to give

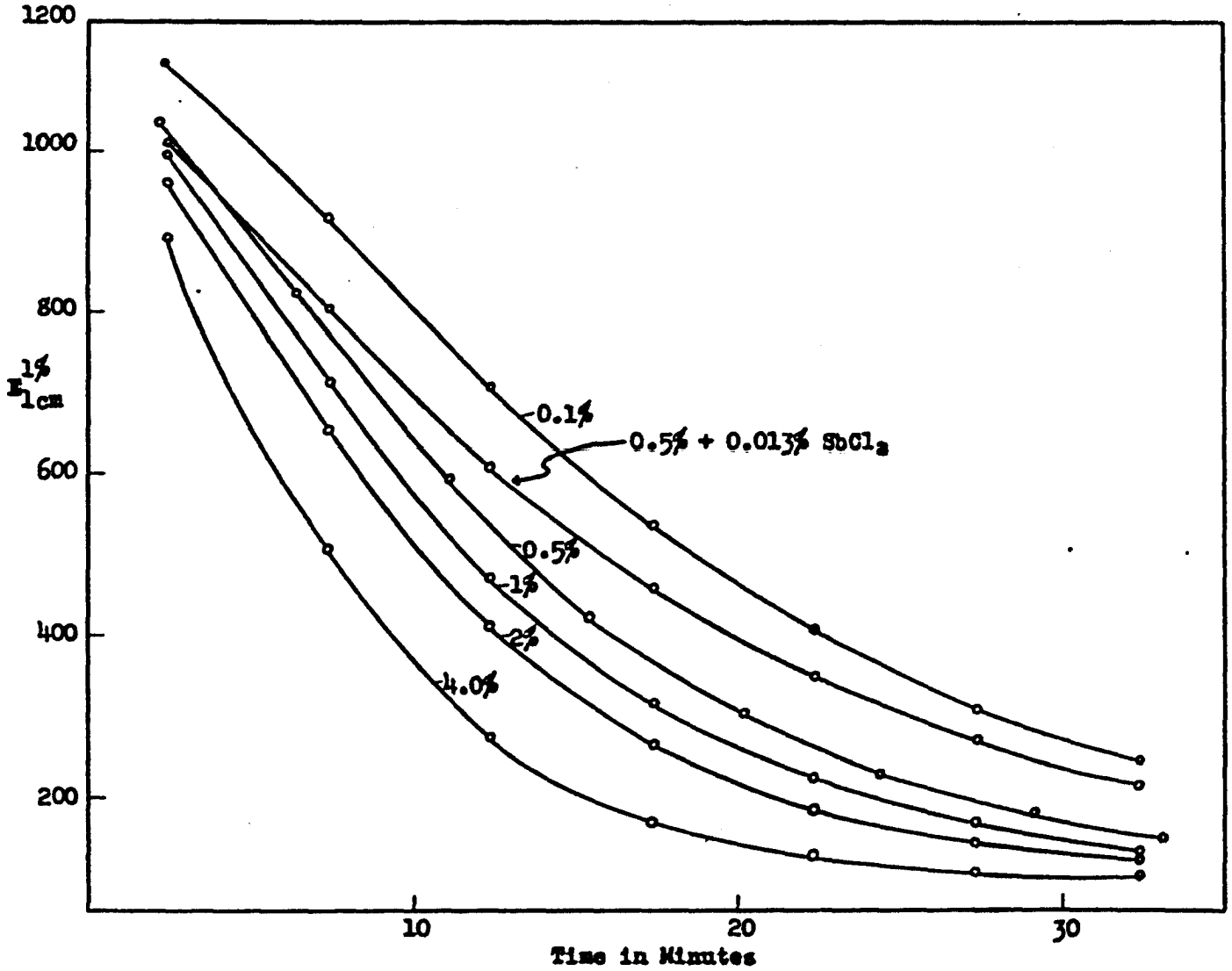


Figure 3. Stability of the Color, at 555 mμ, Produced by the Reactions of Vitamin A with Shell GDH Activated by Addition of Several Levels of Concentrated Hydrochloric Acid.

reagents which had HCl levels ranging from 0 to 0.367 N. A total of sixteen reagents activated with anhydrous HCl were tested for activity by reaction with standard chloroform solutions of vitamin A. Three sets of data were collected using vitamin A solutions ranging in concentration from 4.33 to 5.16 micrograms per ml. of chloroform. One ml. of vitamin A solution was mixed with 4 ml. of reagent being tested and the optical densities of the colors were determined at 555 mμ with the Beckman spectrophotometer. Readings were made at half-minute intervals between 2 and 5 minutes and an additional reading made at 6 minutes after mixing reagent and vitamin A solution. Optical densities were converted to extinction coefficient values. These data are summarized in Table 3. In practically all cases it was observed that the colors faded after the initial 2 minute reading. Table 4 summarizes the 2 minute extinction coefficients and includes the logarithm of the normality to assist in the graphic representation of these data (see Figure 4). Optimum activation was obtained when the reagent was approximately 0.01 N with respect to HCl. Good activity was obtained with reagents containing from 0.002 to 0.08 N HCl. Activity of the reagents were very poor when the concentration of HCl was less than 0.003 N. On the other hand, when the HCl concentration increased above 0.08 N the activity rather slowly decreased with increasing HCl

Table 3

Stability of the Color Produced by the Reaction of Vitamin A with Shell Glycerol Dichlorohydrin Activated with Anhydrous Hydrogen Chloride

Normality HCl in reagent	Time after mixing (minutes) 2.0	2.5	3.0	3.5	4.0	4.5	5.0	6.0
			$E^{1\%}_{1cm}$ at 555 mμ					
0.874	446	455	455	455	446	446	417	397
0.691	562	562	562	552	532	514	514	475
0.433	650	650	620	601	581	552	543	514
0.367	693	693	683	664	644	615	595	566
0.145	873	843	814	775	746	717	688	640
0.083	940	901	872	824	785	736	698	620
0.045	1028	988	950	901	853	804	756	678
0.0307	1108	1062	1004	958	912	854	808	716
0.0175	1064	1005	947	898	839	771	722	615
0.00915	1074	1005	976	868	800	742	673	556
0.0044	1020	947	878	800	712	654	576	459
0.00245	993	912	843	773	693	623	566	439
0.00089	15	15	15	20	20	20	20	20
0.00021	6	6	12	12	12	12	12	12
0.000018	0	0	0	0	0	0	0	0
0.00	0	0	0	0	0	0	0	0

levels. It was observed that reagents containing high levels of HCl would produce a deep blue color when vitamin A was first added. This color rapidly faded, changed to a violet color and finally appeared to be nearly colorless

Table 4

The Activation of Shell Glycerol Dichlorohydrin with Anhydrous Hydrogen Chloride

Normality HCl	Log Normality	$E^{1\%}_{1cm}$ 555 mμ[a]	Normality HCl	Log Normality	$E^{1\%}_{1cm}$ 555 mμ[a]
0.874	-0.058	446	0.0175	-1.757	1064
0.691	-0.161	562	0.00915	-2.039	1074
0.433	-0.364	650	0.0044	-2.357	1020
0.367	-0.435	693	0.00245	-2.611	993
0.145	-0.839	873	0.00089	-3.051	15
0.083	-1.081	940	0.00021	-3.680	6
0.045	-1.347	1028	0.000018	-4.740	0
0.0307	-1.513	1108	0.0		0

[a] All optical density readings were made 2 minutes after mixing the reagent with a chloroform solution of vitamin A.

when the 2 minute reading was made.

A few of the HCl-activated reagents were permitted to stand for several weeks in clear, glass-stoppered volumetric flasks. The activity of the reagents was determined after three weeks storage using a vitamin A test solution containing 4.7 micrograms per ml. and after five weeks storage using a test solution containing 5.71 micrograms vitamin A

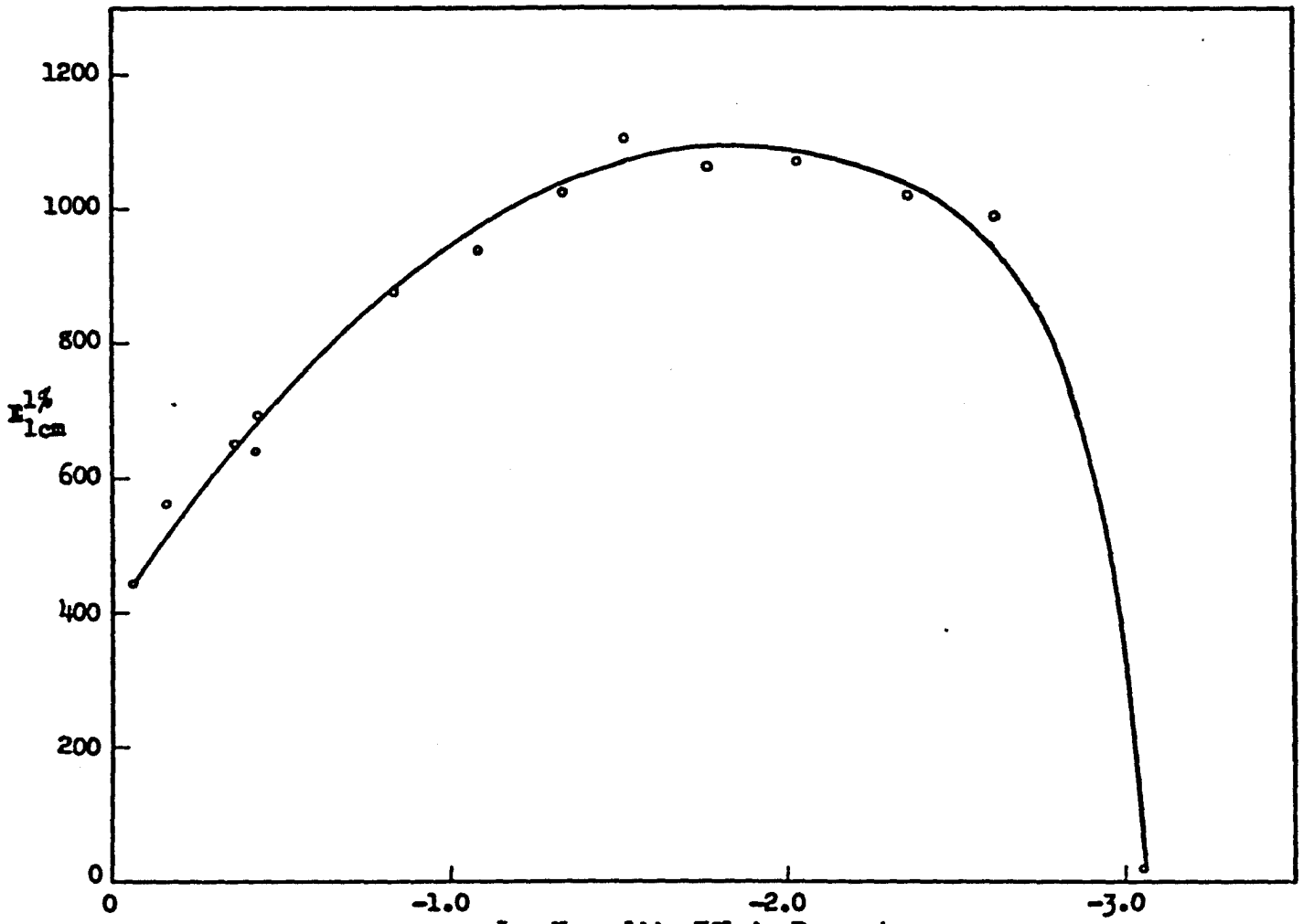


Figure 4. Activation of Shell GDH by Anhydrous Hydrogen Chloride.

per ml. In each case 1 ml. of the vitamin A solution was mixed with 4 ml. of reagent. The data collected (see Table 5) indicated that the activity of highly active HCl-activated GDH decreased considerably on standing for five weeks. On the other hand, the reagent which initially contained a

Table 5

Effect of Aging on the Activity of Shell GDH Activated with Anhydrous Hydrogen Chloride

Normality HCl in Reagent	Freshly Prepared	Three Weeks Storage	Five Weeks Storage
		$E^{1\%}_{1cm}$ at 555 mμ[a]	
0.0307	1108	1016	613
0.0174	1064	846	-
0.00915	1074	857	438
0.0044	1020	756	499
0.0089	15	516	315

[a] All values based on the optical density readings at 2 minutes after mixing a chloroform solution of vitamin A with the reagents.

very low level of HCl and had little activity became fairly active after standing in the light for three weeks. Two weeks later its activity had decreased. It was also observed that the color formed when these "stored" reagents reacted with vitamin A was quite unstable, i.e., after 5 minutes the extinction coefficients were only one-third the

2 minute values.

Sulfuric acid activation. Redistilled, Nuchar-deactivated GDH was activated by the addition of concentrated sulfuric acid to give reagents with 0.1, 0.5 and 1.0% by volume. The activity of these reagents and the absorption spectrum of the color resulting from reaction with vitamin A were determined by using the Cary recording spectrophotometer. One part of a chloroformic vitamin A solution containing 13.57 micrograms per ml. was mixed with four parts of reagent and the resulting blue solution placed in the 50 mm. absorption cell. An absorption spectral curve over the range of 330 to 700 mμ was traced every five minutes using a scanning speed of 2 mμ per second. Each curve was begun at the red end of the spectrum and tracings were made until seven curves were obtained.

The first spectral absorption curve for each reaction mixture showed absorption maxima at 622, 548-552 and 452 mμ. By the time the second absorption curve was traced, the 622 mμ band had practically disappeared while the central absorption band had increased and shifted about 5 or 6 mμ toward the shorter wavelengths. Figure 5 shows the second spectral absorption curve for the three sulfuric acid-activated reagents after their reactions with vitamin A. In each case the tracing was started at 700 mμ at 6.5 minutes after the reactants were mixed. As the sulfuric acid con-

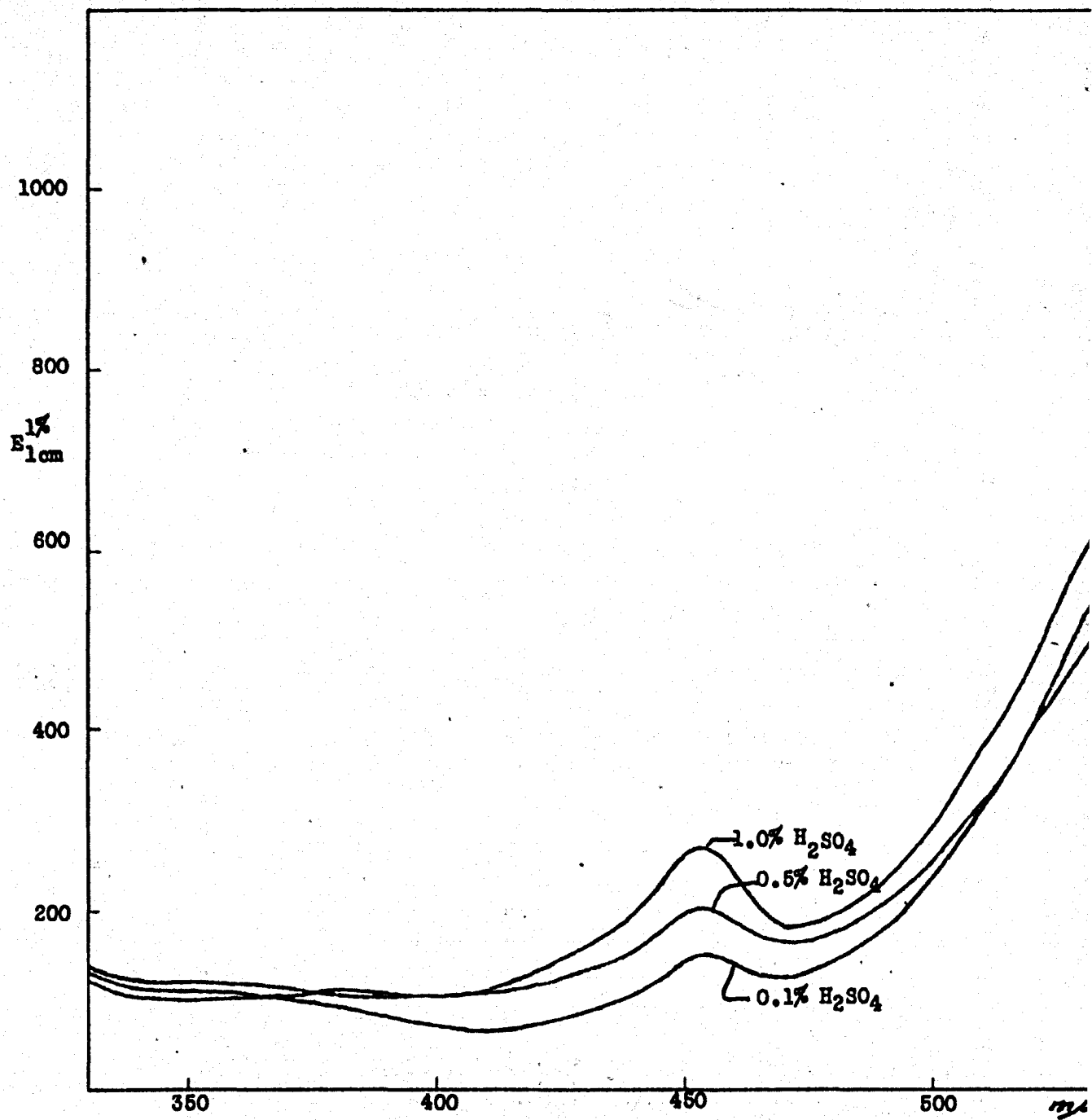
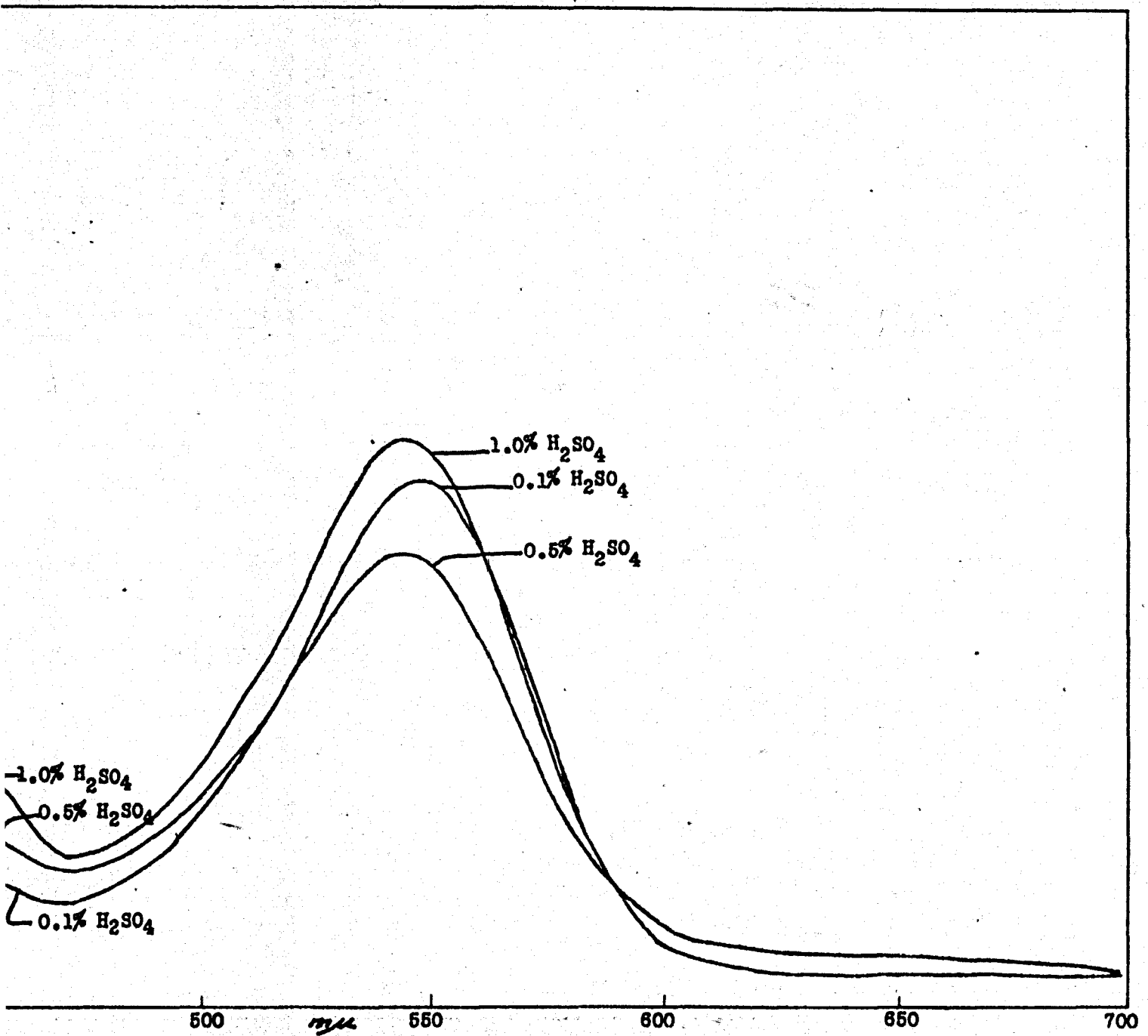


Figure 5. Absorption Spectra of the Colors 7-8 Mi
with GDH Activated by the Addition of T



Spectra of the Colors 7-8 Minutes after the Reactions of Vitamin A
ctivated by the Addition of Three Levels of Concentrated Sulfuric Acid.

tent in GDH was increased the absorption at 452 mμ increased. However, at 545 mμ the 0.5% sulfuric acid reagent had the lowest extinction coefficient and the 1.0% sulfuric acid reagent had the highest. No explanation for this peculiarity has been advanced.

The stability of the absorption maxima could be seen from the tracings made with the recording spectrophotometer. Table 6 summarizes the extinction coefficients of the three reaction mixtures at the two principal absorption maxima as the reactions progressed. These data are graphically shown in Figure 6. Activity of reagent and stability of the 545 mμ band was greatest in the case of the GDH activated with 1% sulfuric acid; maximum absorption occurred between 10 and 12 minutes after mixing reagent with vitamin A. In the case of the 0.5% reagent, the absorption was maximal at about 8 minutes and then decreased rather rapidly. At 452 mμ the 1.0% sulfuric acid-activated reagent exhibited the greatest initial absorption but the stability of this band was less than that for the other reagents.

Chlorosulfonic acid activation. The stability at the 548 mμ maxima in the case of the sulfuric acid-activated GDH was observed to be greater than the 555 mμ maxima in the case of the hydrochloric acid-activated GDH (compare Figures 3 and 5). It was postulated that perhaps one could use chlorosulfonic acid as the activating agent and obtain a

Table 6

Stability of the Color Developed by the Reaction of Vitamin A with Shell GDH Activated with Several Levels of Concentrated Sulfuric Acid

Time after mixing (minutes	Concentration of H_2SO_4 in GDH		
	0.1%	0.5%	1.0%
	$E^{1\%}_{1cm}$ at 545 mμ		
2.82	516	466	545
7.82	623	540	680
12.82	621	515	687
17.82	604	476	668
22.82	576	444	641
27.82	548	413	617
32.82	517	389	591
	$E^{1\%}_{1cm}$ at 452 mμ		
3.57	147	237	293
8.57	147	203	273
13.57	131	172	236
18.57	116	153	208
23.57	105	145	189
28.57	98	134	175
33.57	97	133	165

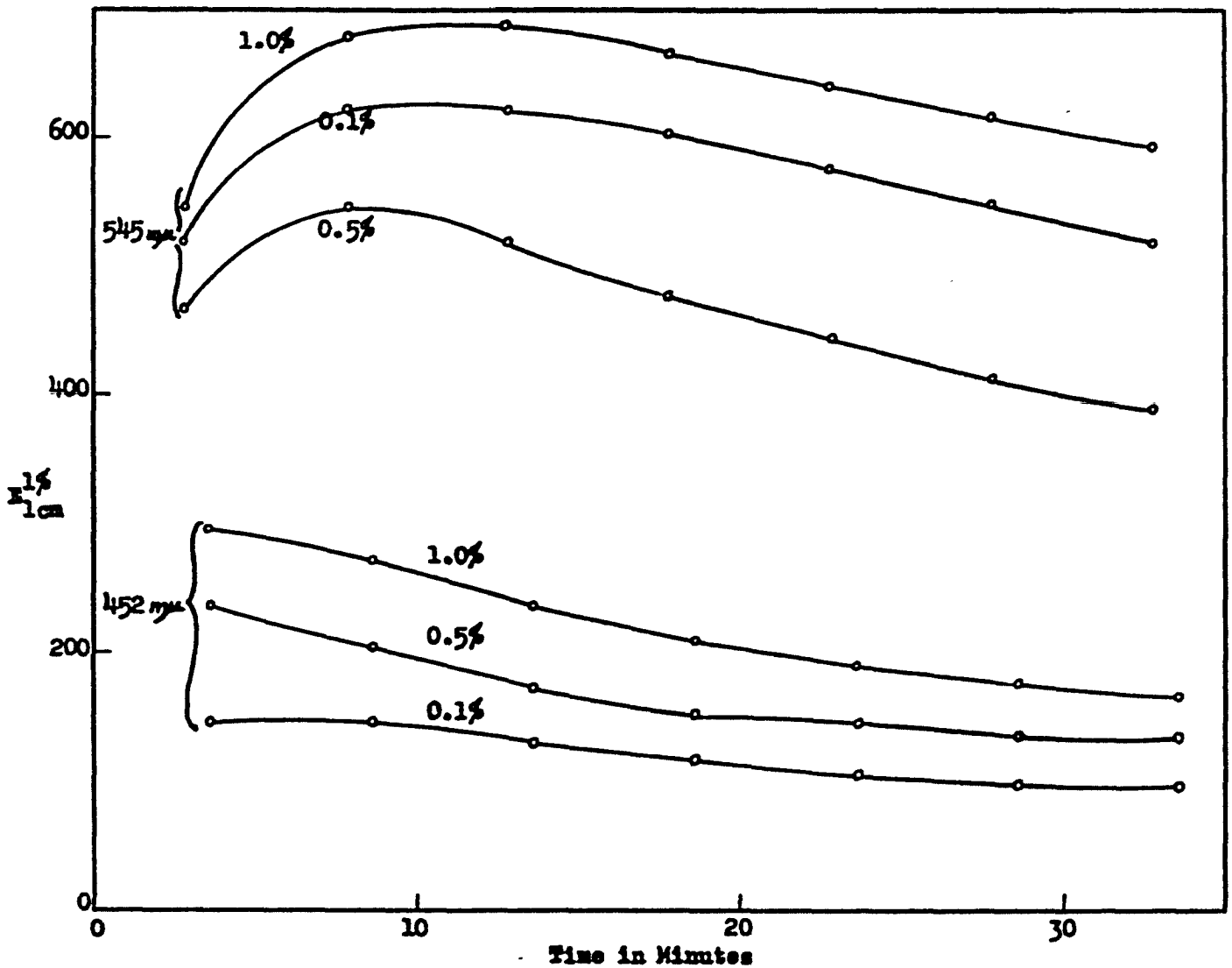


Figure 6. Stability of the Color Maxima at 452 and 545 mμ Vitamin A with GDH Activated by Several Levels of Concentrated Sulfuric Acid.

color with vitamin A that would have increased stability.

Reagents containing 0.046, 0.258 and 0.504% chlorosulfonic acid by weight were prepared using Eastman chlorosulfonic acid, practical grade. Four parts of reagent were mixed with one part of a chloroform solution containing 13.88 micrograms of vitamin A per ml. This mixture was poured into the 50 mm. cells and the absorption spectral curves were determined over the range of 330 to 700 mμ with the Cary recording spectrophotometer. Tracings were made every 5 minutes until seven curves were obtained. Figure 7 shows the initial absorption curve obtained when each reagent was allowed to react with vitamin A. The principal absorption maximum was at 553 mμ, and a rather weak absorption band was observed at 358 mμ. A still weaker band occurred at 452 mμ.

From the absorption curves obtained with the aid of the recording spectrophotometer it was possible to determine the stability at the main absorption bands. Table 7 shows the change in extinction coefficients at 553 and 358 m as the reaction mixtures aged. With each reagent the extinction coefficients at 358 mμ increased with time, and the reagent containing the most acid gave the highest values. The reagent containing the lowest level of chlorosulfonic acid gave the highest initial extinction coefficient at 553 mμ after reaction with vitamin A. A plot of the 553 mμ data (see

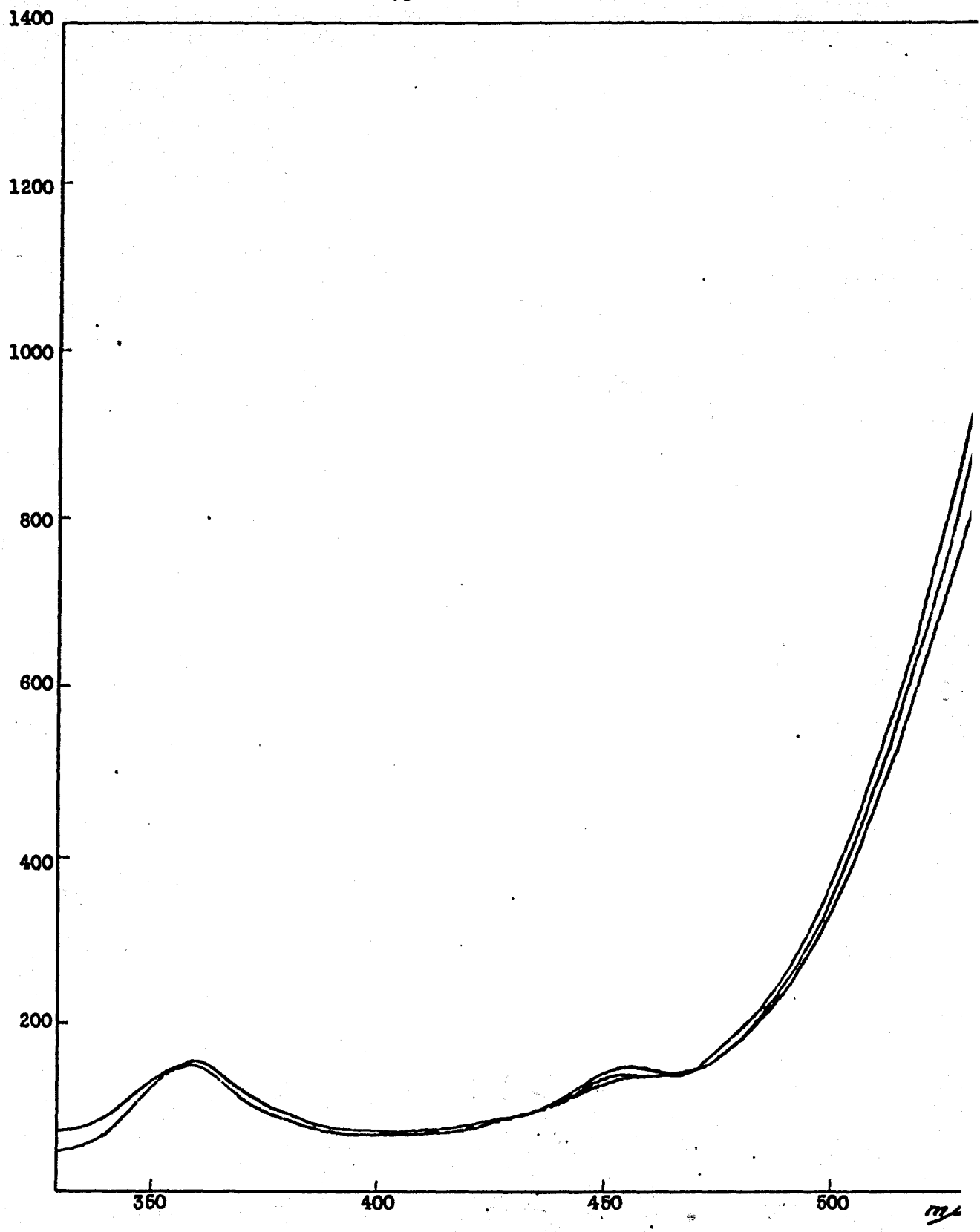
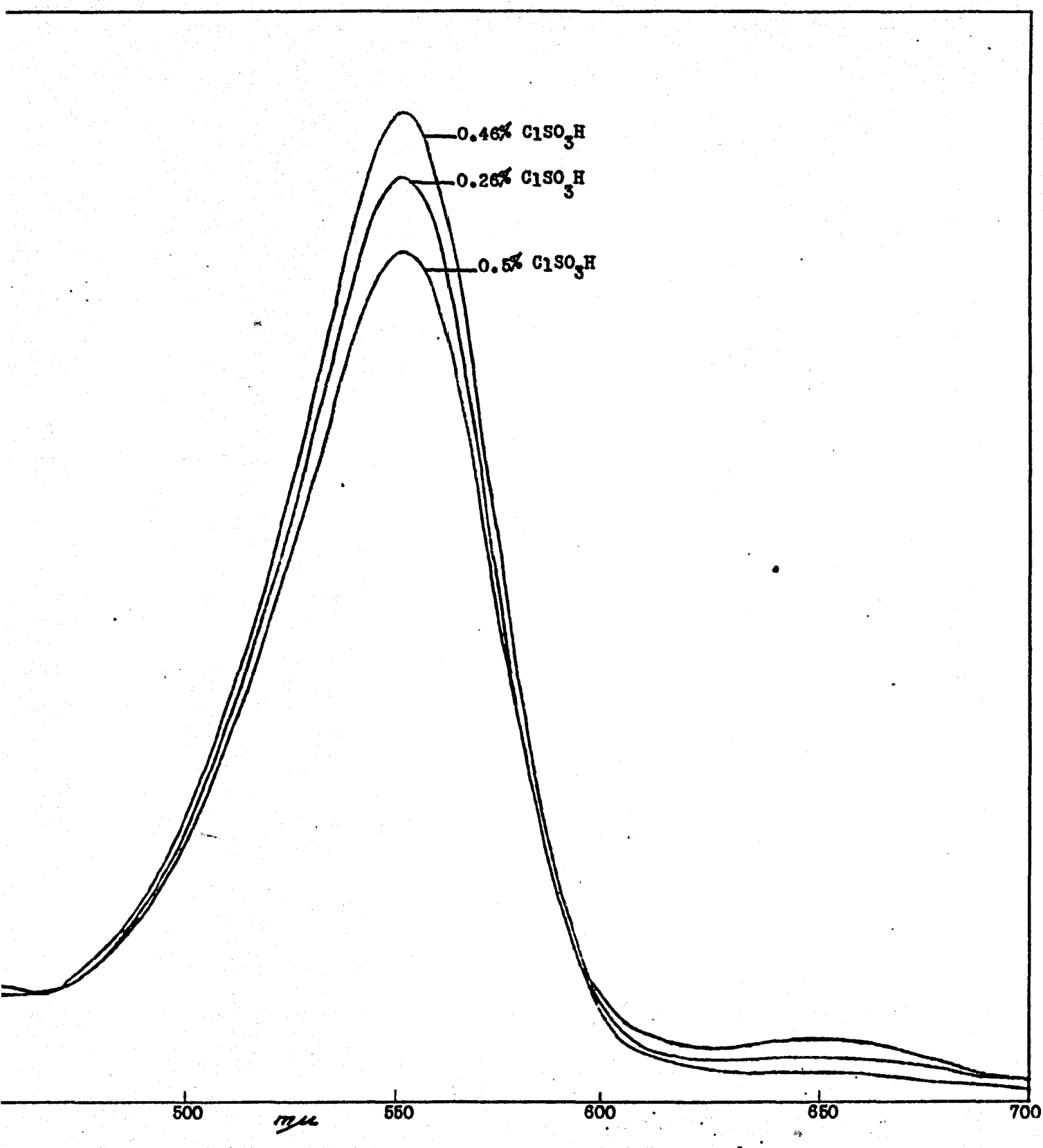


Figure 7. Absorption Spectra of the Color Pro
with GDH Activated by the Addition



tion Spectra of the Color Produced by the Reactions of Vitamin A
DH Activated by the Addition of Several Levels of Chlorosulfonic Acid.

Table 7

Stability of the Color Developed by the Reaction of Vitamin A with Shell GDH Activated with Several Levels of Chlorosulfonic Acid

Time after mixing (minutes)	Concentration of Chlorosulfonic acid in GDH		
	0.046%	0.258%	0.504%
	$E^{1\%}_{1cm}$ at 553 mμ		
2.75	1262	1175	1086
7.75	1138	1030	987
12.75	982	856	862
17.75	834	711	743
22.75	703	591	648
27.75	591	496	563
32.75	490	416	492
	$E^{1\%}_{1cm}$ at 358 mμ		
4.37	153	160	160
9.37	166	175	175
14.37	174	184	187
19.37	179	192	196
24.37	184	197	202
29.37	186	200	207
34.37	188	202	211

Figure 8) shows that the activity of the chlorosulfonic acid-activated reagents increased with decreasing acid content in the reagent. The stability curves indicated that the color obtained with the reagent containing 0.504% chlorosulfonic acid did not fade as rapidly as did those obtained with the reagents containing less acid.

A comparison of Figures 3 and 8 indicates that chlorosulfonic acid-activated CDH produces a more stable color when mixed with a chloroform solution of vitamin A than does hydrochloric acid-activated CDH. The extinction coefficient obtained with the former was also slightly higher. The postulate expressed above has therefore been confirmed.

Another experiment was set up to determine the concentration of chlorosulfonic acid necessary to give maximum activation of CDH. Reagents containing from 0.001 to 2.535% chlorosulfonic acid by weight were prepared by using Eastman chlorosulfonic acid, practical grade, and redistilled, inactive CDH. Three sets of reagents were tested with chloroformic vitamin A solutions containing 4.78, 4.70 and 5.88 micrograms per ml. One part vitamin A solution was mixed with four parts reagent and the solutions poured into calibrated Beckman 1 cm. Corex cells. Optical density readings were made in the Beckman spectrophotometer at 555 mμ every half minute over the 2 to 5 minute interval and a final reading at 6 minutes. Since the concentration of vitamin A was known, the

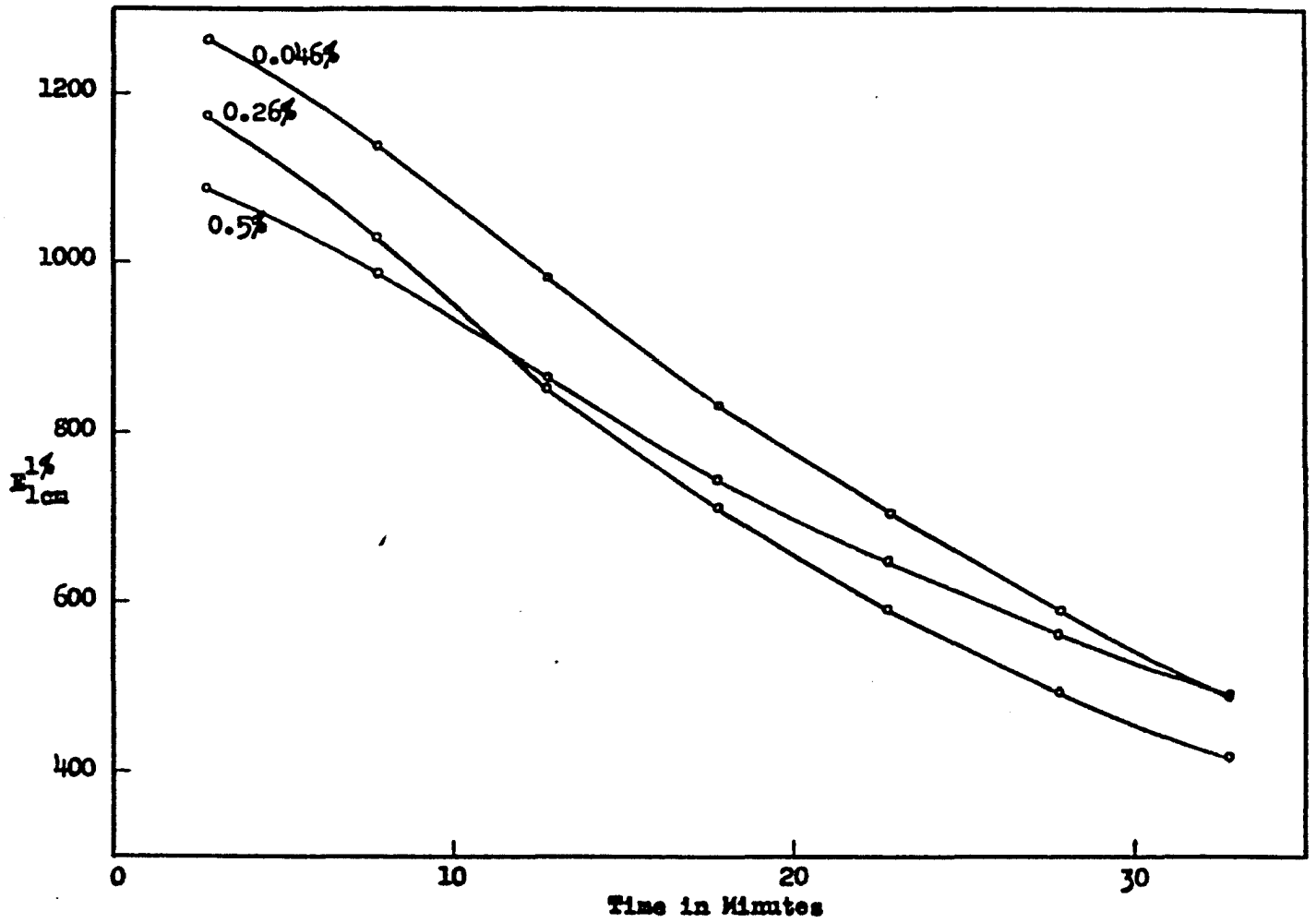


Figure 8. Stability of the Vitamin A-GDH Colors at 555 mμ when the Reagents were Activated with Several Levels of Chlorosulfonic Acid.

extinction coefficients were readily calculated for each reading. Table 8 summarizes the data collected by using reagents activated with fifteen different levels of chlorosulfonic acid. The stability of the 555 mμ absorption band was not especially good in the cases where the initial extinction coefficient was highest, however, reagents with higher acid concentrations gave more stable colors but of lesser intensity.

In order to better describe the range of acid concentration in CDH which gives good activity, the 2 minute extinction coefficients were plotted against the logarithm of the per cent acid (Figure 9). Maximum activation appears to be with approximately 0.1% acid. Good activation was obtained with reagents containing from 0.02 to 0.5% chlorosulfonic acid.

Several of the chlorosulfonic acid-activated reagents were set aside in clear, glass-stoppered flasks. The activity of each reagent was checked after four to five weeks storage in the laboratory by using a chloroform solution containing 5.71 micrograms vitamin A per ml. Readings were made at 555 mμ in the Beckman spectrophotometer as described above. The effect of storage is indicated in Table 9. The activity of reagents that were most active when freshly prepared decreased significantly during storage. Increased activity was observed with the reagents containing relatively

Table 8

Stability of the Color Produced by the Reaction of Vitamin A with Shell Glycerol Dichlorohydrin Activated with Chlorosulfonic Acid

Per Cent Acid in Reagent	Time after mixing (minutes)							
	2.0	2.5	3.0	3.5	4.0	4.5	5.0	6.0
			$E^{1\%}_{1cm}$ at 555 mμ					
2.535	727	736	744	757	761	761	757	757
1.637	816	825	833	833	833	833	825	821
1.263	897	901	906	906	901	901	893	884
0.938	974	978	978	978	965	952	952	923
0.2216	1185	1148	1105	1063	1010	967	925	850
0.1142	1241	1214	1173	1141	1099	1057	1015	942
0.113	1185	1137	1063	1010	946	893	840	744
0.0672	1132	1074	978	914	850	765	712	606
0.0526	1125	1068	1005	932	879	827	775	670
0.0313	1141	1099	1047	984	911	848	785	680
0.0160	1052	988	914	840	765	691	638	500
0.01036	958	900	848	796	733	680	628	534
0.00509	489	500	468	457	425	404	361	308
0.00470	162	168	168	168	157	157	147	147
0.001077	42	52	52	52	52	52	52	52

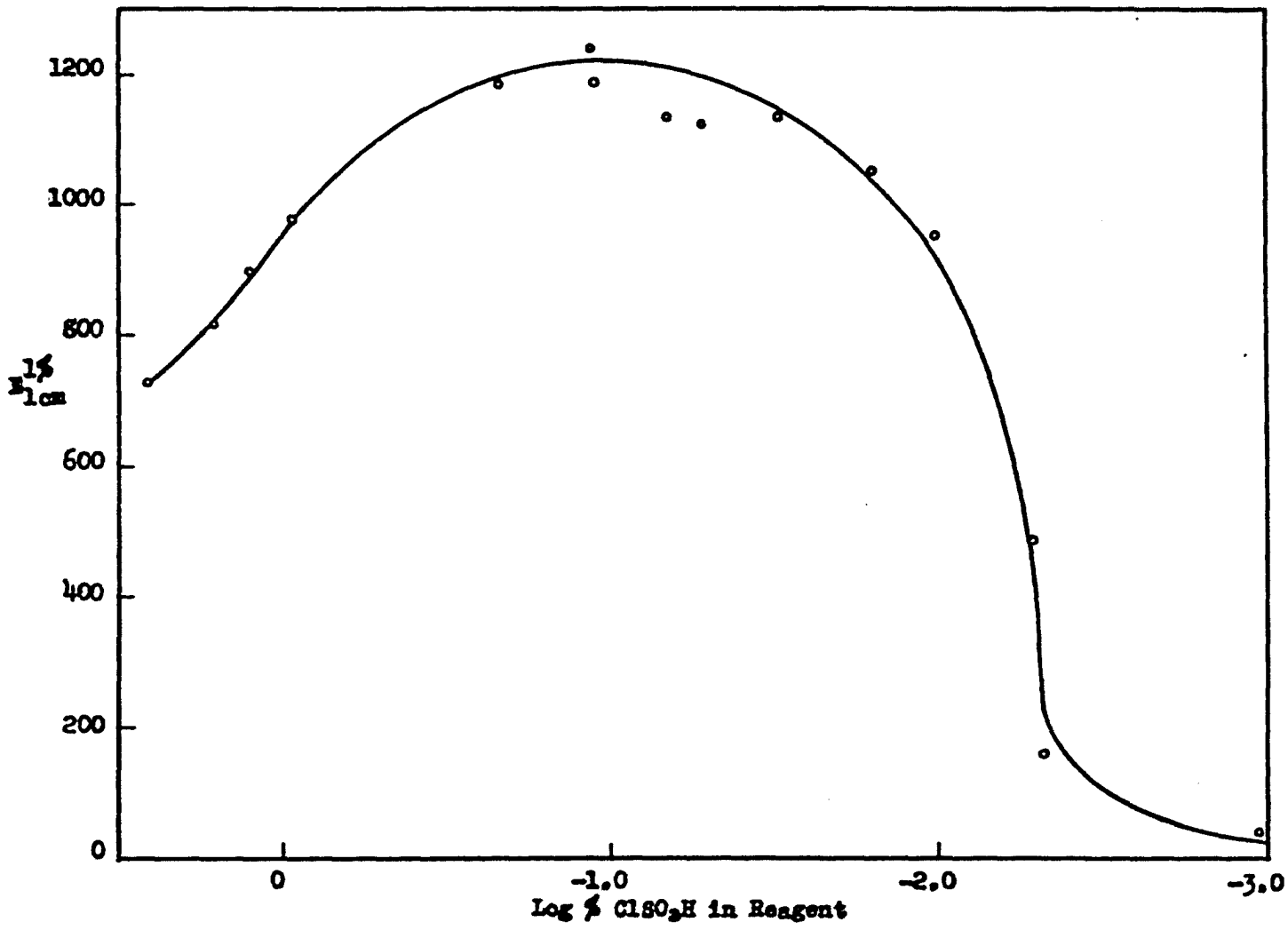


Figure 9. Activation of Shell GDH with Chlorosulfonic Acid.

high or low acid concentrations. This has not been explained.

Activation with antimony trichloride. Two methods of antimony trichloride-activation of redistilled, inactive GDH were investigated. The first involved vacuum distil-

Table 9

Effect of Aging on the Activity of Shell Glycerol Dichlorohydrin Activated with Chlorosulfonic Acid

Per Cent Acid in Reagent	Freshly Prepared	4 to 5 weeks Storage
	$E^{1\%}_{1cm}$ at 555 mμ	
1.263	897	1038
0.2216	1185	946
0.1142	1241	1056
0.0313	1141	574
0.01036	958	525
0.0047	162	421

lation of inactive GDH with 1.06% antimony trichloride according to the method prescribed by Sobel and Werbin (211). After discarding the first portion that boiled below 76°C., the reagent boiling between 76° and 79°C. at 15 mm. pressure was collected (reagent no. 1). Part of this reagent was again subjected to vacuum distillation. The product boiling between 75.5° and 76.5°C. at 15 mm. pressure was

collected (reagent no. 2). Part of reagent no. 2 was redistilled and the fraction distilling at the same conditions of temperature and pressure was collected (reagent no. 3).

These reagents were then tested for activity and stability of color produced by reaction with a chloroform solution containing 10.38 micrograms vitamin A per ml. Four parts of reagent were mixed with one part of vitamin A solution and the absorption spectra over the range of 340 to 700 mμ were determined with the aid of the Cary recording spectrophotometer. Seven tracings were made with each reaction mixture during the 35 minute period after the reactions were initiated.

From the series of absorption spectra of each mixture, the stability of the color could be determined. Table 10 summarizes these data and they are plotted in Figure 10. The stability of the 555 mμ band appeared to be best in the case of the colorimetric reaction involving reagent no. 2.

Ultraviolet absorption spectra of these reagents indicated the presence of considerable antimony trichloride in reagent no. 1, a small amount in no. 2 and practically none in no. 2.

The reactivity of these reagents was checked by using the Beckman spectrophotometer. One ml. of a vitamin A solution (8 micrograms per ml.) was mixed with 4 ml. of rea-

gent and the colored solution poured into 1 cm. Corex cells. Optical density measurements were made at 555 mμ every half minute over the interval of 1.5 to 5 minutes after initiation of the reaction. Two additional reagents were prepared

Table 10

Stability of Activated Shell GDH-Vitamin A Color at 555 mμ

Time after mixing (minutes	Reagent 1[a]	Reagent 2[b]	Reagent 3[c]
		$E^{1\%}_{1cm}$	
2.72	1245	1239	1083
7.72	1180		1049
9.22		1192	
12.72	1091		987
14.72		1107	
17.72	1010		918
19.22		1040	
22.72	932		848
23.72		977	
27.72	859		782
28.25		916	
32.72	800		722

[a] Prepared by one distillation of Shell GDH with $SbCl_3$.
[b] Prepared by redistillation of Reagent 1.
[c] Prepared by redistillation of Reagent 2.

and were tested in the same manner. The first was reagent no. 3 to which was added 0.018% antimony trichloride per se, and the second was a solution of 0.025% antimony trichloride in inactive GDH. Optical density values were converted

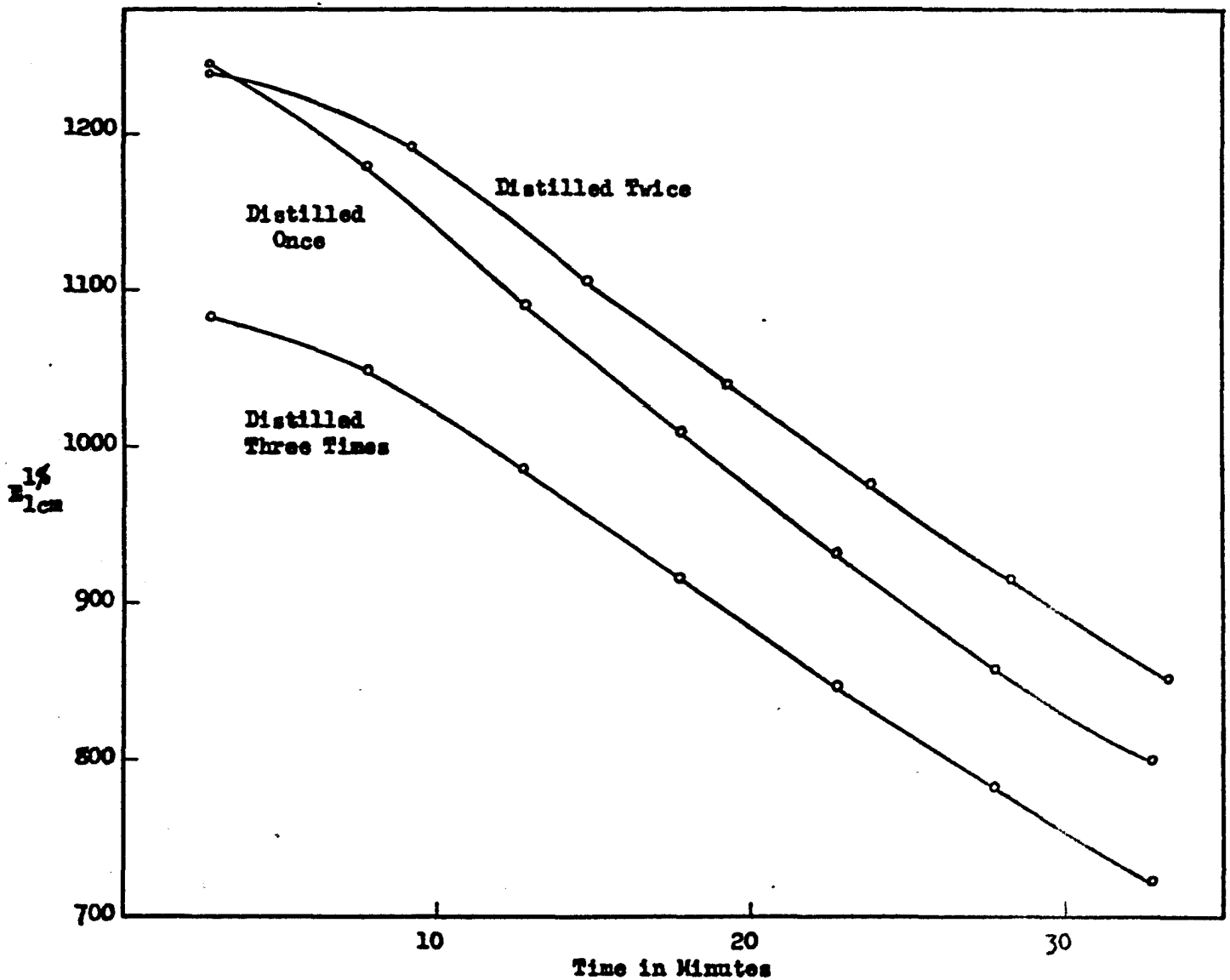


Figure 10. Influence of Activation Method on the Stability of the Vitamin A-Shell GDH Color with 555 mμ as Determined by the Cary Recording Spectrophotometer.

to extinction coefficients and the data plotted (see Figure 11).

The activity and stability of the color produced by reagent no. 2 was better than those of any of the other reagents tested. It was interesting to note that the addition of antimony trichloride *per se* to reagent no. 3 caused a reduction in activity of the reagent and influenced the course of the color development; maximum color was not attained until about 3 minutes after the reaction was initiated. Addition of a small amount of antimony trichloride to inactive GDH did not cause any significant activation, however, the absorption at 555 mμ increased over the 5 minute interval.

The second antimony trichloride-activation procedure involved addition of Baker's analyzed antimony trichloride to inactive GDH, then subjecting the reagents to 80° to 85°C. temperature for varying lengths of time. The GDH used in this experiment was inactivated by treating 500 ml. of active GDH with a solution containing 1 pellet of KOH in 2-3 ml. of water. The aqueous KOH was added to neutralize any free acid which might have been responsible for the activity of GDH which had become active by standing for two or three weeks in a clear, glass-stoppered bottle. Upon vacuum distillation a completely inactive reagent was obtained.

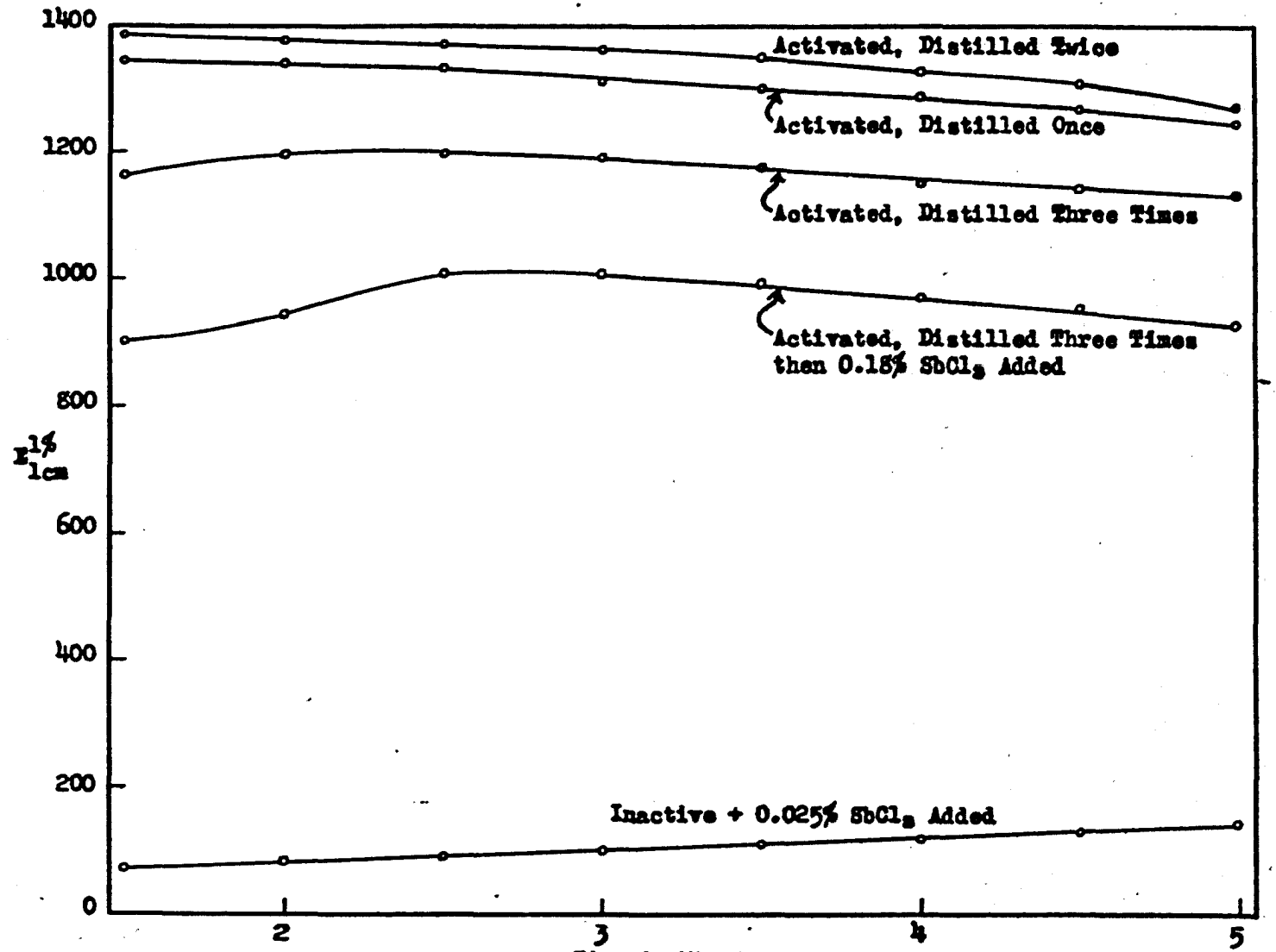


Figure 11. Influence of Activation Method on the Stability of the Vitamin A-Shell GDH Color at 555 mμ.

Reagents containing 0.0948, 0.391 and 1.06% antimony trichloride by weight were prepared. Each reagent was divided into four-10 ml. portions (in glass-stoppered volumetric flasks). The reagents were subjected to 1, 2 and 4 hours heating at 80° to 85° C. in an oven. Inactive GDH was also given the same heat treatment. The resulting reagents were then permitted to stand overnight.

The ultraviolet absorption spectrum of each reagent was determined with the aid of the Cary recording spectrophotometer, using reagent grade methanol as the solvent. The absorption spectra were obtained over a range of 230 to 400 mμ using the 10 mm. absorption cells. Figure 12 indicates the ultraviolet absorption spectra of untreated inactive GDH, GDH containing 0.0948% antimony trichloride, and the latter reagent heated for 1, 2 and 4 hours. The extinction coefficients were based solely on the GDH content in the solutions. It can be readily seen that the absorption at, for example, 270 mμ was increased by simple addition of antimony trichloride per se, and increased to even a greater extent by heating at 85°C. Maximum absorption was observed in the reagent that was heated for 4 hours. Apparently, heating effected some sort of chemical reaction which produced a compound or mixture of compounds which absorbed light in the ultraviolet region. The absorption spectra of the reagents containing higher levels of antimony trichlor-

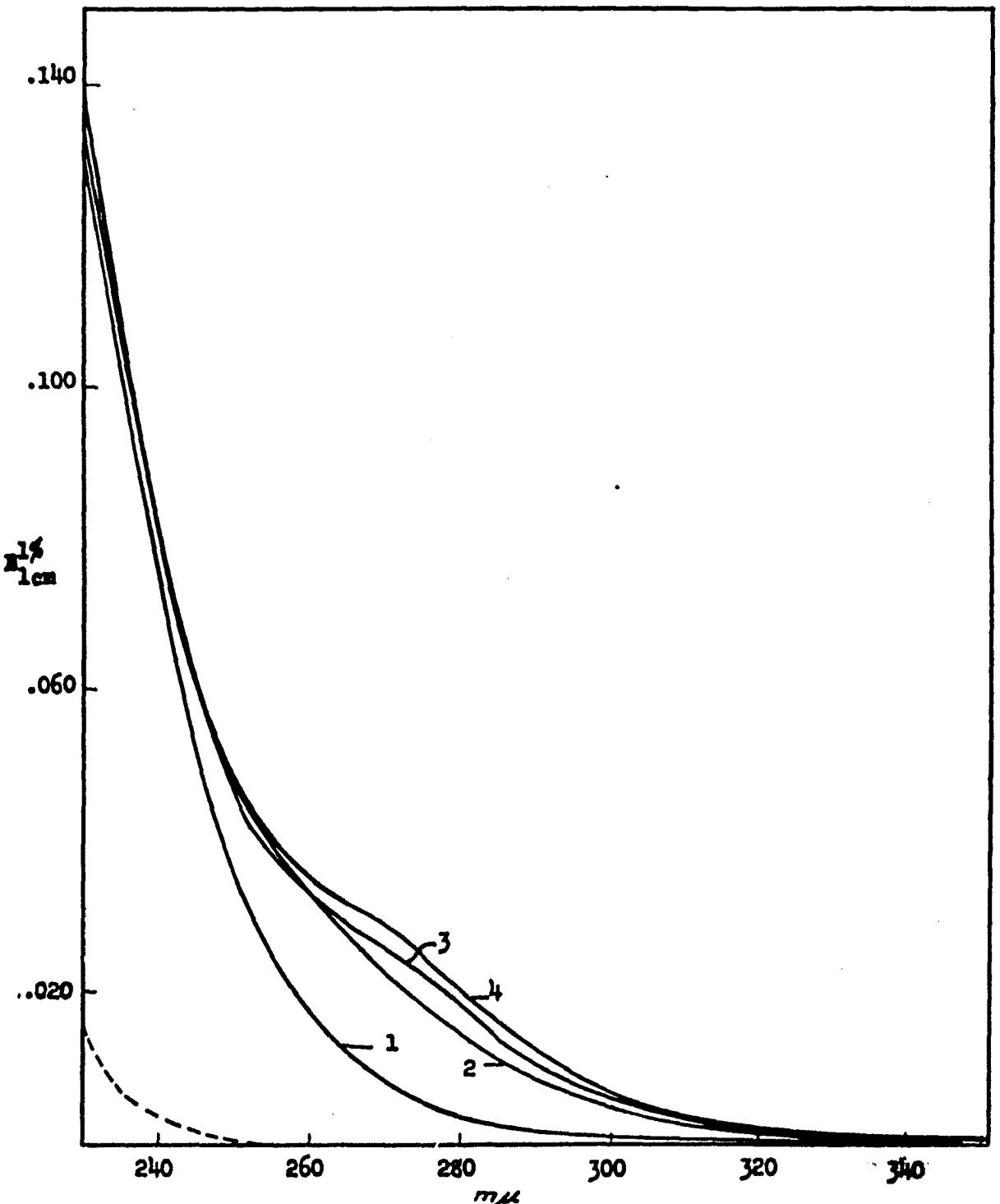


Figure 12. Ultraviolet Absorption Spectra of Glycerol Dichlorohydrin with 0.39% Added Antimony Trichloride: Reagent Not Heated (Curve 1), Heated 1 Hour (Curve 2), Heated 2 Hours (Curve 3) and Heated 4 Hours (Curve 4). The Dotted Line Indicates the Absorption by Inactive GDH.

ide indicated similar changes resulting from heating except that the changes were less pronounced. As the antimony trichloride content increased the absorption increased in the lower-wavelength region. Changes in absorption resulting from the heating process were almost negligible in the reagent containing 1.06% antimony trichloride.

That the change in the ultraviolet absorption spectra which results on mere addition of antimony trichloride to GDH is due entirely to antimony trichloride per se was shown by determining the absorption spectrum of a solution of antimony trichloride in reagent grade methanol. Summation of optical density of GDH absorption and antimony trichloride absorption was equivalent to the absorption of the mixture.

The activity of the above antimony trichloride-activated reagents was tested with a chloroform solution containing 5.08 micrograms of vitamin A per ml. Four parts of reagent were mixed with one part of vitamin A solution and poured into calibrated 1 cm. Corex cells. Optical density readings were made at 555 mμ in the Beckman spectrophotometer. Eight readings were made between 2 and 6 minutes after mixing the reactants. The extinction coefficients were calculated and listed in Table 11. The activity of each batch of reagent increased with increased heating time and maximum activation was attained with the reagent containing

Table 11

Stability of the Color Developed by the Reaction of Vitamin A with Shell Glycerol Dichlorohydrin Activated by Heating with $SbCl_3$ at 80° to 85°C.

Per Cent $SbCl_3$ in Reagent	Heating Time	Time after mixing (minutes)							
		2.0	2.5	3.0	3.5	4.0	4.5	5.0	6.0
		$E^{1\%}_{1cm}$ at 555 mμ							
0.0948	none	403	413	403	394	364	354	325	285
	1 hour	689	659	620	600	560	531	502	443
	2 hours	945	915	876	846	797	767	738	669
	4 hours	1072	1043	1004	974	945	905	866	797
0.3913	none	364	413	433	433	413	394	384	335
	1 hour	689	669	640	610	581	551	521	462
	2 hours	876	846	817	777	748	718	689	620
	4 hours	886	856	826	800	758	728	699	620
1.0566	none	689	807	876	905	905	905	895	861
	1 hour	748	748	738	718	699	679	659	610
	2 hours	915	895	876	866	836	807	787	748
	4 hours	1102	1082	1063	1053	1023	1004	974	925
none	none	0	0	0	0	0	0	0	0
	1 hour	0	0	0	0	0	0	0	0
	2 hours	148	177	207	216	236	216	216	187
	4 hours	826	767	718	659	610	550	512	413

1.06% antimony trichloride and had been heated for 4 hours. Curiously enough, even the inactive control became slightly active on heating for 2 hours and quite active when heated for 4 hours. To get a better understanding of the effect of heat on the activation with antimony trichloride, the 2 minute extinction coefficients were plotted against heating time of reagent (Figure 13).

Activation by heating. Activation of GDH by heat alone appeared to be rather odd, so another experiment was set up to recheck the first work. GDH that had been used, recovered, and deactivated by treatment with aqueous KOH prior to final vacuum distillation was used in this study. About 9 ml. of this GDH was placed in several 10-ml. glass-stoppered volumetric flasks. After being tightly stoppered the flasks were placed in an oven kept at 84° to 88°C. The heat treatment ranged from 1 to 6 hours after which the flasks were removed and allowed to cool. Two days later, the reagents were checked for activity by testing with a chloroform solution containing 6.16 micrograms vitamin A per ml. One ml. vitamin A solution was mixed with 4 ml. of reagent and the optical density of the colors were measured at 555 mμ in the Beckman spectrophotometer. Readings were made at half-minute intervals from 2 to 4 minutes after initiation of the reactions. Figure 14 is a plot of the two minute extinction coefficients calculated from the observed optical density readings. GDH

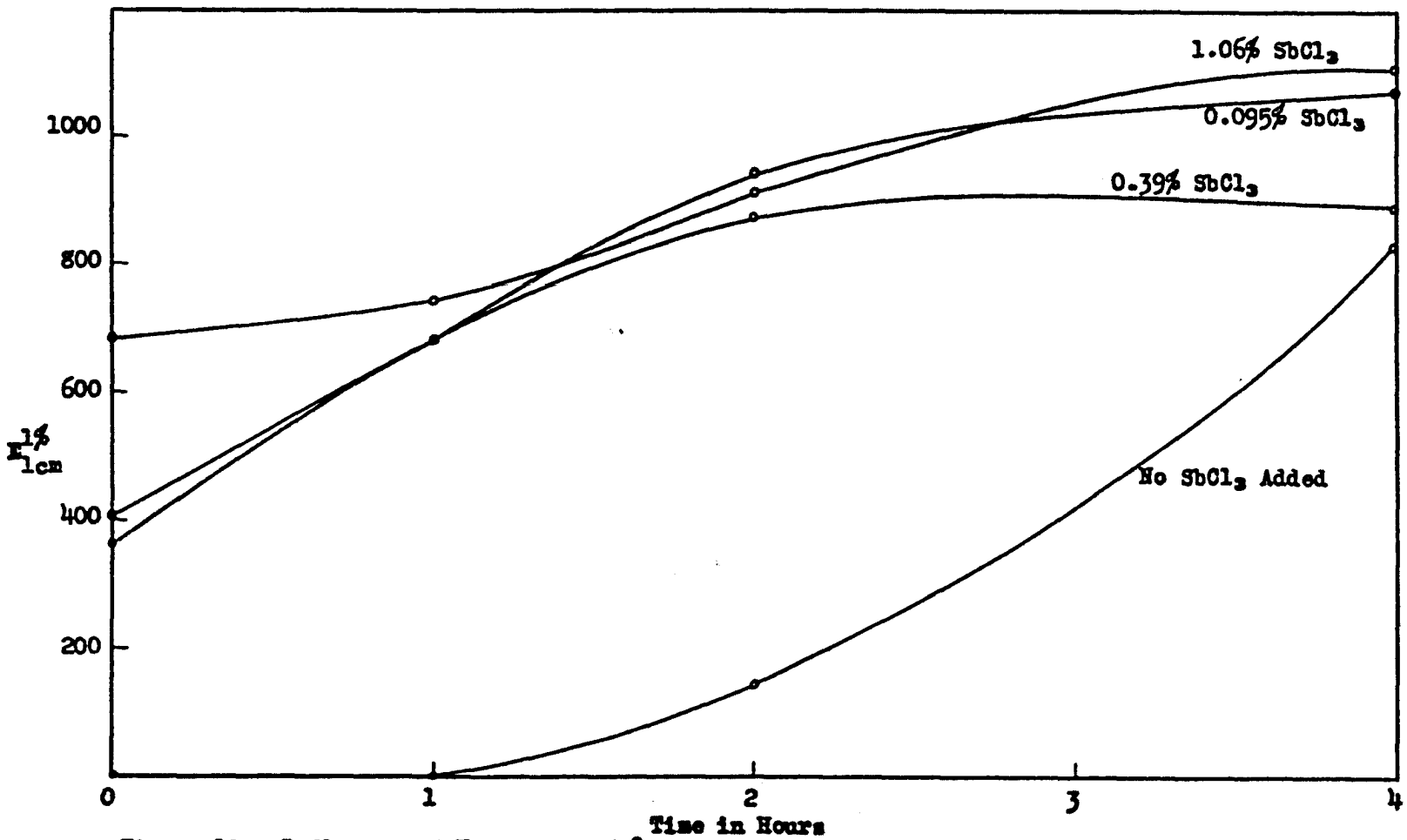


Figure 13. Influence of Heating at 85°C. on the Activation of Glycerol Dichlorohydrin with and without Antimony Trichloride.

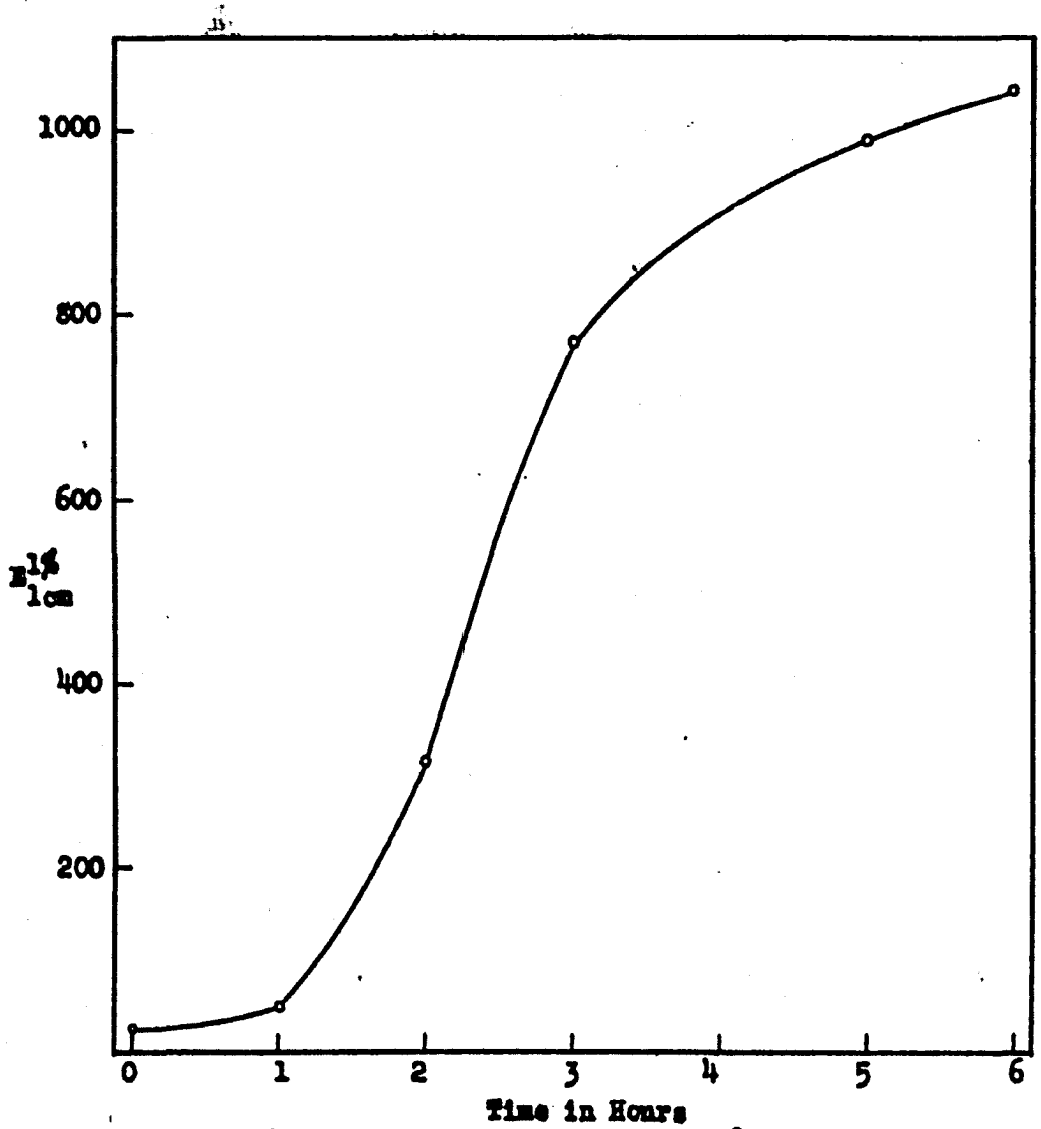


Figure 14. Influence of Heating at 85°C. on the Activation of Previously Deactivated GDH.

appears to decompose, a reaction which seems to be catalyzed by heat and perhaps light, to form some substance which causes activation. These findings confirmed the original observation.

Activated Shohan Glycerol Dichlorohydrin.

Activated GDH purchased from Shohan Laboratories, Newark, New Jersey, was used without further treatment unless otherwise indicated. When received the reagent was clear but had a faint yellowish-green color. It was stored in brown bottles and kept under refrigeration until a few hours before use.

Deactivation. Five grams of activated charcoal (Nuchar W) were mixed with 500 grams of activated Shohan GDH in a 600 ml. beaker. The mixture was warmed on a steam plate, with occasional stirring, until the temperature had reached 60°C. The charcoal was removed by filtration through a Buchner funnel. The GDH was refiltered three times to assure removal of all charcoal, then placed in a clear, glass-stoppered bottle to cool. The product was transferred to a 1-liter Claisen flask and vacuum distilled. The first material to distill was a two-phase liquid that probably contained some water. This was followed by a clear, colorless, single phase. When the distillation temperature reached 76°C. at 16 mm. pressure, the receiver was changed and clear, colorless GDH was collected over the temperature range of 76.2 to

78.2°C. at 16 mm. pressure.

This GDH was tested with a chloroform solution of vitamin A and found to be faintly active. Redistillation produced a reagent that was still somewhat active. This GDH was treated with aqueous KOH and then distilled, with discarding of the first fraction and residue. The clear, colorless and completely inactive GDH was collected; boiling point, 76.2° to 78.2°C. at 16 mm. pressure.

Activation with heat. Since inactive Shell GDH became active when heated, it seemed advisable to test Shohan GDH in the same manner. The heat treatment was the same as described for the Shell product. When the cooled reagents were tested with vitamin A they were found to be completely inactive. Table 12 summarizes all the heat activation data. Shohan GDH is, therefore, much more stable than the Shell GDH that had been previously used and recovered. However, the inactive Shohan product became active when allowed to stand in a clear, glass-stoppered bottle for six weeks. Apparently GDH will decompose to some extent under the influence of heat and light to form a substance which causes activation. A qualitative test for the chloride ion in these products was positive. It seems likely then that one of the decomposition products is HCl.

Solvent-reagent ratio study. Although Sobel and Werbin (213) advocated the use of a 1:4 ratio, no report of any in-

Table 12

Effect of Heating at 85°C. on the Activation of Glycerol Dichlorohydrin

Glycerol Dichlorohydrin	Heating time	Time after mixing (minutes) 2.0	2.5	3.0	3.5	4.0
		$E^{1\%}_{1cm}$ at 555 mμ				
Used, recovered Shell						
Trial 1	none	0	0	0	0	0
	1 hour	0	0	0	0	0
	2 hours	148	177	207	216	236
	4 hours	826	767	718	659	610
Trial 2	none	24	24	24	24	24
	1 hour	49	65	73	81	81
	2 hours	317	365	365	349	325
	3 hours	772	755	690	650	569
	5 hours	991	918	828	747	674
	6 hours	1040	958	877	780	690
Inactivated Shohan	none	0	0	0	0	0
	1 hour	0	0	0	0	0
	2 hours	0	0	0	0	0
	3 hours	0	0	0	0	0
	4 hours	0	0	0	0	0
	5 hours	0	0	0	0	0
	6 hours	0	0	0	0	0

vestigation of the proper choice of solvent-reagent ratio has been published. Ratios ranging from 1:9 to 9:1 were chosen for study. Activated Shohan GDH was mixed with standard solutions of vitamin A in redistilled U.S.P. chloroform. The reaction mixtures were transferred to the 50 mm. absorption cells and the absorption spectra obtained with the aid of the Cary recording spectrophotometer. The spectral tracings were always begun at 700 mμ at 1.5 minutes after the reactants were mixed. The spectral range covered by these tracings was 325 to 700 mμ. Eight tracings were made over a period of 35 minutes using a scanning speed of 2 mμ per second. From these curves, which were actually plots of optical density at each wavelength, the extinction coefficients were calculated. The initial absorption spectra of the reaction products obtained when the 9:1, 5:1, 1:1, 1:5 and 1:9 solvent-reagent ratios were used are shown in Figure 15. As the quantity of solvent in the reaction mixture is increased, the absorption at 555 mμ decreased whereas the absorption at 353 mμ increased. In addition it was observed that with high solvent-reagent ratios, additional absorption maxima appeared at 338, 372, 397 and 422 mμ.

From the series of absorption spectral curves for each reaction mixture, the effect of aging on the extinction coefficients at the various absorption maxima was indicated. Table summarizes these data and Figures 16 and 17 show the

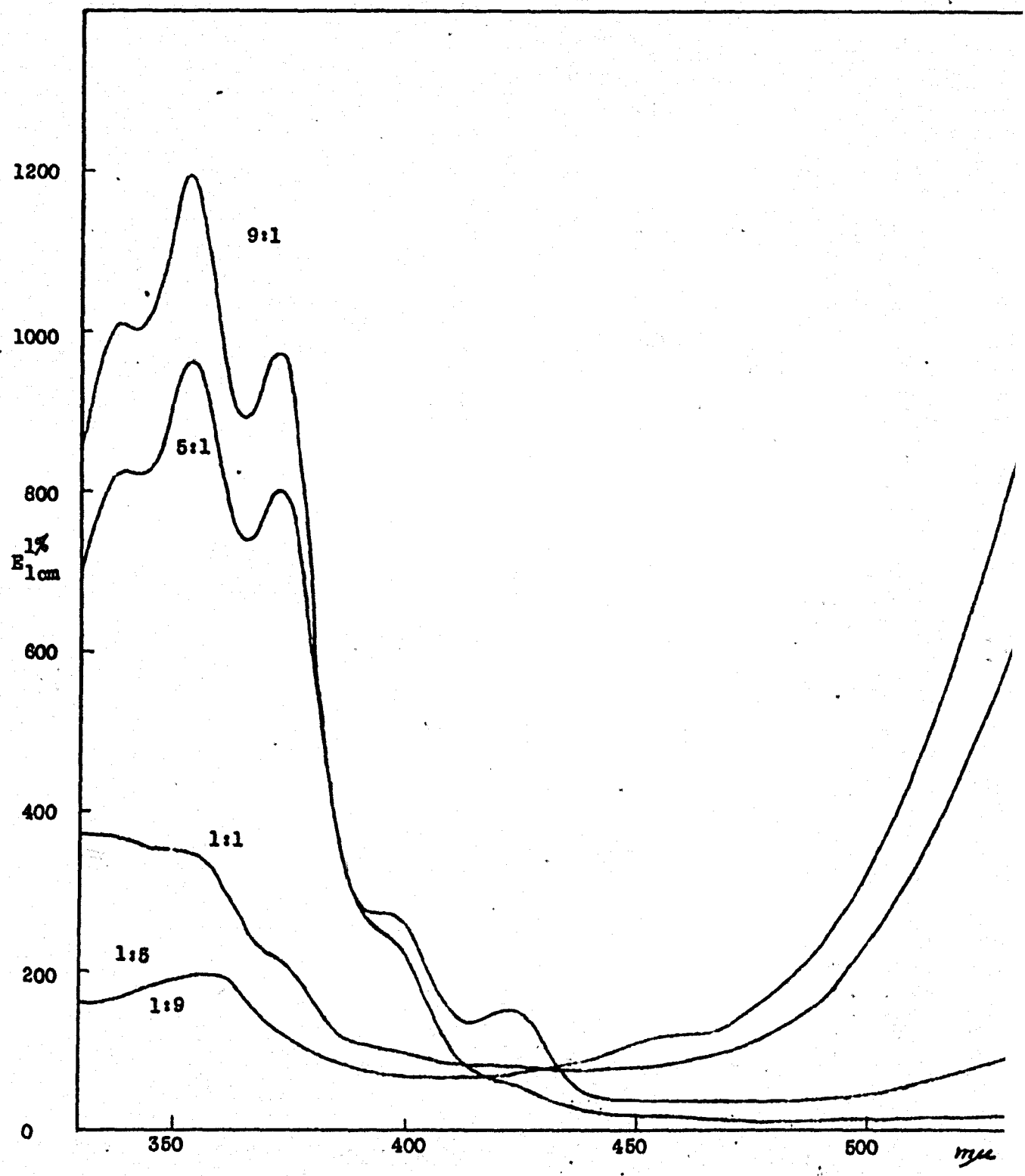
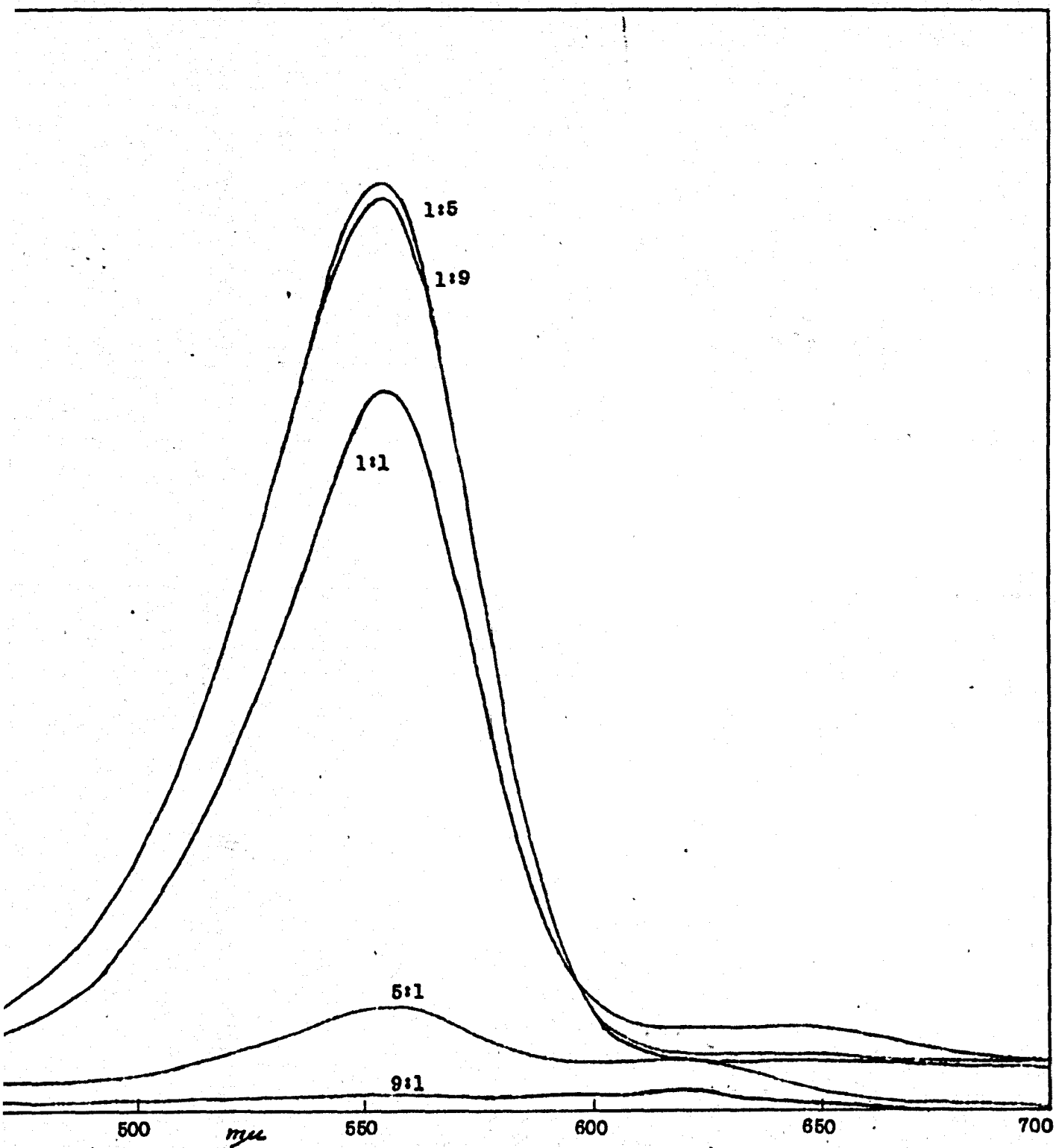


Figure 15. Absorption Spectra of the Products
Vitamin A when Several Solvent-Reag



Spectra of the Products of the Reaction of Shohan GDH with hen Several Solvent-Reagent Ratios were Employed.

Table 13

Stability of the Color Developed by the Reaction of Vitamin A with Shohan GDH when Several Solvent-Reagent Ratios are employed

Time after mixing (minutes)	Solvent-Reagent Ratio								
	1:9	1:7	1:5	1:3	1:1	3:1	5:1	7:1	9:1
	$E^{1\%}_{1cm}$ at 555 mμ								
2.72	1159	1165	1178	1171	919	357	150	52	25
7.22	1052	1050	1060	1036	753	238	162	108	66
11.72	930	922	926	899	599	159	103	80	66
16.22	818	809	809	775	473	117	80	60	55
20.72	718	705	704	667	372	94	68	52	46
25.22	621	619	611	571	295	81	63	48	43
29.72	548	541	529	491	239	75	57	46	42
34.22	481	473	457	421	194	72	54	44	40
	$E^{1\%}_{1cm}$ at 353 mμ								
4.37	196	200	202	213	352	793	1080	1325	1486
8.87	203	203	208	214	298	634	887	1093	1277
13.37	212	210	216	224	291	530	785	980	1151
17.87	219	218	225	232	290	465	698	911	1074
22.37	226	228	233	240	288	413	633	845	1016
26.87	234	234	239	248	287	384	565	785	966
31.37	239	240	245	251	285	355	522	736	916
35.87	245	246	249	254	285	341	478	689	870
	$E^{1\%}_{1cm}$ at 338 mμ								
4.53					371		926		1249
9.03					315		804		1062
13.53					299		758		970
18.03					292		722		928
22.53					288		680		899
27.03					283		650		871
31.53					278		617		843
36.03					273		591		823

Table 13
(Continued)

Time after mixing (minutes)	Solvent-Reagent Ratio								
	1:9	1:7	1:5	1:3	1:1	3:1	5:1	7:1	9:1
					$E^{1\%}_{1cm}$ at 372 mμ				
4.23					217	634	901	1092	1209
8.73					186	464	715	917	1071
13.23					183	362	606	811	970
17.73					178	304	513	727	896
22.23					174	261	452	658	832
26.73					171	239	400	599	776
31.23					167	225	358	549	725
35.73					163	217	330	508	676
					$E^{1\%}_{1cm}$ at 397 mμ				
4.05							306		309
8.55							280		330
13.05							226		340
17.55							196		320
22.05							183		295
26.55							174		271
31.05							172		252
35.55							170		236
					$E^{1\%}_{1cm}$ at 422 mμ				
3.82							174		70
8.32							205		186
12.82							174		230
17.32							155		235
21.82							148		223
26.32							146		208
30.82							146		195
35.32							146		188

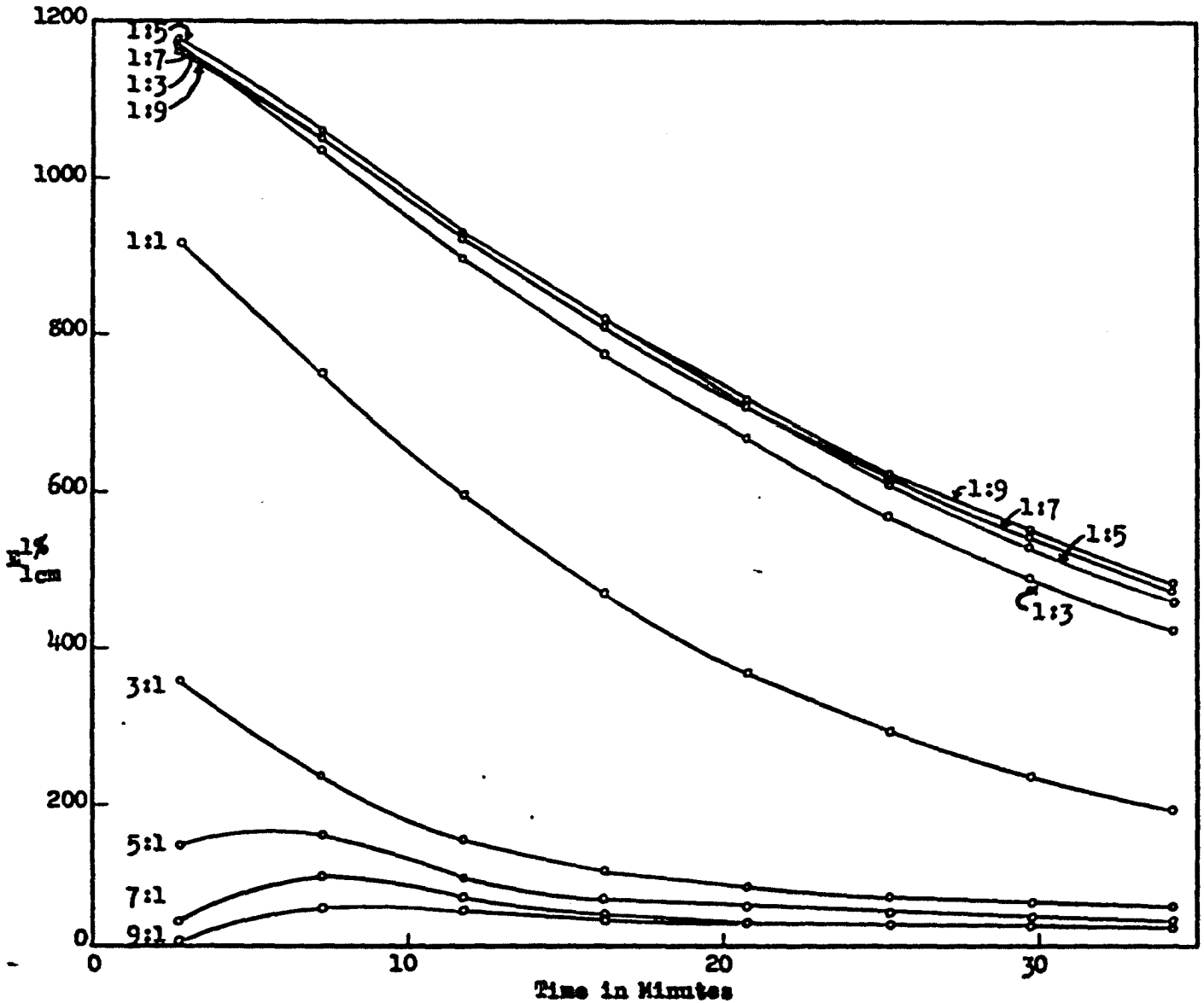


Figure 16. Influence of the Solvent-Reagent Ratio on the Absorption at 555 mμ by the Product of the Vitamin A-Shohan GDH Reaction.

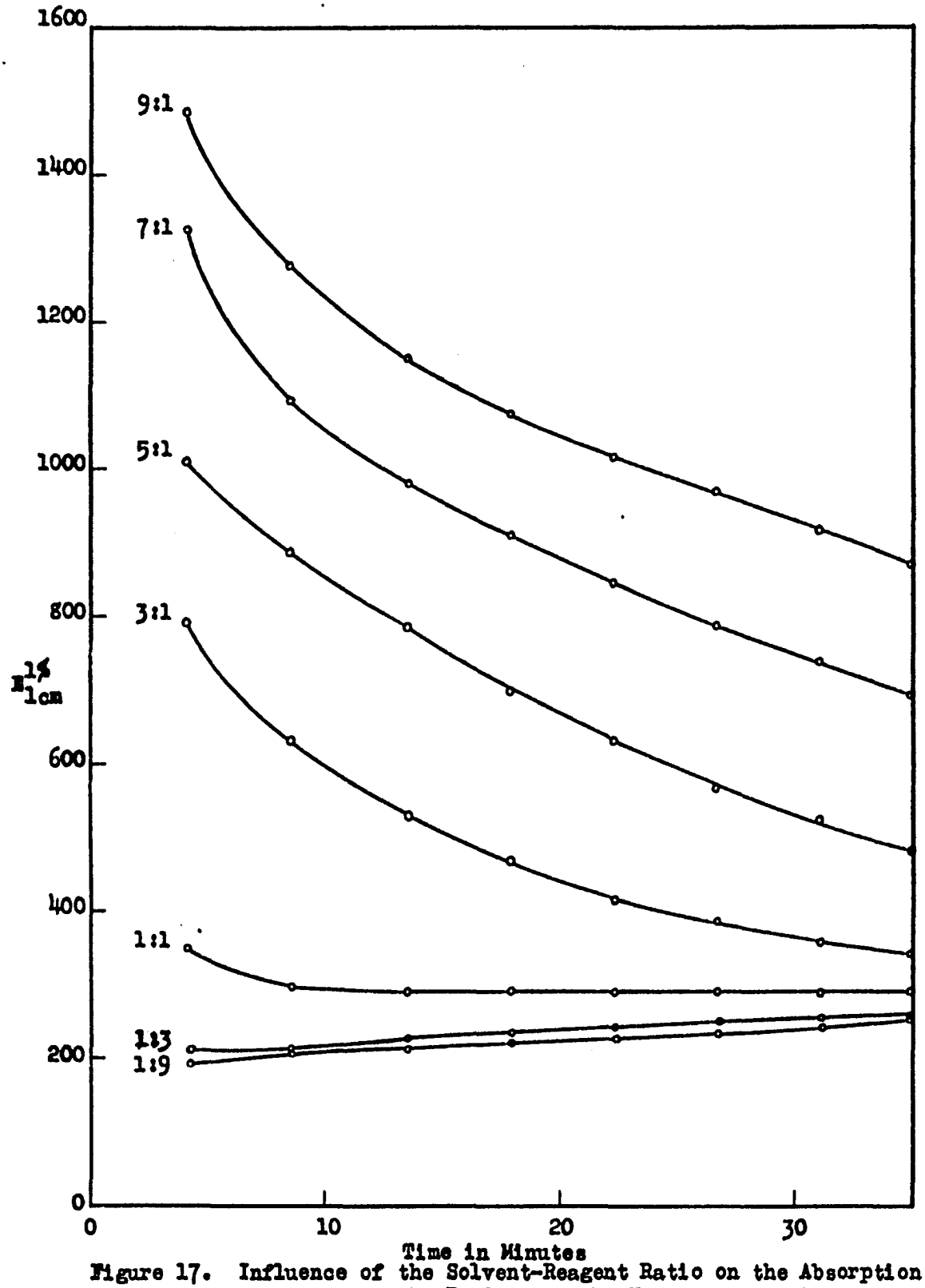


Figure 17. Influence of the Solvent-Reagent Ratio on the Absorption at 353 mμ by the Product of the Vitamin A-Shohan GDH Reaction.

effect of time on the stability of the 555 and 353 mμ maxima with the several solvent-reagent ratios studied. The stability and intensity of the 555 mμ maximum was improved by using low solvent-reagent ratios, whereas the use of high solvent-reagent ratios increased the extinction coefficient but did not improve the stability at 353 mμ.

The effect of the solvent-reagent ratio on the absorption spectra was also investigated with Shohan GDH which had been previously used in blood vitamin A analysis then recovered and reactivated by vacuum distillation with antimony trichloride. This reagent was clear and colorless and gave a good color reaction with vitamin A. The following ratios were investigated: 1:4, 2:3, 3:2 and 4:1. The method of obtaining the absorption spectra of the products resulting from reaction of GDH with vitamin A was the same as described above. The initial absorption spectral curve for each reaction mixture is shown in Figure 18. These curves are like those obtained with original Shohan GDH except for the magnitude of the extinction coefficients at 353 mμ when higher solvent-reagent ratios were used. A 4:1 ratio in this case gave an extinction coefficient of 1520 as compared to a value of approximately 900 when original Shohan GDH was used.

The extinction coefficients at the absorption maxima and the manner in which they change as the reaction mixtures

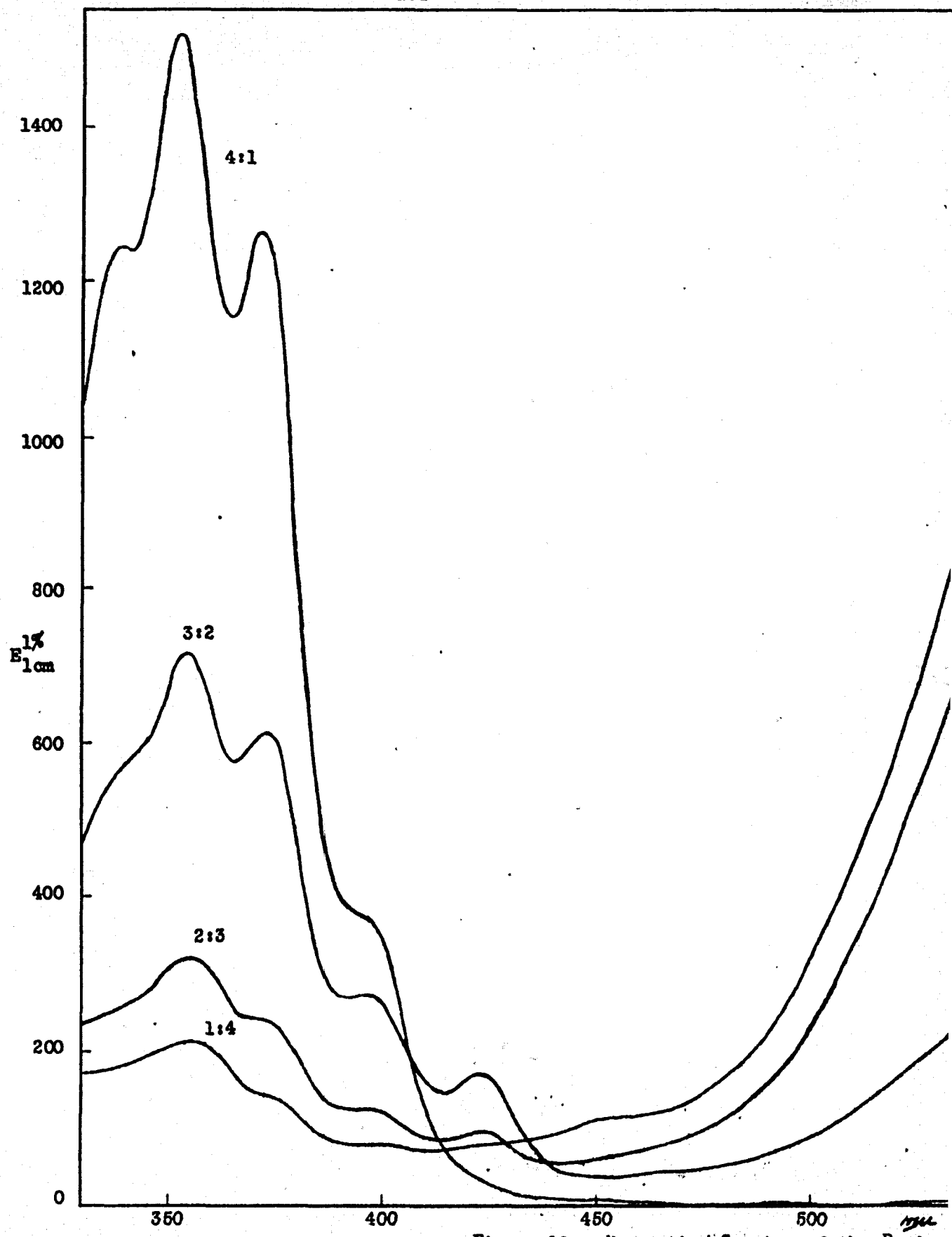
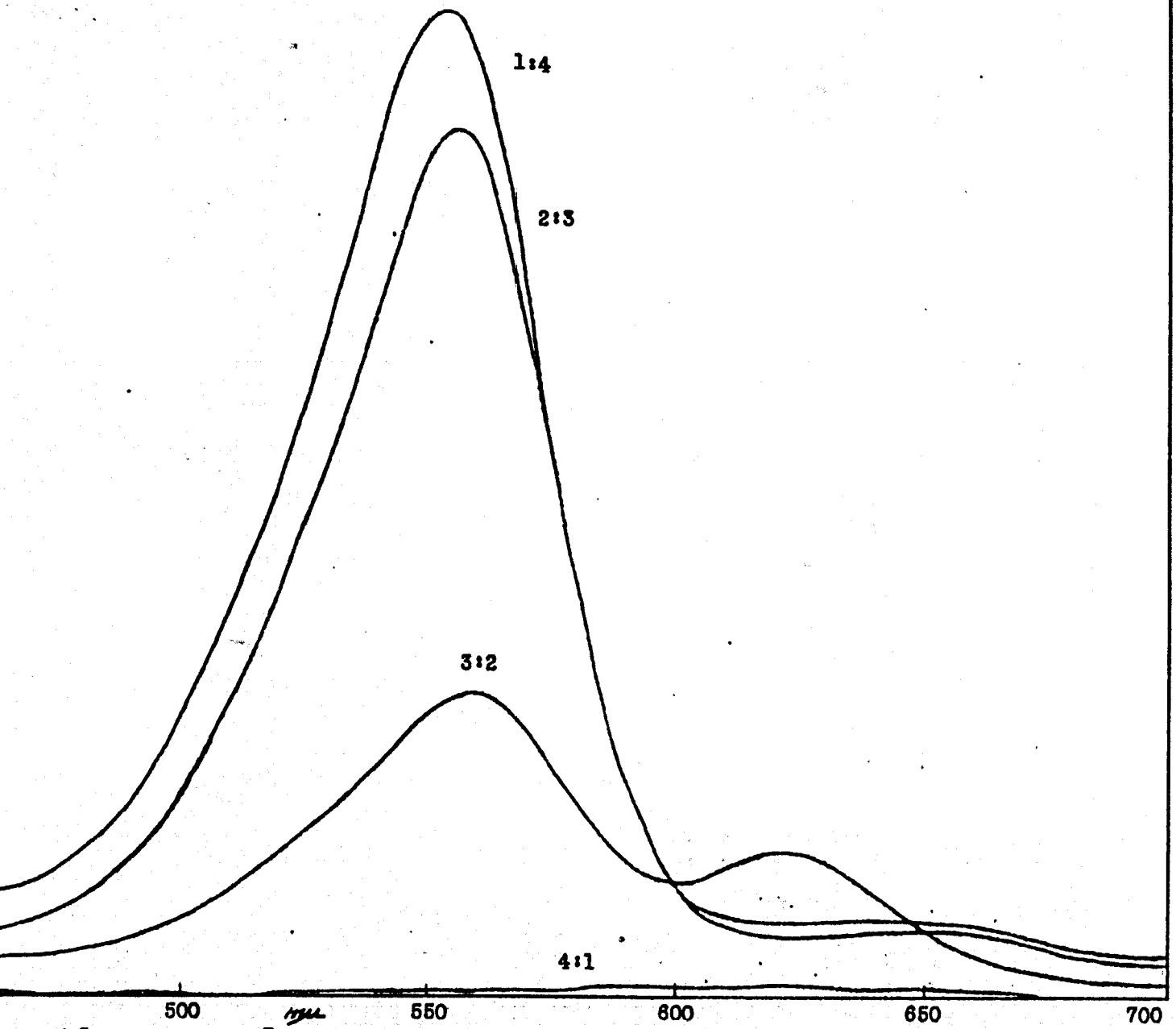


Figure 18. Absorption Spectra of the Produc
Recovered, Reactivated GDH when



ption Spectra of the Products of the Reactions of Vitamin A with
ered, Reactivated GDH when Several Solvent-Reagent Ratios were Employed.

age are shown in Table 14. The stability of the 555 mμ absorption maxima is demonstrated in Figure 19. The 1:4 reaction mixture produced a color which was fairly stable for several minutes. This was not the case when the original Shohan product was tested (see Figure 16). The stability of the 353 mμ maxima is indicated in Figure 20. Here again the reactivated, used GDH produced a more stable 353 mμ maximum than did the new product.

Reaction with β-carotene. The interference of carotene in the measurement of vitamin A at 555 mμ has been adequately described by Sobel and Werbin (213). These workers have also described the absorption spectrum of the GDH-carotene reaction and it was shown that the absorption increased markedly from 750 to 900 mμ. No mention was made of the stability of the reaction product at the longer wavelengths. Since carotene is almost invariably present in blood plasma, it seemed important to check on the reaction of GDH with carotene.

A solution containing 9.6 micrograms of crystalline β-carotene (General Biochemicals, Inc.) per ml. of reagent grade chloroform was prepared. One ml. of this solution was mixed with 4 ml. of Shohan GDH and the optical density was measured at various intervals up to 6 minutes in the Beckman spectrophotometer. Measurements were made at 800, 850, 900, 950, 1000, 1050 and 1100 mμ. The validity of the readings

Table 14

Stability of the Color Developed by the Reaction of Vitamin A with Used, Recovered, $SbCl_3$-Activated GDH when Several Solvent-Reagent Ratios were Employed

Time after mixing (minutes)	Solvent-Reagent Ratio			
	1:4	2:3	3:2	4:1
		$E^{1\%}_{1cm}$ at 553 mμ		
2.75	1110	983	344	7
7.75	1088	868	330	21
12.75	1026	759	253	28
17.75	954	653	210	30
22.75	881	560	184	30
27.75	818	479	164	30
32.75	753	410	149	30
		$E^{1\%}_{1cm}$ at 353 mμ		
4.37	217	321	715	1520
9.37	187	262	556	1420
14.37	181	228	479	1255
19.37	181	222	418	1132
24.37	184	222	370	1056
29.37	187	222	333	997
34.37	193	222	299	953
		$E^{1\%}_{1cm}$ at 422 mμ		
3.82		96	172	37
8.82		82	202	140
13.82		75	184	251
18.82		76	169	314
23.82		79	155	338
28.82		82	147	343
33.82		83	140	338

Table 14
(Continued)

Time after mixing (minutes)	Solvent-Reagent Ratio			
	1:4	2:3	3:2	4:1
		$E^{1\%}_{1cm}$ at 397 mμ		
4.02		126	275	372
9.02		100	262	379
14.02		90	233	432
19.02		86	209	456
24.02		87	192	468
29.02		88	175	460
34.02		88	162	439
		$E^{1\%}_{1cm}$ at 372 mμ		
4.23		245	618	1266
9.23		187	482	1234
14.23		164	411	1115
19.23		149	356	1032
24.23		143	314	972
29.23		143	277	920
34.23		143	253	875

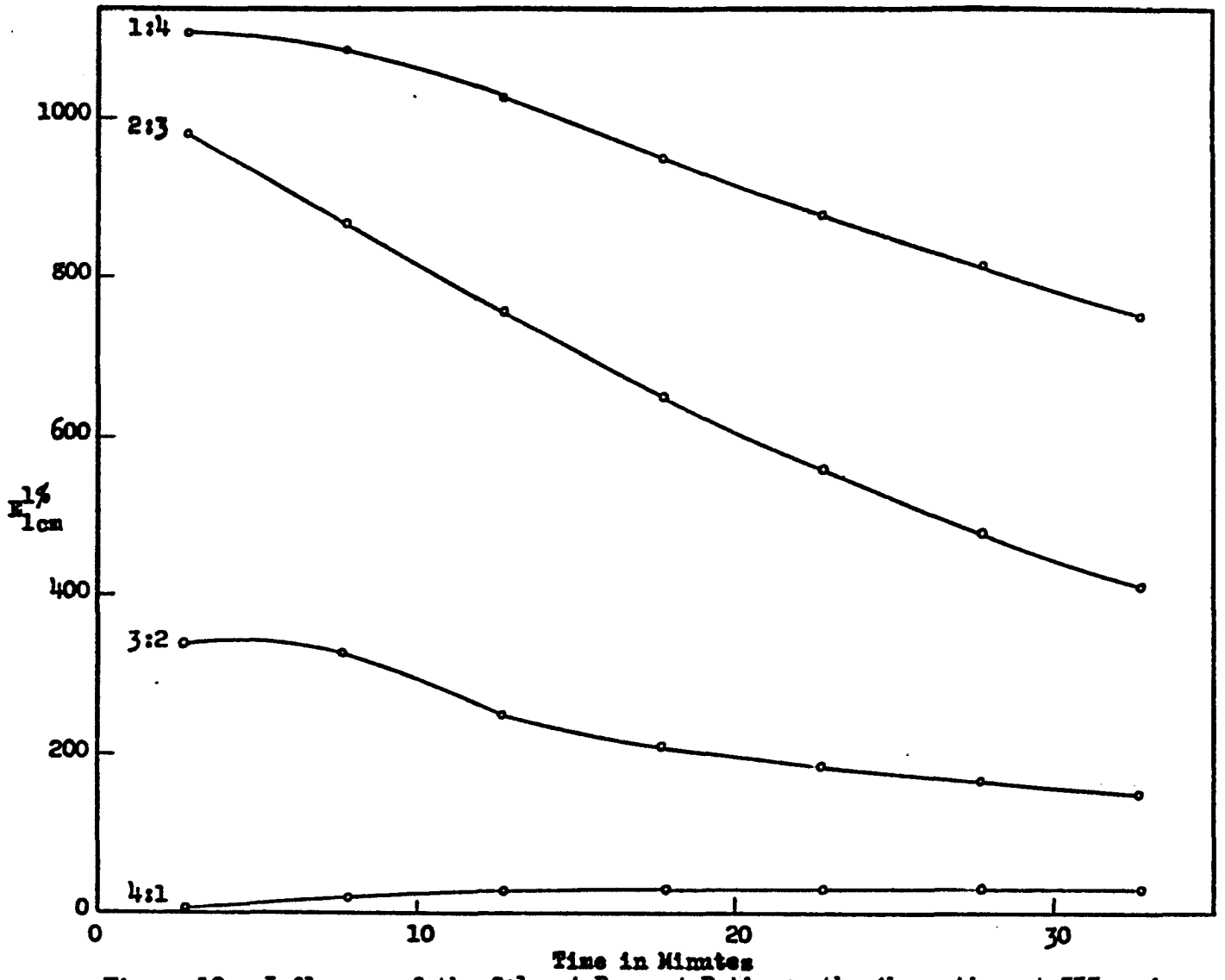


Figure 19. Influence of the Solvent-Reagent Ratio on the Absorption at 555 mμ by the Product of the Reaction of Vitamin A with Reactivated, Once-Used GDH.

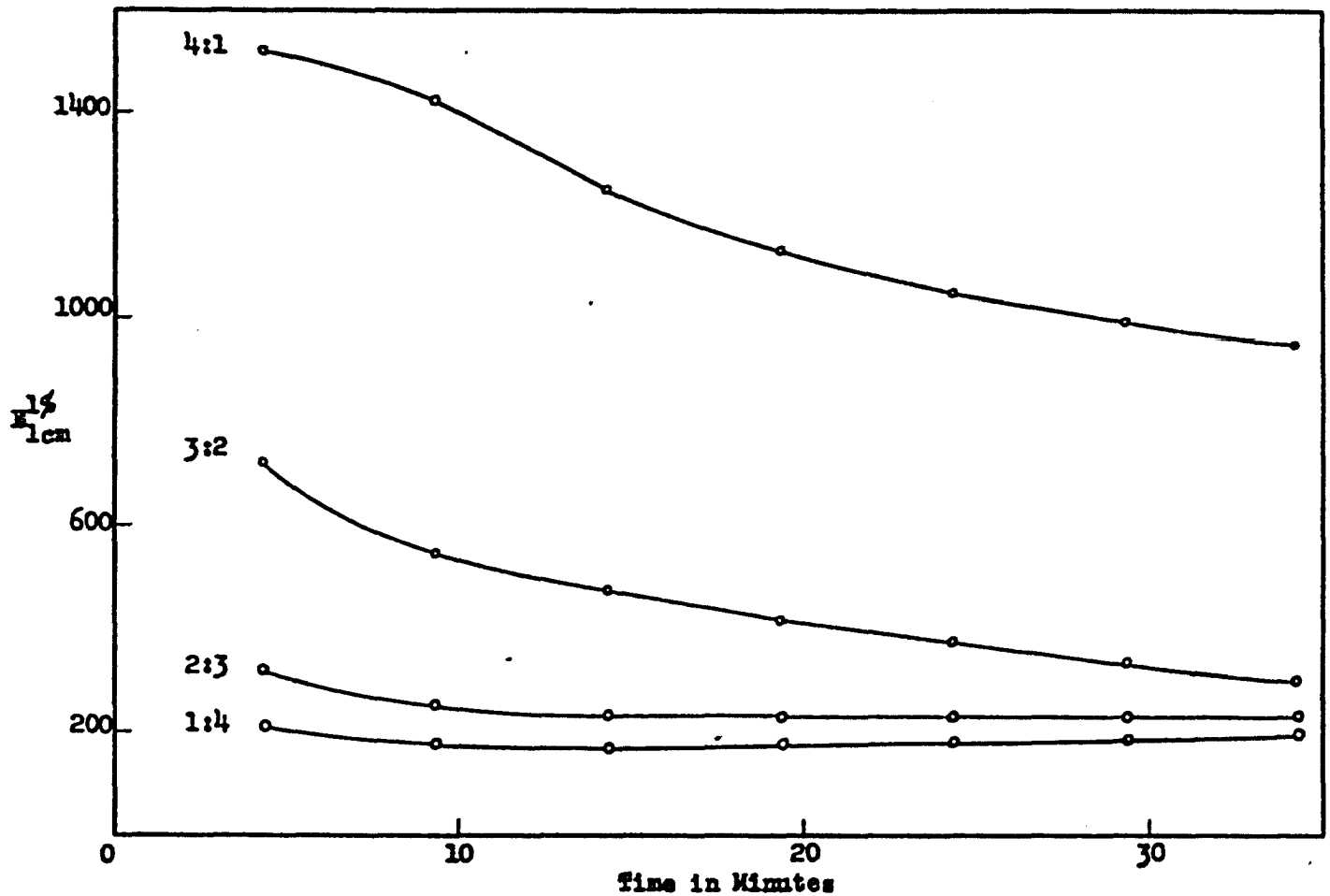


Figure 20. Influence of the Solvent-Reagent Ratio on the Absorption at 353 mμ by the Product of the Reaction of Vitamin A with Reactivated, Once-Used GDH.

at the last two wavelengths is not certain but they seemed to indicate that an absorption maximum occurs at approximately 1030 mμ.

Extinction coefficients were calculated from the optical density measurements and are listed in Table 15. The

Table 15

Stability of the Color Produced by the Reaction of β-Carotene with Shohan Glycerol Dichlorohydrin

Wave Length mμ	Time after mixing (minutes)						
	1.0	1.5	2.0	3.0	4.0	5.0	6.0
				$E^{1\%}_{1cm}$			
800	344	354	365	375	396	417	437
850	784	802	807	812	828	839	844
900	1573	1578	1573	1568	1552	1526	1521
950	2547	2552	2542	2490	2427	2359	2297
1000	3604	3583	3526	3422	3297	3141	3021
1050	3875	3802	3719	3536	3391		
1100	2792	2724	2656	2552			

stability of the GDH-carotene reaction product as measured at several wave lengths is shown in Figure 21. The extinction coefficients increased with the wavelength at which they were measured, at least up to 1000 mμ and apparently even higher. However, the stability of the extinction coefficients at the higher wavelengths was not as good as those

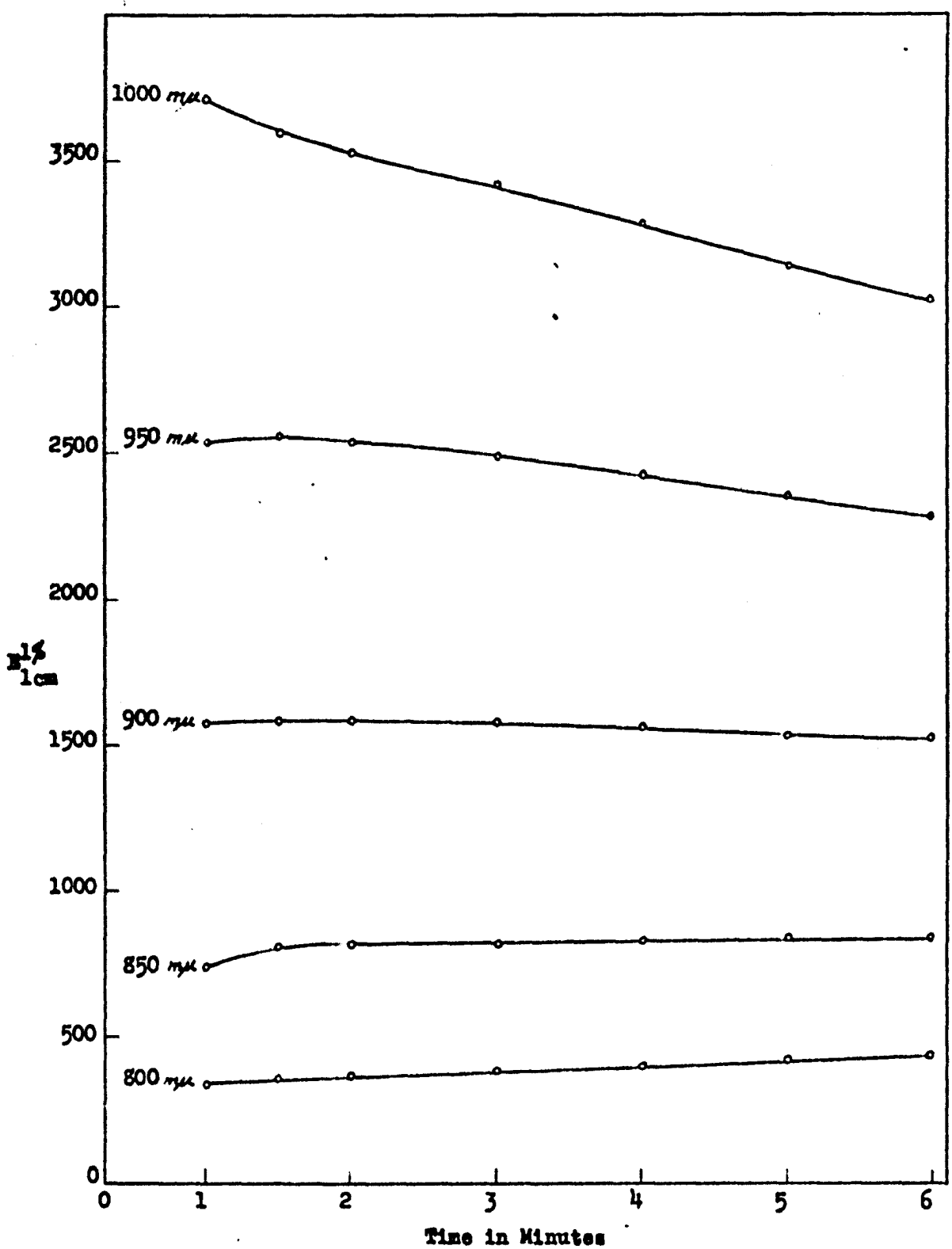


Figure 21. Absorption at Several Wavelengths of the Product of the Reaction of β-Carotene with Shohan GDH.

measured at lower wavelengths. An isobestic point appeared to be at approximately 880 mμ.

The effect of added impurities on the GDH-vitamin A color reaction. If the mechanism of the GDH-vitamin A reaction is similar to that of the Carr-Price reaction, compounds such as alcohols, water, ethers and amines should cause the color to rapidly disappear. A series of test tubes each containing the same quantity of vitamin A and GDH were placed in a test tube rack. Two minutes after adding the GDH, one drop of the compound to be tested was added to a tube and mixed well with the colored solution. The effect on the color was noted. Pyridine and quinoline caused rapid disappearance of the violet color. Diethyl ether and dioxane caused the color to fade quite rapidly. Water caused some fading but was not as effective as ether or dioxane. Ethanol and acetone had only slight effects on the rate of color fading.

The problem was then investigated quantitatively. Solutions containing known quantities of impurity were prepared. One ml. of standard chloroformic vitamin A was mixed with 4 ml. of GDH being tested. Eight measurements of optical density of the resulting color were made in the Beckman spectrophotometer at 555 mμ between 2 and 6 minutes after the reaction was initiated. Extinction coefficients were calculated.

Part of the data are given in Table 16. The added impurities, for the most part, did not appear to influence the rate of color fading, but did display their effect in reduction of total color formed at a given time after mixing the reactants. Figure 22 gives one a better picture of the influence of various levels of several added impurities on the 555 mμ absorption at 2 minutes after initiation of the GDH-vitamin A reaction.

Since pyridine had such a profound effect on the colorimetric reaction, an aliphatic amine, an aromatic amine and aqueous ammonia were tested. All were found to hinder color formation when present in very low concentration. Figure 23 shows the effect of the amines on the formation of the GDH-vitamin A color. All levels of ammonia tested completely inhibited color formation.

Table 17 summarizes the influence of the amines, ammonia, epichlorohydrin, absolute ethanol and dioxane on the 2 minute reading of the GDH-vitamin A color reaction. It appears that any substance that will react with HCl is very effective in prevention of the color reaction. Other substances such as water, dioxane and alcohol have lesser effects on the reaction.

Recovery and reactivation of used reagent. It has been shown (211) that GDH which had been used in the analysis of vitamin A could be recovered and reactivated to give a re-

Table 16

Effect of Added Impurities on the Color Reaction of Shohan Glycerol Dichlorohydrin with Vitamin A

Per Cent Impurity in Reagent	Time after mixing (minutes)							
	2.0	2.5	3.0	3.5	4.0	4.5	5.0	6.0
	$E^{1\%}_{1cm}$ at 555 mμ							
95% Ethanol								
0.000	1256	1218	1180	1112	1044	998	946	855
0.134	1218	1180	1120	1067	1021	976	923	817
0.268	1195	1165	1112	1074	1029	983	938	847
0.670	991	946	877	817	756	696	643	537
1.339	836	787	741	688	643	598	560	484
Water								
0.000	1324	1293	1263	1232	1191	1151	1120	1039
0.144	1115	1079	1049	1008	967	927	886	815
0.289	947	906	866	815	764	723	682	601
0.722	586	540	458	397	356	305	255	204
1.444	71	81	92	92	71	71	61	41
Aqueous 0.448 N KOH								
0.000	1334	1324	1293	1263	1222	1202	1171	1100
0.180	998	947	896	845	794	743	713	621
0.298	794	743	692	631	570	530	468	387
0.788	107	112	92	71	61	51	41	31
1.524	0	0	0	0	0	0	0	0
Pyridine								
0.000	1320	1309	1278	1256	1210	1188	1165	1089
0.00645	1124	1074	1021	953	893	855	787	696
0.0129	1048	1014	976	930	885	847	809	726
0.0323	0	0	0	0	0	0	0	0
0.0645	0	0	0	0	0	0	0	0

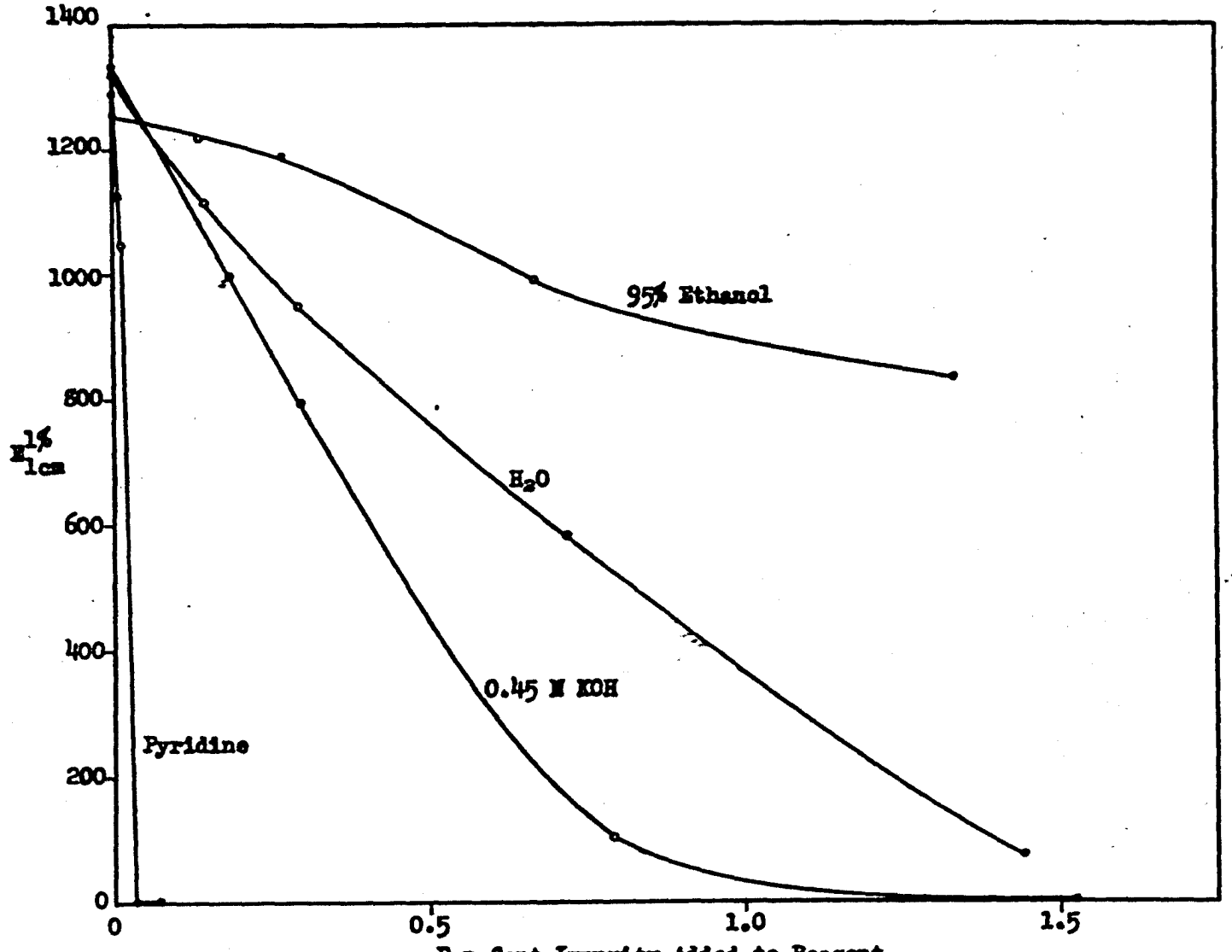


Figure 22. Influence of Added Impurities on the Activity of Shohan GDH.

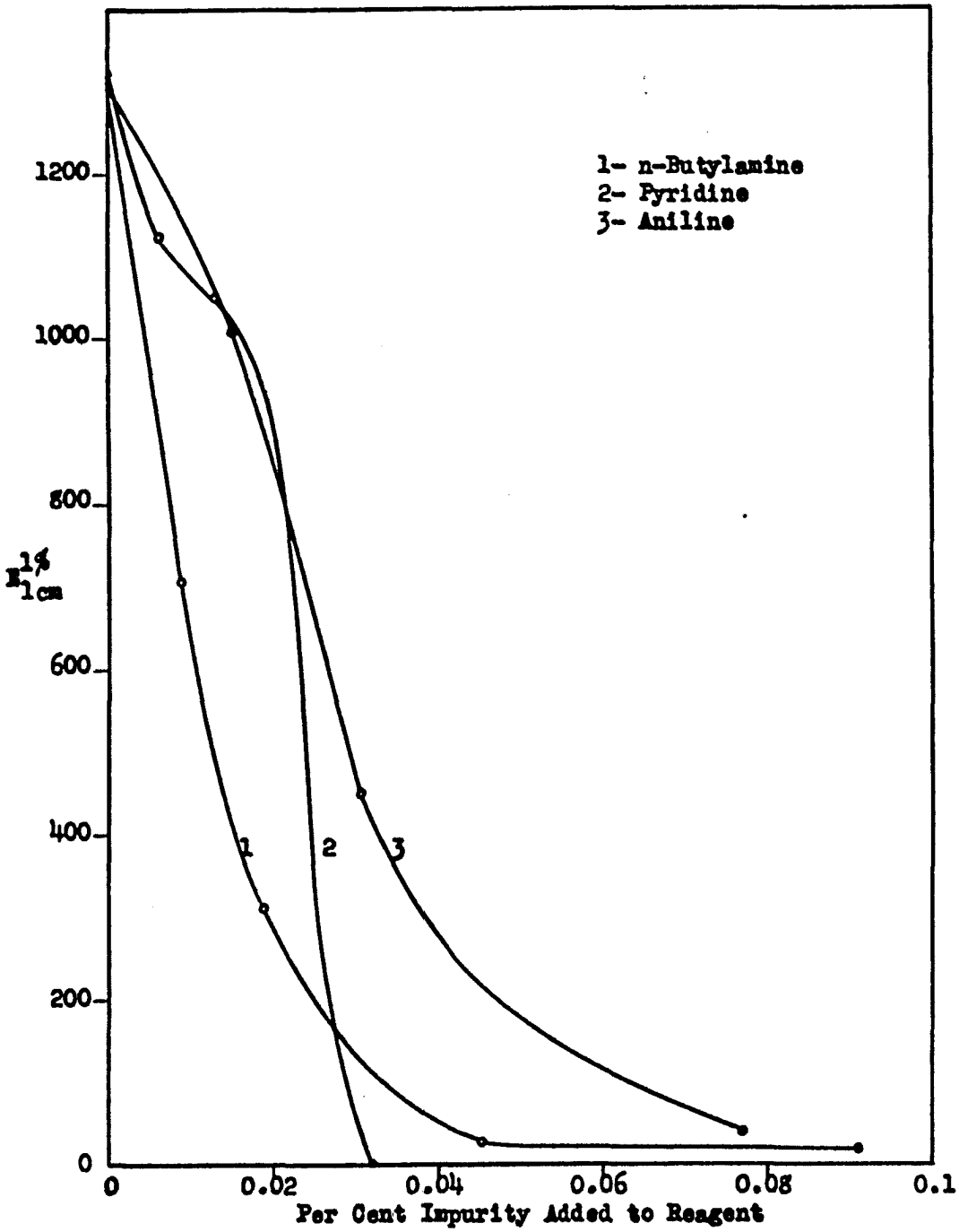


Figure 23. Influence of Certain Amines on the Activity of Shohan GDH.

Table 17

Effect of Added Impurities on the Color Reaction of Shohan Glycerol Dichlorohydrin with Vitamin A

Per Cent Impurity in Reagent	$E^{1\%}_{1cm}$ 555 mμ[a]	Per Cent Impurity in Reagent	$E^{1\%}_{1cm}$ 555 mμ[a]
n-Butylamine		**Epichlorohydrin**	
0.00	1310	0.00	1260
0.0091	705	0.0178	1219
0.0182	309	0.0354	1094
0.0454	29	0.089	10
0.0908	25	0.178	0
Aniline		**Absolute Ethanol**	
0.00	1310	0.00	1294
0.0153	1009	0.110	987
0.0307	445	0.220	828
0.0767	41	0.549	847
0.0796	0	1.098	701
Conc. NH_4OH		**Dioxane**	
0.00	1294	0.00	1294
0.142	0	0.202	878
0.284	0	0.404	693
0.711	0	1.011	597
1.422	0	2.022	272

[a] All values based on the optical density readings at 2 minutes after mixing a chloroform solution of vitamin A with the reagents.

agent with good activity. No mention was made of the procedure used in this process.

Three methods have been tried in the present investigation. The first involved vacuum distillation to remove the chloroform fraction followed by collection of GDH at the proper boiling range (76°-78°C. at 16 mm. pressure). The dark residue was discarded. On standing for a day or so the GDH was observed to turn a light yellow-green color. A clear and colorless reagent was obtained on redistillation under vacuum and was sufficiently active without the antimony trichloride treatment. However, after this reagent had been used in vitamin A analysis, recovery by the same method gave a clear, colorless product but insufficiently active for most analytical work.

The second method was similar to the first except that a few crystals of antimony trichloride (about 0.5%) were added prior to the final vacuum distillation. The product was clear and colorless and had good activity.

The third method involved addition of approximately 0.5% antimony trichloride directly to the used reagent. The chloroform fraction was removed under vacuum and the GDH collected at the proper temperature and pressure. This product turned a light yellow-green color within a few days. Redistillation under vacuum gave a clear, colorless reagent with good activity. After GDH was used three times, re-

covery and reactivation produced a reagent with diminished activity. Apparently enough impurities accumulate during repeated use and recovery to make continued use impractical when this method was used. Further investigation of methods of purifying GDH would seem advisable.

Eastman Glycerol (75%α,β-; 25%α,γ-) Dichlorohydrin.

The product was a yellow-orange color when received and gave no color reaction with vitamin A. Vacuum distillation was used as the method of purification. At 18 mm. pressure a two-phase liquid system began to distill at 55°C. The temperature slowly increased to 75°C. and during this time the distillate became a single phase. GDH was then collected between 76° and 79°C. at 16 mm. pressure. The first part of the distillate had a light yellow color, but as the distillation was continued the GDH became more colorless. The dark brown residue and the first fraction collected were discarded.

Qualitative activation tests. Table 18 summarizes the results obtained. The following substances appeared to produce good activity: concentrated hydrochloric acid, 60% perchloric acid, concentrated sulfuric acid, chlorosulfonic acid, methyl sulfate, acetyl chloride, benzoyl chloride, phosphorus trichloride, phosphorus oxychloride and arsenic trichloride. As observed with Shell GDH, no activity could be obtained by using nitric or trichlor-

Table 18

Activation of Redistilled Eastman Glycerol (75%α,β-;25%α,γ-), Dichlorohydrin technical grade, with Various Agents

Activating Agent	Color Change when Vitamin A was added	Remarks
Aqueous HF (52%)	Light blue	Color unstable
Concentrated HCl	Blue changing to violet	Color quite stable
Aqueous HBr (40%)	Blue changing to violet	Color rather unstable
Aqueous HI (47%)	No perceptible change	Reagent turned yellow
$HClO_4$ (60%)	Bright blue changing to violet	Color quite stable
Concentrated HNO_3	No color	
Concentrated H_2SO_4	Bright blue changing to rose	Color quite stable
$ClSO_3H$	Bright blue changing to violet	Color quite stable
Methyl Sulfate	Bright blue changing to light violet	Slow color change
Naphthalene-β-sulfonic acid	Light blue	Color quite unstable
CCl_3COOH	No color	
CH_3COCl	Bright blue changing to violet	Color quite stable
Benzoyl chloride	Bright blue changing to violet	Color quite stable

Table 18
(Continued)

Activating Agent	Color Change when Vitamin A was Added	Remarks
PCl_3	Bright blue changing to violet	Color quite stable
$POCl_3$	Bright blue changing to violet	Color quite stable
$AsCl_3$	Light blue changing to violet	Color quite stable
$AlCl_3$	Bright blue changing to violet	

acetic acid.

Activation with antimony trichloride. Three grams of Baker's analyzed antimony trichloride were added to 300 grams redistilled GDH and the mixture was vacuum distilled. The first portion of distillate was discarded even though it had good activity when tested with vitamin A. Activated GDH was collected between 75.7° and 76.8°C. at 15 mm. Pressure. It was clear but had a faint yellow color.

The absorption spectra of the products of reaction between vitamin A and activated reagent were determined with the aid of the Cary recording spectrophotometer. Solvent-reagent ratios of 1:5 and 5:1 were employed and the concentration of vitamin A in the mixtures was kept at 13.63 micrograms per 6 ml. of solution. Spectral absorption tracings were begun at 1.5 minutes after initiation of the reactions. A tracing was made every 5 minutes over the spectral range of 340 to 700 mμ until seven curves were made for each reaction mixture. The initial absorption curve for each solvent-reagent ratio is shown in Figure 24.

Absorption maxima were found at 553, 452, 383 and 358 mμ when a ratio of one part solvent to five parts reagent was employed. The 383 mμ maximum was rather weak but is of interest since no other GDH has shown this absorption band. Table 19 lists the extinction coefficients at these maxima and indicates the change in absorption as the reaction

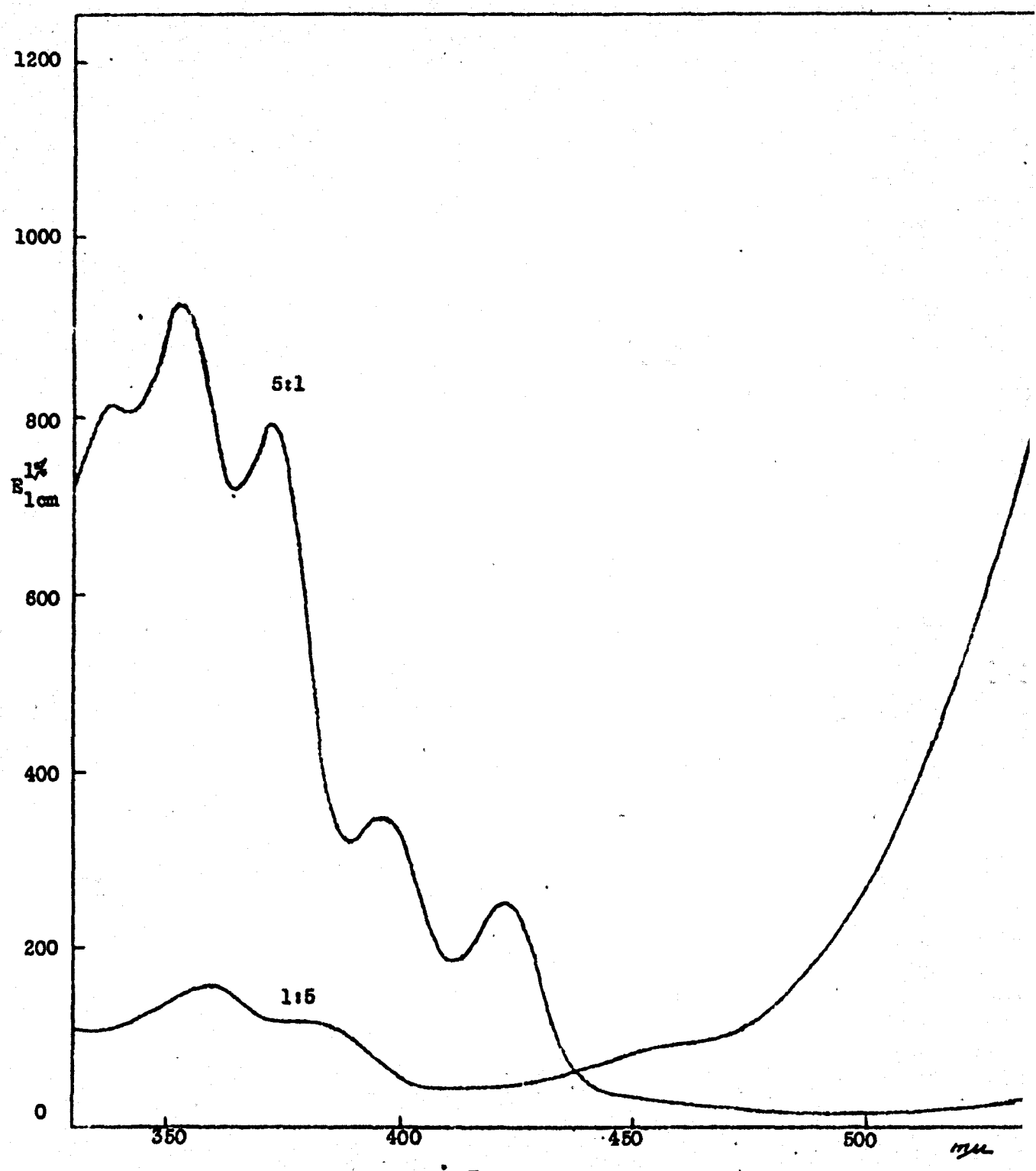
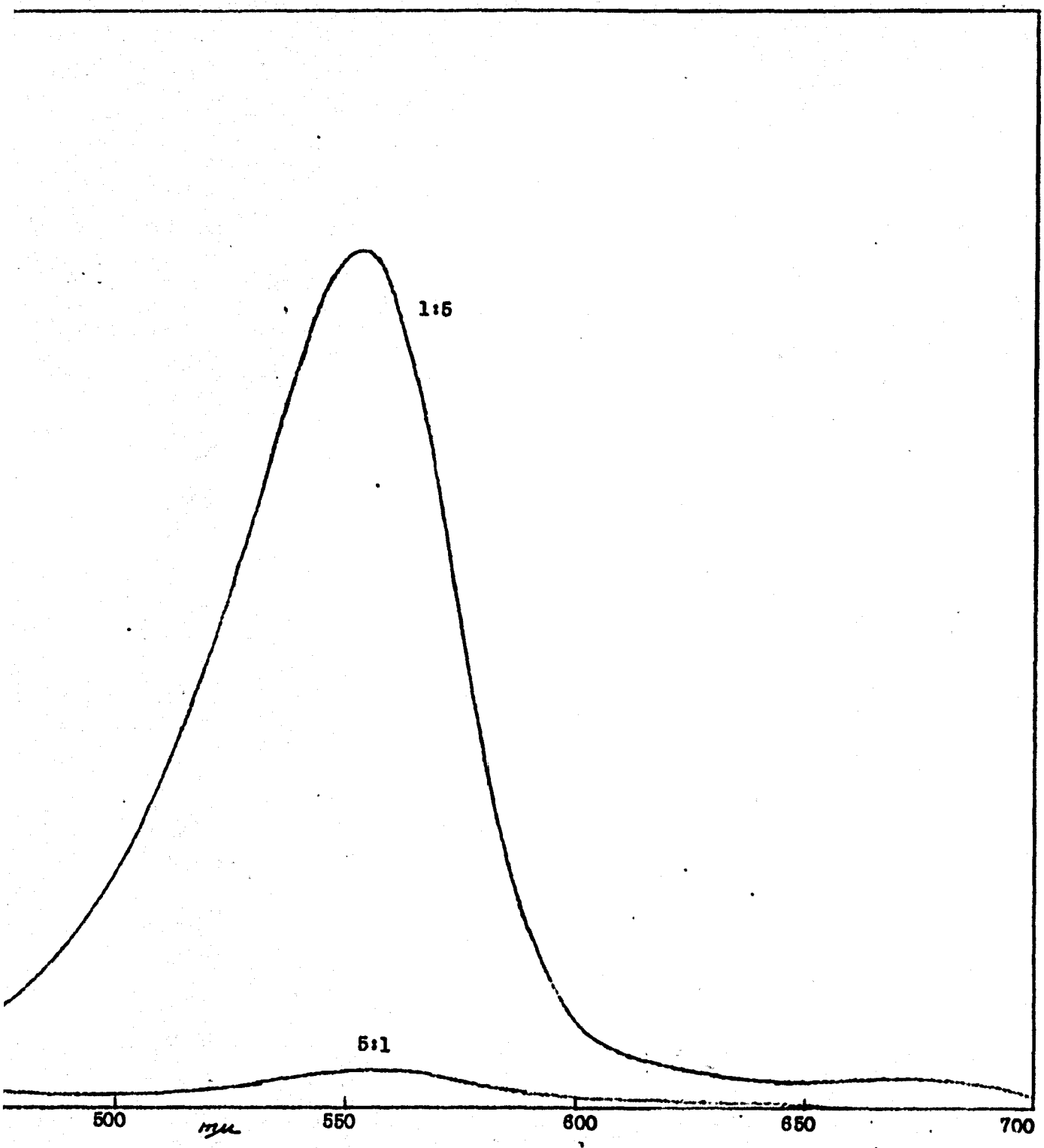


Figure 24. Absorption Spectra of the Products
Activated Eastman Glycerol (75%,
5:1 Solvent-Reagent Ratios were Emp



pectra of the Products of the Reactions of Vitamin A with $SbCl_3$-
stman Glycerol (75%α,β-; 25%α,γ-) Dichlorohydrin when 1:5 and
Reagent Ratios were Employed.

Table 19

Stability of the Color Developed by the Reaction of Vitamin A with Activated Eastman GDH(75% α,β-; 25% α,γ-) when a 1:5 Solvent-Reagent Ratio was Employed

Time after mixing (minutes)	$E^{1\%}_{1cm}$ 358 mμ	Time after mixing (minutes)	$E^{1\%}_{1cm}$ 383 mμ
4.35	202	4.15	150
9.35	218	9.15	130
14.35	235	14.15	113
19.35	248	19.15	104
24.35	262	24.15	99
29.35	275	29.15	95
34.35	284	34.15	88
	452 mμ		553 mμ
3.57	120	2.73	1231
8.57	122	7.73	1108
13.57	135	12.73	973
18.57	150	17.73	857
23.57	156	22.73	757
28.57	161	27.73	662
33.57	169	32.73	587

mixture aged. A clearer picture of these changes may be seen in Figure 25.

When a 5:1 ratio was used, maxima were observed at 338, 353, 372, 397 and 422 mμ. The observed extinction coefficients at these maxima are recorded in Table 20. The rate of change in absorption at the several absorption maxima are best represented in graphic form (Figure 26).

Paragon Glycerol α,γ-Dichlorohydrin.

The product had a light yellow color when received from the Mathison Chemical Company. It gave no color reaction when tested with vitamin A dissolved in chloroform. Several qualitative tests were made with this GDH which indicated that activity was readily obtained by adding small quantities of concentrated sulfuric acid, concentrated hydrochloric acid, 60% perchloric acid, dimethyl sulfate and benzoyl chloride. The yellow color was found objectionable in colorimetric work and thus purification was attempted.

The yellow GDH was subjected to vacuum distillation. All liquid distilling below 75° C. at 15 mm. pressure was discarded. The fraction boiling between 76° and 79°C. at 15 mm. was collected. The distillate was clear and very nearly colorless, and gave no test with vitamin A.

Qualitative activation tests. Table 21 summarizes the results of these tests and the following substances caused good activation: concentrated hydrochloric acid, 60% per-

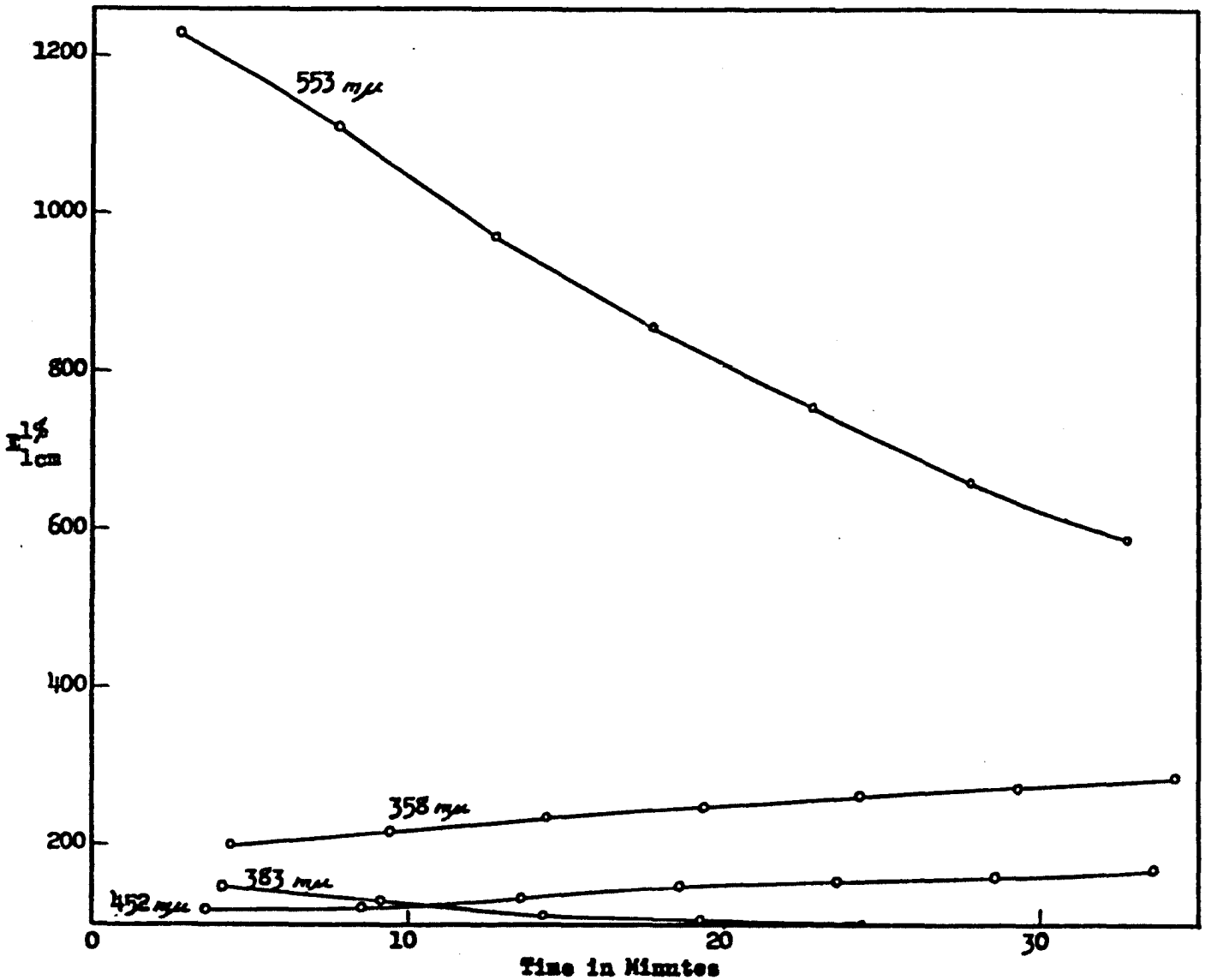


Figure 25. Changes in the Extinction Coefficients with Time at the Absorption Maxima of the Vitamin A-Eastman GDH Reaction Product when a 1:5 Solvent-Reagent Ratio was Used.

Table 20

Stability of the Color Developed by the Reaction of Vitamin A with Activated Eastman GDH (75% α,β-; 25% α,γ-) when a 5:1 Solvent-Reagent Ratio was Employed

Time after mixing (minutes)	$E^{1\%}_{1cm}$ 338 mμ	Time after mixing (minutes)	$E^{1\%}_{1cm}$ 353 mμ	Time after mixing (minutes)	$E^{1\%}_{1cm}$ 372 mμ
4.52	1012	4.40	1160	4.23	993
9.52	816	9.40	880	9.23	757
14.52	680	14.40	703	14.23	593
19.52	575	19.40	571	19.23	482
24.52	500	24.40	476	24.23	395
29.52	433	29.40	405	29.23	335
34.52	380	34.40	358	34.23	283
	397 mμ		422 mμ		555 mμ
4.03	440	3.82	319	2.72	55
9.03	430	8.82	333	7.72	45
14.03	385	13.82	307	12.72	45
19.03	342	18.82	276	17.72	45
24.03	298	23.82	246	22.72	45
29.03	247	28.82	219	27.72	45
34.03	225	33.82	194	32.72	45

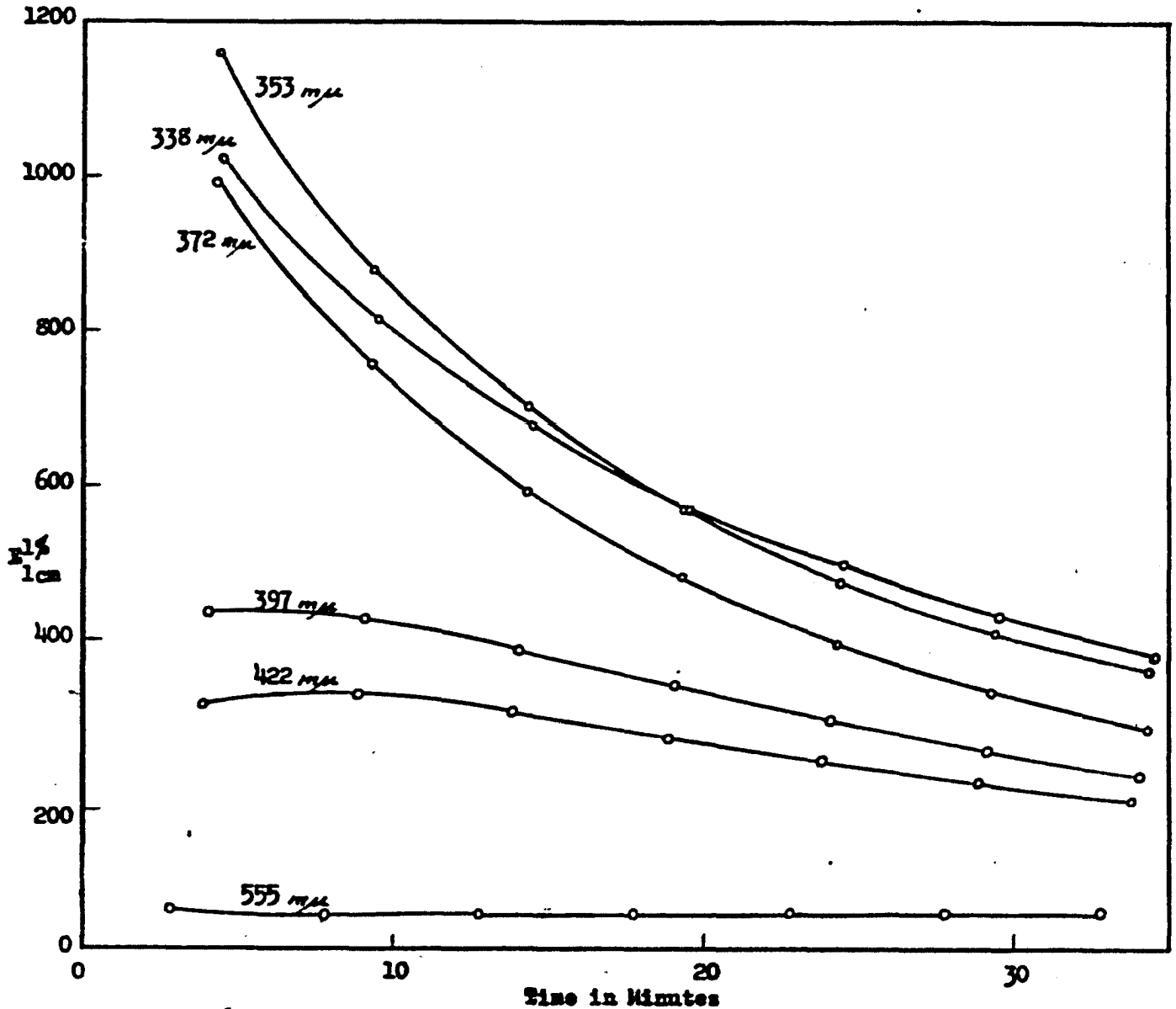


Figure 26. Changes in the Extinction Coefficients with Time at the Absorption Maxima of the Vitamin A-Eastman GDH Reaction Product when a 5:1 Solvent-Reagent Ratio was Used.

Table 21

Activation of Paragon Glycerol α,β-Dichlorohydrin with Various Agents

Activating Agent	Color Change when Vitamin A was Added	Remarks
Aqueous HF (52%)	Light blue	Color rather unstable
Concentrated HCl	Bright blue changing to violet	Color quite stable
Aqueous HBr (40%)	Bright blue changing to violet	Color rather unstable
Aqueous HI (47%)	Orange	Reagent turned yellow when acid was added
$HClO_4$ (60%)	Bright blue changing to violet	Color quite stable
Concentrated HNO_3	No color	
Concentrated H_2SO_4	Bright blue changing to rose	Color rather stable
$ClSO_3H$	Bright blue changing to violet	Color rather stable
Methyl Sulfate	Blue changing to violet	Color quite stable
Naphthalene-β-sulfonic	Light blue changing to violet	Color quite stable
CCl_3COOH	No color	
CH_3COCl	Blue changing to violet	Color quite stable

Table 21
(Continued)

Activating Agent	Color Change when Vitamin A was Added	Remarks
Benzoyl chloride	Bright blue changing to violet	Color quite stable
PCl_3	Bright blue changing to violet	Color quite stable
$POCl_3$	Bright blue changing to violet	Color quite stable
$AsCl_3$, liquid	Blue green changing to violet	Color quite stable
$AlCl_3$, anhydrous	Faint violet	
$SnCl_2$	Light blue	Color quite unstable
$CH_2ClCOOH$	No color	
Citric acid	No color	

chloric acid, concentrated sulfuric acid, chlorosulfonic acid, methyl sulfate, acetyl chloride, benzoyl chloride, phosphorus trichloride, phosphorus oxychloride and arsenic trichloride. No color was observed with nitric, monochloracetic, trichloracetic or citric acids as activating agents.

Activation with antimony trichloride. The GDH purified by vacuum distillation was stored in clear, glass-stoppered bottles for three months before antimony trichloride activation was attempted. During that interval it had turned yellow and redistillation appeared to be required. When subjected to vacuum distillation, the first distillate was a two-phase liquid system boiling lower than GDH. As distillation continued, a single phase resulted and GDH was collected between 77.8° and 79.2°C. at 16 mm. pressure. The chemical nature of the substance immiscible in small amounts of GDH that appears in the first fraction is not known. It was observed that the GDH fraction collected was slightly active when tested with vitamin A.

Activation with antimony trichloride was accomplished by mixing 3 grams of antimony trichloride with 300 grams redistilled GDH in a 1-liter Claisen flask and vacuum distilling the mixture. The initial low-boiling fraction was collected and found to have good activity when tested with vitamin A. The main activated GDH fraction was collected

at 77° to 77.5°C. and 16 mm. pressure. It was clear and colorless and had good activity when tested with vitamin A.

The spectral absorption curves of the product of the reaction of vitamin A with the main fraction of activated GDH were obtained by using the Cary recording spectrophotometer. Solvent-reagent ratios of 1:5 and 5:1 were employed. The quantity of vitamin A in each reaction mixture was 13.63 micrograms per 6 ml. of solution. A series of seven curves were traced for each ratio during the 35-minute period after the reactants were mixed.

The initial absorption spectral curves for each reaction mixture are shown in Figure 27. In the case of the 1:5 mixture, absorption maxima were observed at 358, 455 and 553 mμ, the latter being by far the most prominent. Extinction coefficients were calculated at the absorption maxima and listed in Table 22. The stability of the color at these maxima can best be seen when the data are plotted (see Figure 28).

When the 5:1 solvent-reagent mixture was employed, absorption maxima were found at 338, 353, 372, 397 and 422 mμ. The effect of aging on the extinction coefficients at these maxima is indicated in Table 23. The change in the absorption of light at the various maxima is clearly seen in Figure 29.

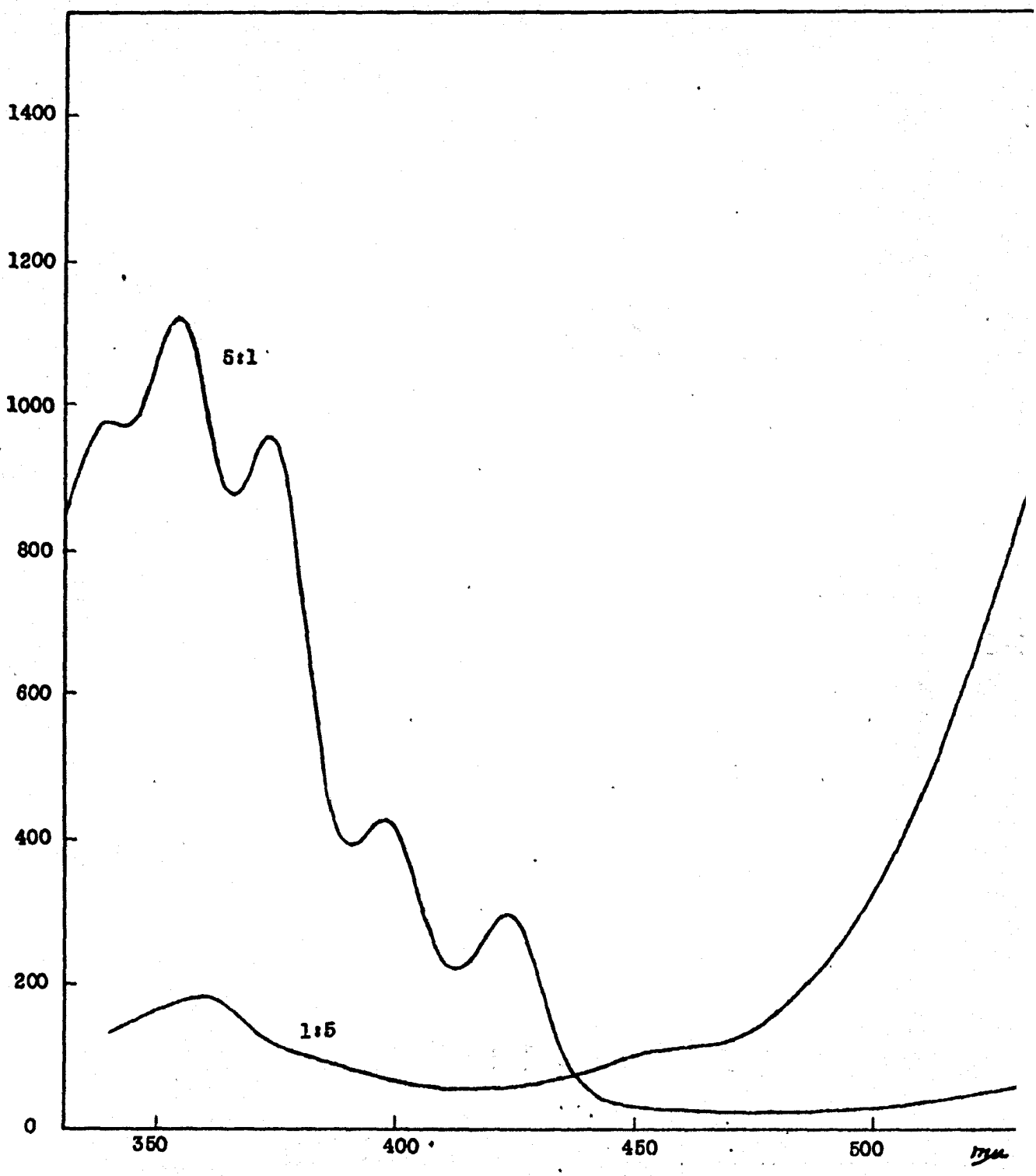
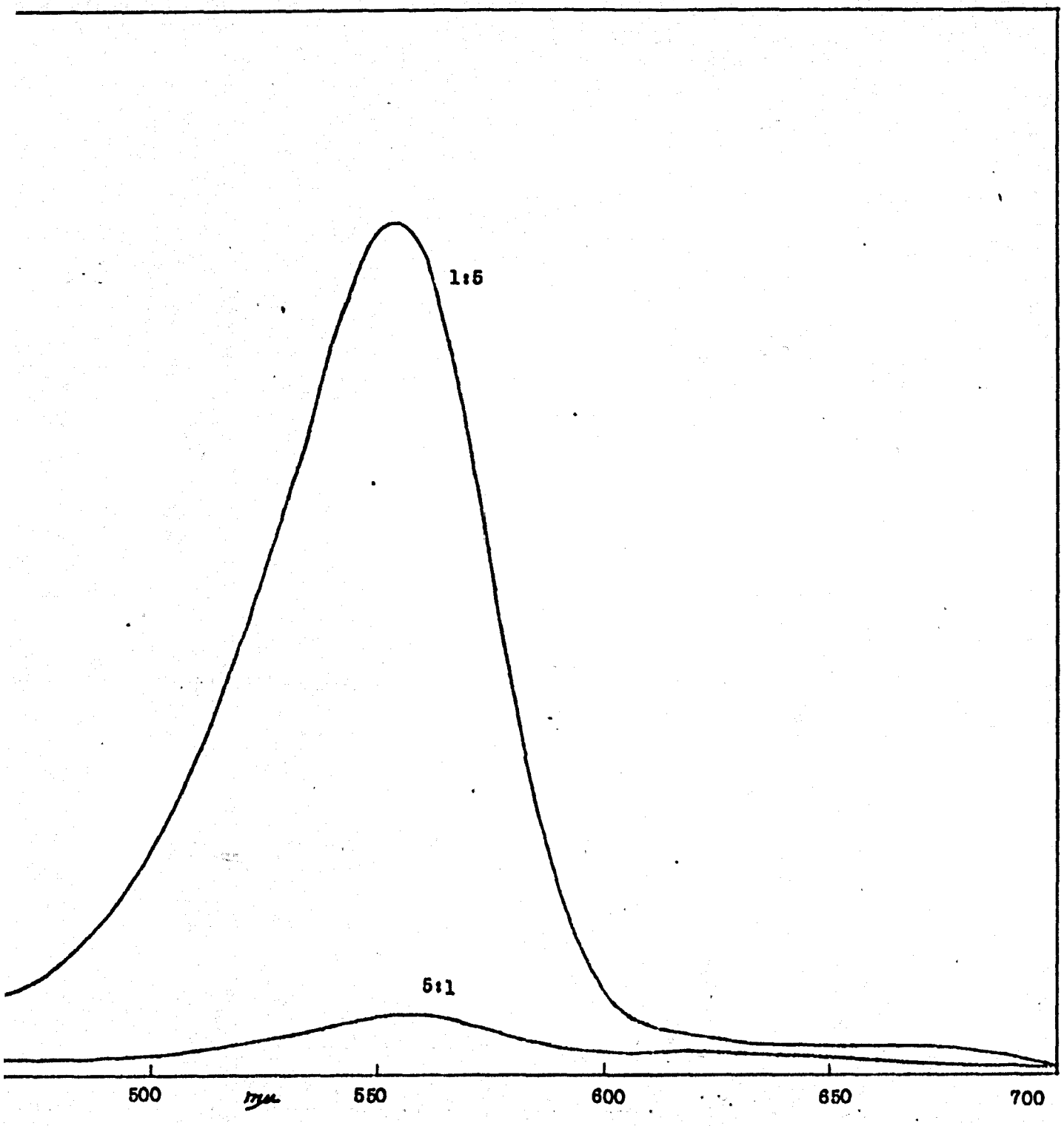


Figure. 27. Absorption Spectra of the Products of Activated Paragon GDH when 1:5 and 5:1



ectra of the Products of the Reactions of Vitamin A with $SbCl_3$-
agon GDH when 1:5 and 5:1 Solvent-Reagent Ratios were Employed.

Experiments with Two Closely Related Glycerol Derivatives

Glycerol Monochlorohydrin

Eastman glycerol monochlorohydrin, practical grade, was a rather viscous, clear but slightly yellow-green liquid when

Table 22

Stability of the Color Developed by the Reaction of Vitamin A with Activated Paragon Glycerol α,γ-Dichlorohydrin when a 1:5 Solvent-Reagent Ratio was Employed

Time after mixing (minutes)	$E^{1\%}_{1cm}$ 358 mμ	Time after mixing (minutes)	$E^{1\%}_{1cm}$ 455 mμ	Time after mixing (minutes)	$E^{1\%}_{1cm}$ 553 mμ
4.35	183	3.55	110	2.74	1241
9.35	204	8.55	106	7.74	1152
14.35	222	13.55	120	12.74	1042
19.35	239	18.55	132	17.74	942
24.35	249	23.55	141	22.74	846
29.35	265	28.55	148	27.74	763
34.35	275	33.55	156	32.74	684

received. It gave a negative color reaction with vitamin A.

Qualitative activation tests. Glycerol monochlorohydrin was tested before any purification experiments were performed. The results of these tests are summarized in Table 24. No activating agent tested produced a reagent that would give more than a weak color when tested with vitamin A. However, most of the substances which activate GDH also

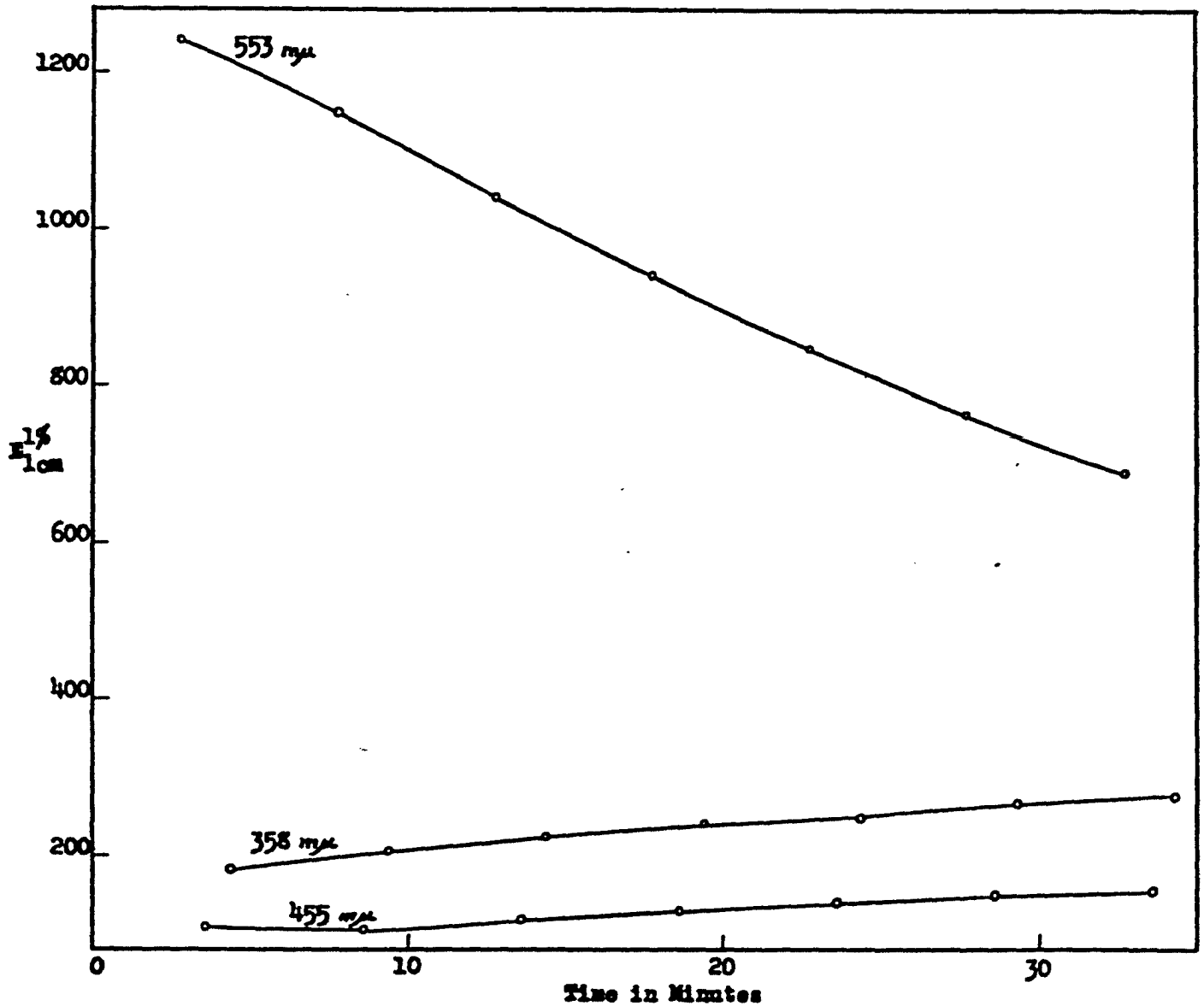


Figure 28. Changes in the Extinction Coefficients with Time at the Absorption Maxima of the Vitamin A-Paragon GDH Reaction Product when a 1:5 Solvent-Reagent Ratio was Used.

Table 23

Stability of the Color Developed by the Reaction of Vitamin A with Activated Paragon GDH (α,γ-dichlorohydrin) when a 5:1 Solvent-Reagent Ratio was employed

Time after mixing (minutes)	$E^{1\%}_{1cm}$ 338 mμ	Time after mixing (minutes)	$E^{1\%}_{1cm}$ 353 mμ	Time after mixing (minutes)	$E^{1\%}_{1cm}$ 372 mμ
4.52	977	4.40	1126	4.23	962
9.52	777	9.40	856	9.23	729
14.52	649	14.40	679	14.23	574
19.52	554	19.40	556	19.23	465
24.52	475	24.40	468	24.23	380
29.52	423	29.40	403	29.23	324
34.52	379	34.40	355	34.23	282
	397 mμ		422 mμ		555 mμ
4.03	430	3.82	299	2.72	88
9.03	401	8.82	306	7.72	63
14.03	354	13.82	279	12.72	55
19.03	308	18.82	248	17.72	51
24.03	267	23.82	221	22.72	49
29.03	231	28.82	195	27.72	48
34.03	201	33.82	177	32.72	46

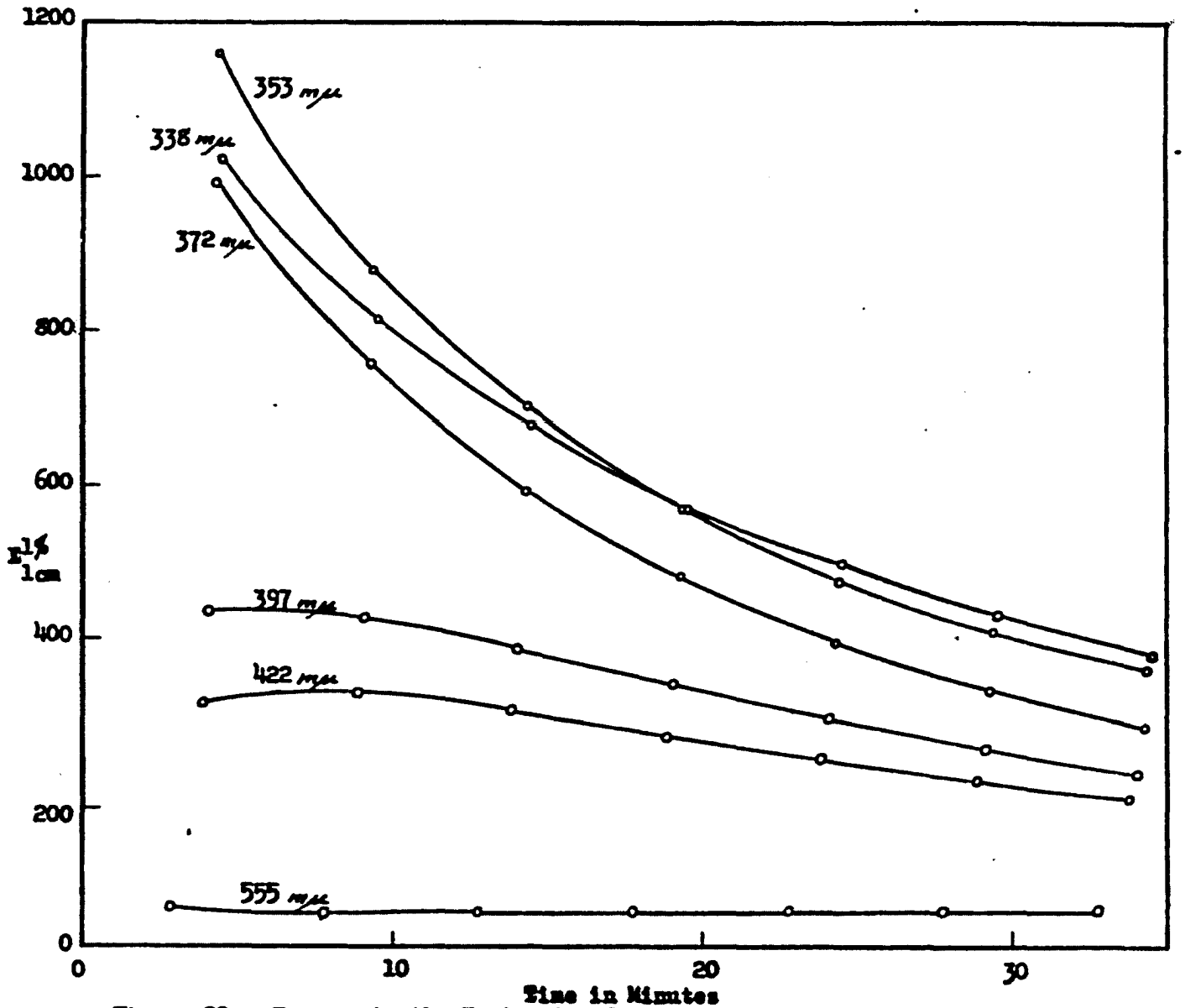


Figure 29. Changes in the Extinction Coefficients with Time at the Absorption Maxima of the Vitamin A-Paragon GDH Reaction Product when a 5:1 Solvent-Reagent Ratio was Used.

Table 24

Activation of Redistilled Eastman, practical grade, Glycerol Monochlorohydrin with Various Agents

Activating Agent	Color Change when Vitamin A was added	Remarks
Aqueous HF (52%)	No color	
Concentrated HCl	Blue changing to violet	Color rather unstable
Aqueous HBr (40%)	Faint violet	Color unstable
Aqueous HI (47%)	Faint rose color	Reagent turned yellow when HI was added
$HClO_4$ (60%)	Light blue changing to violet	
Concentrated HNO_3	No color	
Concentrated H_2SO_4	Light blue changing to violet	Color quite stable
$ClSO_3H$	Light blue changing to violet	Color rather unstable
Methyl sulfate	Faint violet after a few minutes	
Naphthalene-β-sulfonic acid	Faint violet	Color unstable
CCl_3COCl	Light blue changing to violet	Color quite stable
CCl_3COOH	No color	
Benzoyl chloride	Faint violet	Color quite unstable

Table 24

(Continued)

Activating Agent	Color Change when Vitamin A was added	Remarks
PCl_3	Light blue changing to violet	Color quite stable
$POCl_3$	Blue changing to violet	Color fairly stable
$AsCl_3$, liquid	Light blue changing to violet	Color fairly stable
$AlCl_3$, anhydrous	Faint violet	

weakly activated glycerol monochlorohydrin. It was postulated that perhaps the reagent contained enough glycerol dichlorohydrin to give the color tests. Consequently, a purification study was made.

Purification. Glycerol monochlorohydrin was subjected to vacuum distillation. A fraction boiling at 90° to 115°C. at 15 to 20 mm. pressure was collected. All during this collection period the temperature was slowly rising along with a slow decrease in pressure. The main fraction was collected over the temperature range of 116° to 120°C. at 16 mm. pressure. The Eastman catalogue of organic compounds lists the boiling point as 115° to 120°C. at 15 mm. pressure. Because of the great difference in the boiling points of glycerol dichlorohydrin and glycerol monochlorohydrin, it does not seem likely that the former would be a contaminant in the above collected main fraction of the monochlorohydrin.

Qualitative activation tests were made on these fractions and on the original product by mixing one drop of concentrated hydrochloric acid with 1 ml. of material being tested followed by the addition of 0.25 ml. of a chloroform solution of vitamin A. The first (low boiling) and main fractions gave essentially the same color tests with vitamin A. In each case a faint blue color developed and changed to a fairly stable weak violet color. The residue in the distilling flask gave a very weak color test. Since the

first and main fractions gave practically the same color tests, the possibility that GDH was the cause of color formation does not seem likely.

Ultraviolet absorption spectra of the various fractions were next determined in hopes of establishing the purity of the products. The following quantities of reagent were weighed and sufficient reagent grade methanol added to make 10 ml. of solution: original product, 0.8854 g.; low boiling fraction, 0.7288 g.; main fraction, 0.8971 g.; and antimony trichloride-activated reagent (to be discussed later), 0.5179 g. The ultraviolet absorption spectra were determined with the aid of the Cary recording spectrophotometer over a spectral range of 235 to 400 mμ. The extinction coefficients at the various wavelengths were calculated and plotted (see Figure 30). From this it is obvious that some substance absorbing at 256 and 332 mμ could be concentrated in the low boiling fraction. The main fraction showed very weak absorption maxima at 252 and 330 mμ. The antimony trichloride-activated reagent appeared to contain some substance or substances absorbing at 255 and 330 mμ. These absorption spectra data do not prove or disprove the presence of glycerol dichlorohydrin as an impurity.

Activation with antimony trichloride. The original Eastman glycerol monochlorohydrin was vacuum distilled with antimony trichloride in the following manner. Five grams of

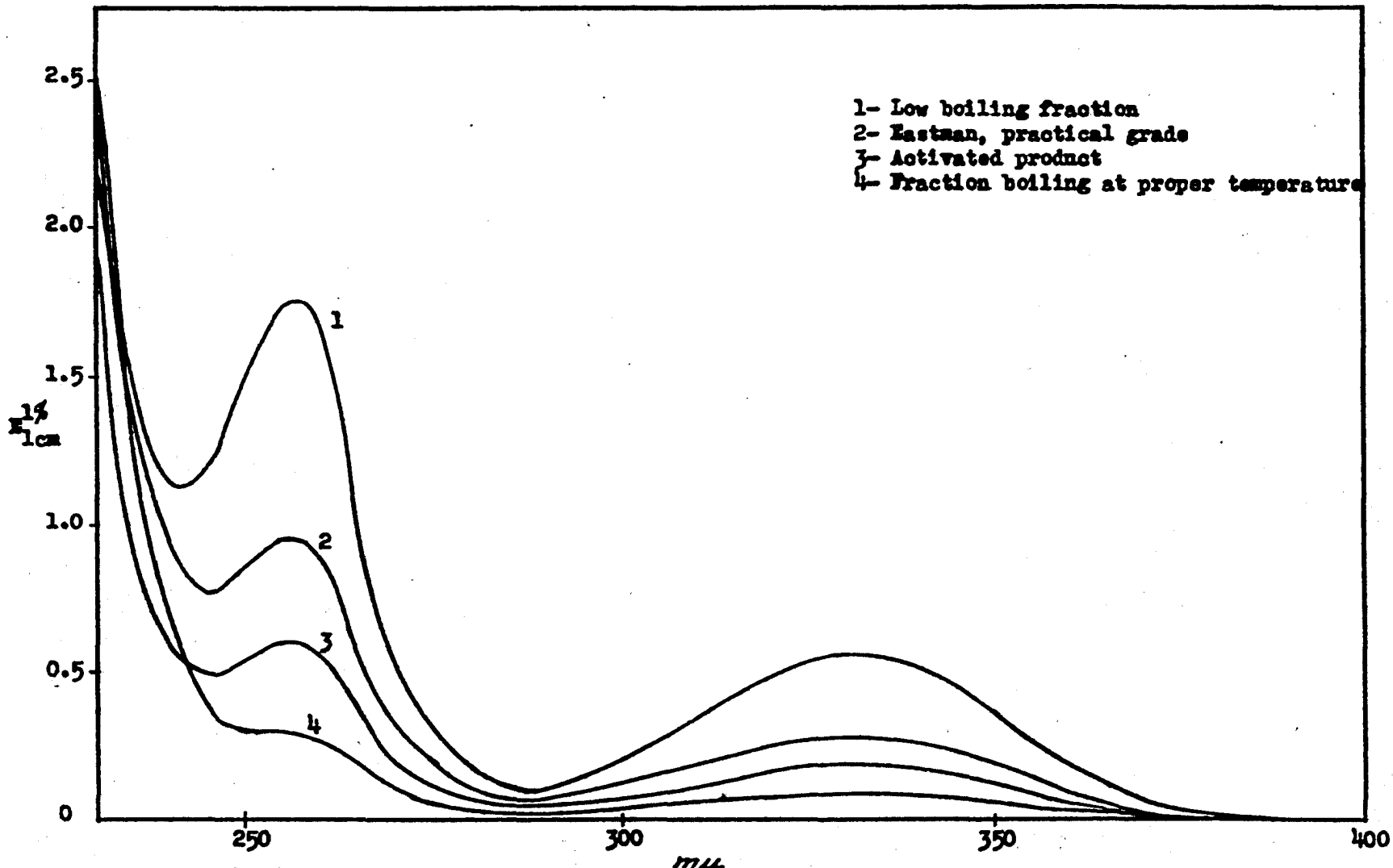


Figure 30. Ultraviolet Absorption of Eastman Glycerol α-Monochlorohydrin and Products Obtained During the Purification Process.

antimony trichloride, Baker's analyzed, was mixed with 500 grams of glycerol monochlorohydrin in a 1-liter Claisen flask. Upon subjecting the mixture to vacuum distillation, the initial distillate was a two-phase liquid system. This was soon followed by a single-phase, clear liquid. At 15 mm. pressure the temperature slowly rose from 74° to 95°C. after which the receiver was changed and the fraction boiling at 96° to 117°C. at 14 mm. pressure was collected. Tests with a chloroform solution of vitamin A indicated that both the low-boiling and main fractions were somewhat active. The activated main fraction was stored in a brown glass bottle.

The absorption spectra of the reaction products of activated glycerol monochlorohydrin with standard vitamin A solutions were obtained with the aid of the Cary recording spectrophotometer. Chloroform solutions of vitamin A were prepared such that the concentrations of vitamin A were 13.62 micrograms per 6 ml. of reaction mixture. Solvent-reagent ratios of 1:5, 1:1 and 5:1 were employed. Spectral absorption tracings were begun at 1.5 minutes after the reactions were initiated and covered the spectral range of 350 to 700 mμ. In each case the tracings were begun at the red end of the spectrum.

The general shape of these curves was not unlike those of the GDH-vitamin A color. The magnitude of absorption was,

however, quite different. Absorption maxima occurred at 376, 395, 421, 553 and 640 mμ when a solvent-reagent ratio of 1:5 was employed. Figure 31 indicates the change in absorption at these maxima as the reaction mixture aged. The 553 mμ band fell off quite rapidly whereas the 640 mμ band increased rapidly at first then became quite stable. The other maxima changed to lesser extents.

In the case of the 1:1 ratio, absorption maxima were observed at 373, 397, 422, 555 and 640 mμ. Figure 32 shows the change in the various maxima at several time intervals up to about 35 minutes. The magnitude of absorption at the 555 and 373 mμ bands was essentially the same at first, but the 555 mμ maxima fell off more rapidly as the color aged. Again the 640 mμ band increased quite rapidly at first then leveled off.

The effect of aging of the 5:1 ratio reaction mixture is shown in Figure 33. The principal absorption bands occurred at 355, 373, 397 and 422 mμ, the first being the most intense. The 355 and 373 mμ absorption maxima were not very stable, particularly during the first 10 or 15 minutes after the reaction was initiated.

1,2,3-Trichloropropane

Qualitative activation tests. Table 25 summarizes the results of the tests made. Eastman 1,2,3-trichloropropane gave color tests with vitamin A only when "activated" with

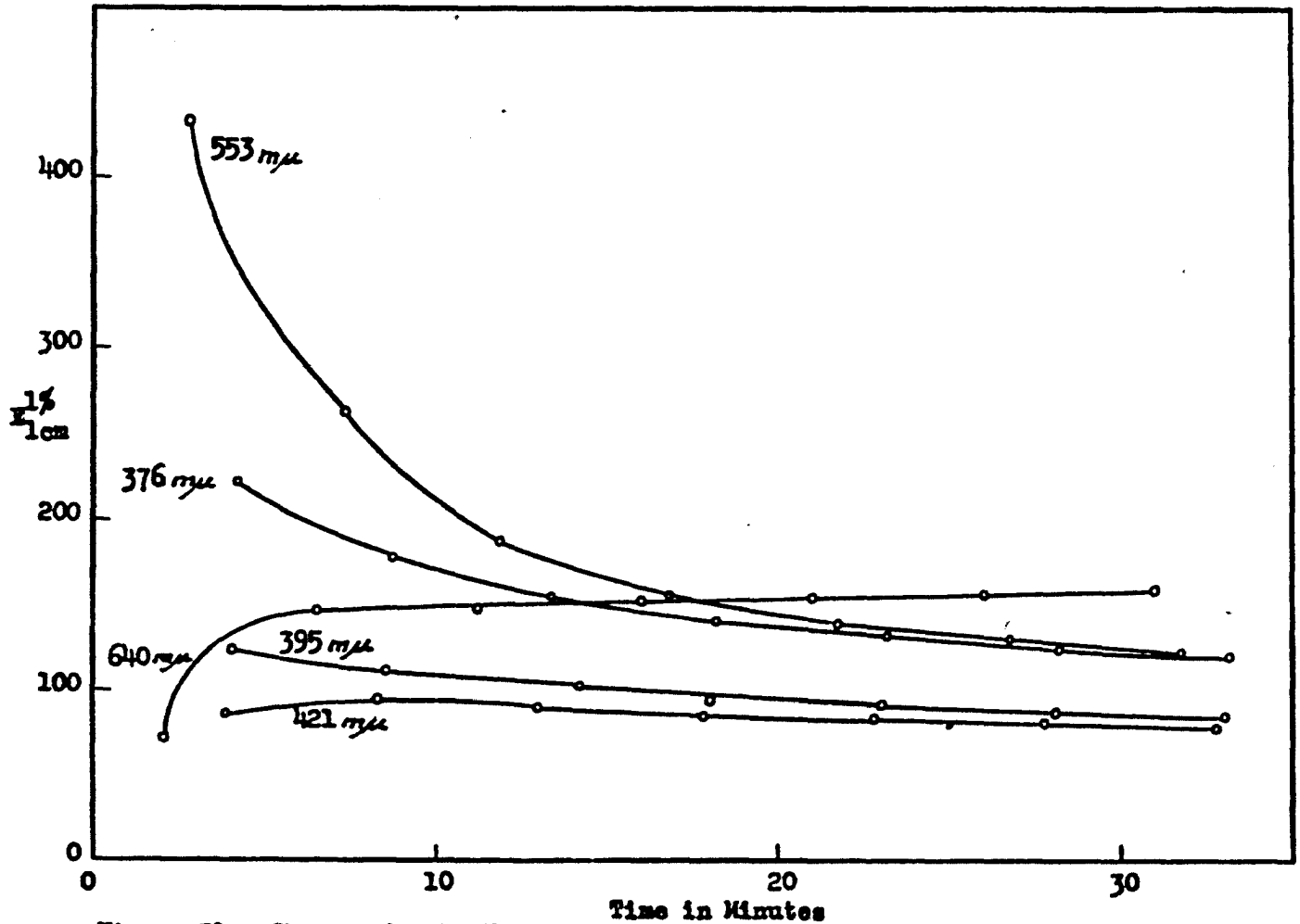


Figure 31. Changes in the Extinction Coefficients with Time at the Absorption Maxima of the Product of the Reaction of Vitamin A with Activated Glycerol Monochlorohydrin when a 1:5 Solvent-Reagent Ratio was Used.

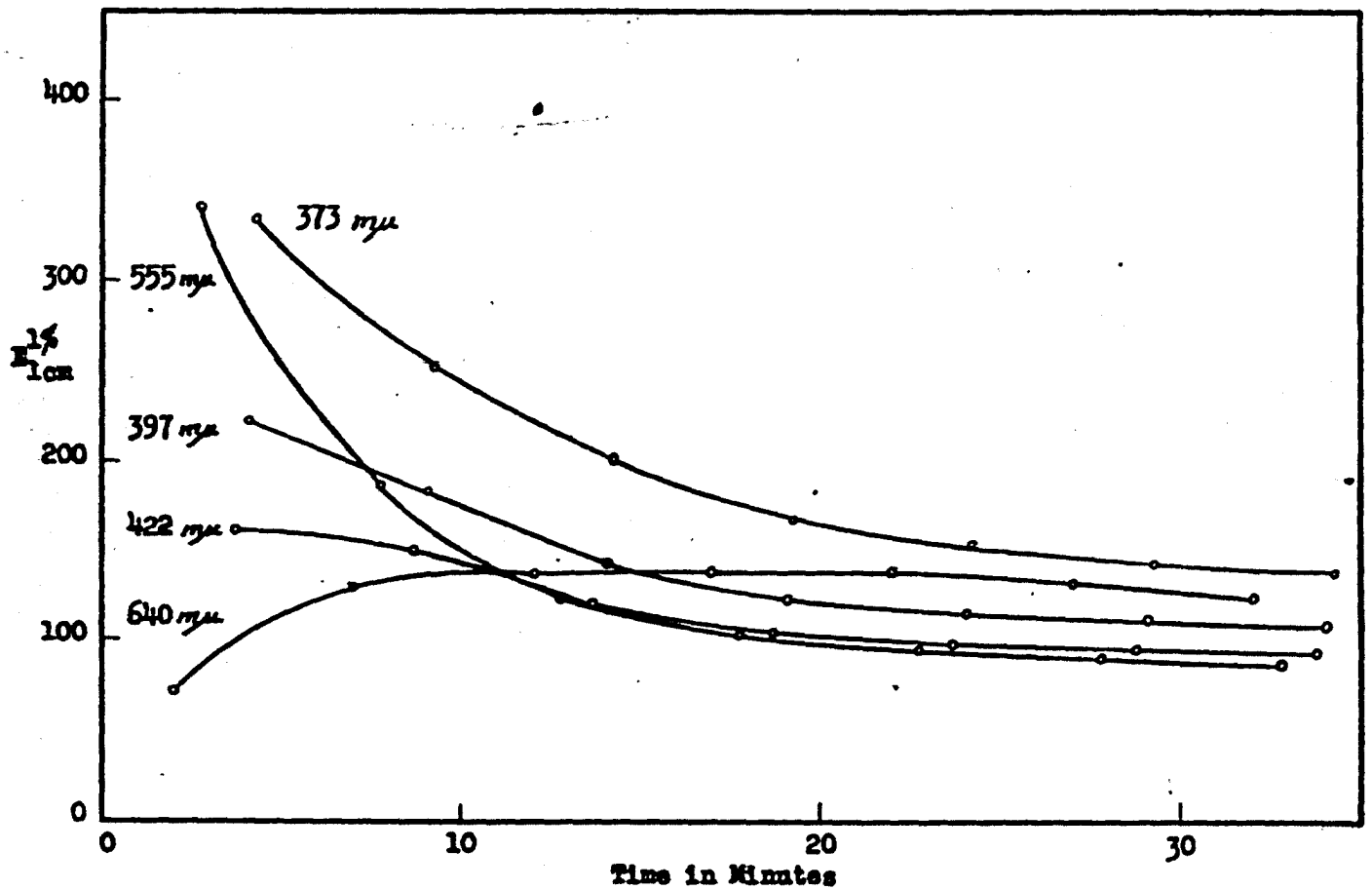


Figure 32. Changes in the Extinction Coefficients with Time at the Absorption Maxima of the Product of the Reaction of Vitamin A with Activated Glycerol Monochlorohydrin when a 1:1 Solvent-Reagent Ratio was Used.

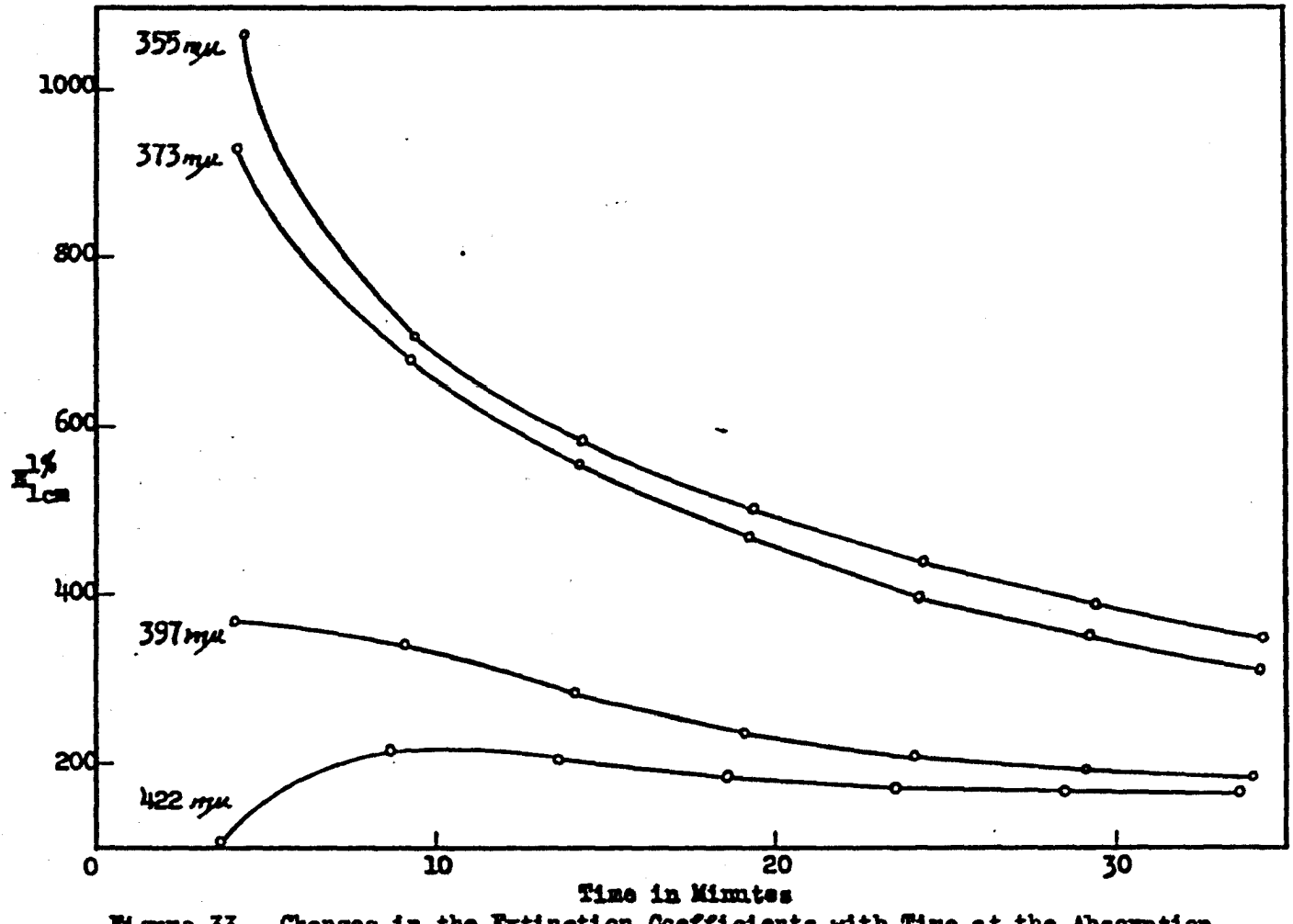


Figure 33. Changes in the Extinction Coefficients with Time at the Absorption Maxima of the Product of the Reaction of Vitamin A with Activated Glycerol Monochlorohydrin when a 5:1 Solvent-Reagent Ratio was Used.

Table 25

Activation of Eastman 1,2,3-Trichloropropane with Various Agents

Activating Agent	Color Change when Vitamin A was Added	Remarks
Aqueous HF (52%)	No color	
Concentrated HCl	No color	
Aqueous HBr (40%)	No color	
Aqueous HI (47%)	No color	
$HClO_4$ (60%)	Bright blue	Color rather unstable. Light yellow color when acid added to reagent
Concentrated HNO_3	No color	
Concentrated H_2SO_4	Bright blue changing to brown	Reagent turned yellow when acid was added
$ClSO_3H$	No color	Reagent turned yellow when acid was added
Methyl sulfate	No color	
Naphthalene-β-sulfonic acid	No color	
CCl_3COOH	No color	
CH_3COCl	No color	
Benzoyl chloride	No color	
PCl_3	No color	
$POCl_3$	No color	
$AsCl_3$, liquid	No color	
$AlCl_3$, anhydrous	No color	Reagent turned light yellow when salt added

small quantities of concentrated sulfuric acid and 60% perchloric acid. This should probably not be termed activation since both sulfuric and perchloric acids added to chloroform solutions of vitamin A also produced blue colors. It was, however, observed that methyl sulfate and chlorosulfonic acids would produce colors alone, but did not activate 1,2,3-trichloropropane.

Attempted antimony trichloride activation. After several crystals of antimony trichloride were dissolved in 1,2,3-trichloropropane, reaction of the product with vitamin A produced a blue color which soon faded to colorless. After standing in a test tube for about an hour the mixture turned milky white.

Activation by vacuum distillation with 1% antimony trichloride was attempted. The first fraction was discarded. The fraction boiling between 50° and 52°C. at 15 mm. pressure was collected. The reagent was clear and colorless, and gave no color test with a chloroform solution of vitamin A. On standing for several hours in a brown-glass bottle, the reagent turned milky white. After several days a white precipitate collected on the bottom of the bottle. The clear supernatant liquid gave no color test with vitamin A.

Investigation of the Mechanism of the GDH-Vitamin A Reaction

Formation of Anhydrovitamin A.

Several investigators (48, 79, 84, 89, 134, 181, 205) have demonstrated the conversion of vitamin A alcohol to anhydrovitamin A by the action of alcoholic HCl. The same substance has been produced by the action of toluene sulfonylchloride of the potassium salt of vitamin A (100) and on vitamin A alcohol and vitamin A methyl ether (78). Vitamin A esters may also be converted to the anhydro compound by the action of acid (50, 198) and by refluxing with alcohol (68). One of the most recent reports (89) lists the absorption maxima of this compound at 352, 370 and 391 mμ. Anhydrovitamin A has been found in certain vitamin A concentrates (27, 137), certain fish liver oils (50) and in the products obtained by a high vacuum distillation of the non-saponifiable fraction from halibut liver oil (82).

Since the absorption spectra of the GDH-vitamin A reaction when high solvent-reagent ratios were used resembles that of anhydrovitamin A to some extent, the formation of anhydrovitamin A by the action of N/30 HCl in alcohol was followed spectrophotometrically. Figure 34 shows the change in the ultraviolet absorption spectrum as 52.97 micrograms of vitamin A (as the acetate in cottonseed oil) in 1 ml. of chloroform reacted with 6 ml. of N/30 HCl in ethanol. Curves 1, 2, 3 and 4 were started at 1.5, 14.5, 34 and 91.5 minutes

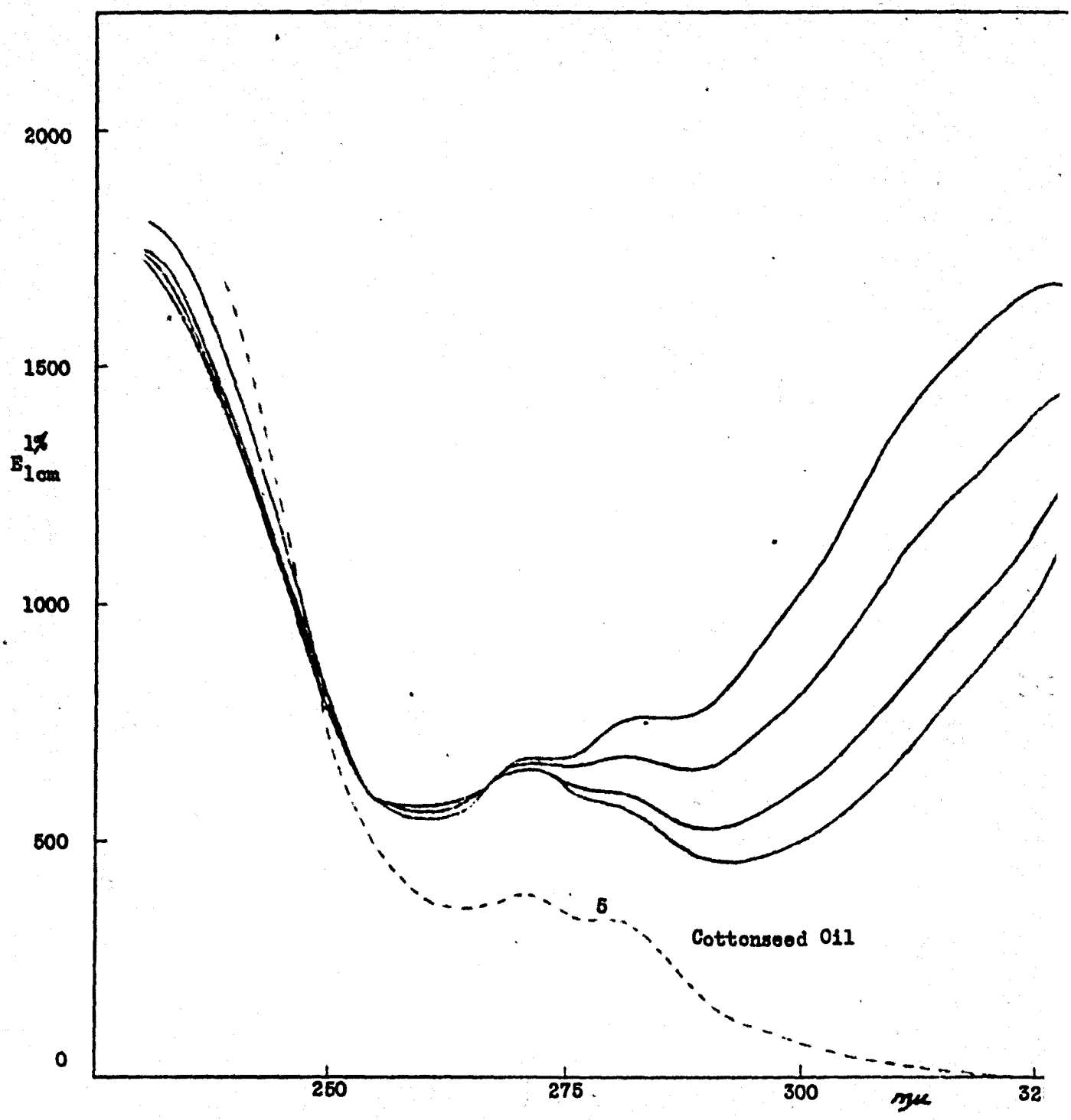
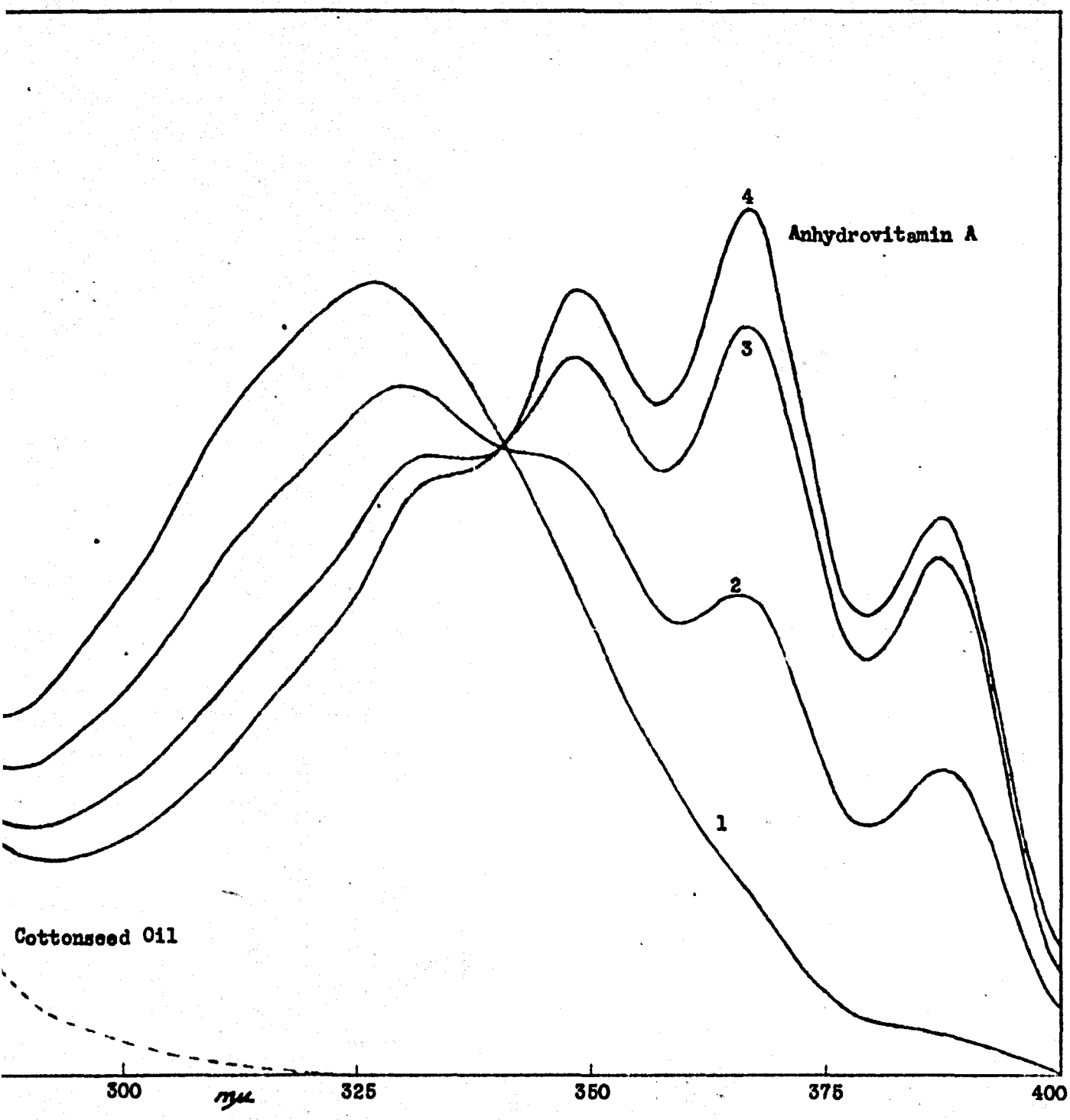


Figure 34. Spectrophotometric Study of the Formation of Anhydrov Cottonseed Oil with N/30 HCl in Ethanol. Curves 1, 2 utes respectively, after Mixing the Reactants. Curve N/30 HCl in Ethanol.



dy of the Formation of Anhydrovitamin A by the Reaction or Vitamin A Acetate in
30 HCl in Ethanol. Curves 1, 2, 3 and 4 were Traced at 1.5, 14.5, 34 and 91.5 min-
er Mixing the Reactants. Curve 5 is the Absorption Spectrum of Cottonseed Oil in

after the reaction was begun. Curve 5 shows the absorption spectrum of a mixture of 6 ml. alcoholic HCl and 1 ml. of a chloroform solution containing a quantity of cottonseed oil essentially the same as that present in the vitamin A test solution.

Table 26 gives a complete summary of the change in the extinction coefficients (based on the original vitamin A content) at the absorption maxima as the reaction progressed. Figure 35 graphically shows the change at each absorption maxima.

The position of these absorption bands (348, 367, 388 and an inflection at 332 mμ) seems to agree quite closely with those of anhydrovitamin A published by other workers. A decrease in the absorption at the 387 mμ band and the stability of the 333 mμ maximum may indicate the beginning of the formation of isoanhydrovitamin A which has absorption maxima at 330, 350 and 370 mμ (205) or at 330, 347 and 367 mμ (89).

Quenching Experiments.

The ultraviolet absorption spectrum of the GDH-vitamin A reaction mixture after the color had been quenched was determined with the aid of the Cary recording spectrophotometer.

The first quenching agent tried was n-butylamine. It was chosen because of its great inhibitory power for the GDH-vitamin A color reaction. One ml. of a chloroform solution

Table 26

Stability of the Product of the Reaction between Vitamin A Acetate and Alcoholic HCl

Time after mixing (minutes)	mμ	$E^{1\%}_{1cm}$	Time after mixing (minutes)	$E^{1\%}_{1cm}$ 348 mμ
2.72	327	1681	2.37	1004
9.20	328	1557	8.87	1081
15.40	330	1458	15.37	1266
22.13	332	1390	21.87	1365
28.55	333	1344	28.37	1450
35.05	333	1312	34.87	1522
41.55	333	1295	41.37	1567
48.05	333	1281	47.87	1612
54.55	333	1269	54.37	1643
61.05	333	1263	60.87	1662
67.55	333	1259	67.37	1674
74.05	333	1259	73.87	1682
80.55	333	1259	80.37	1674
92.55	333	1259	92.37	1662

Time after mixing (minutes)	$E^{1\%}_{1cm}$ 367 mμ	Time after mixing (minutes)	$E^{1\%}_{1cm}$ 387 mμ
2.05	370	1.72	95
8.55	727	8.22	396
15.05	1020	14.72	648
21.55	1258	21.22	846
28.05	1451	27.72	999
34.55	1594	34.22	1096
41.05	1695	40.72	1163
47.55	1768	47.22	1187
54.05	1813	53.72	1195
60.55	1824	60.22	1179
67.05	1842	66.72	1154
73.55	1826	73.22	1117
80.05	1813	79.72	1070
92.05	1758	91.72	979

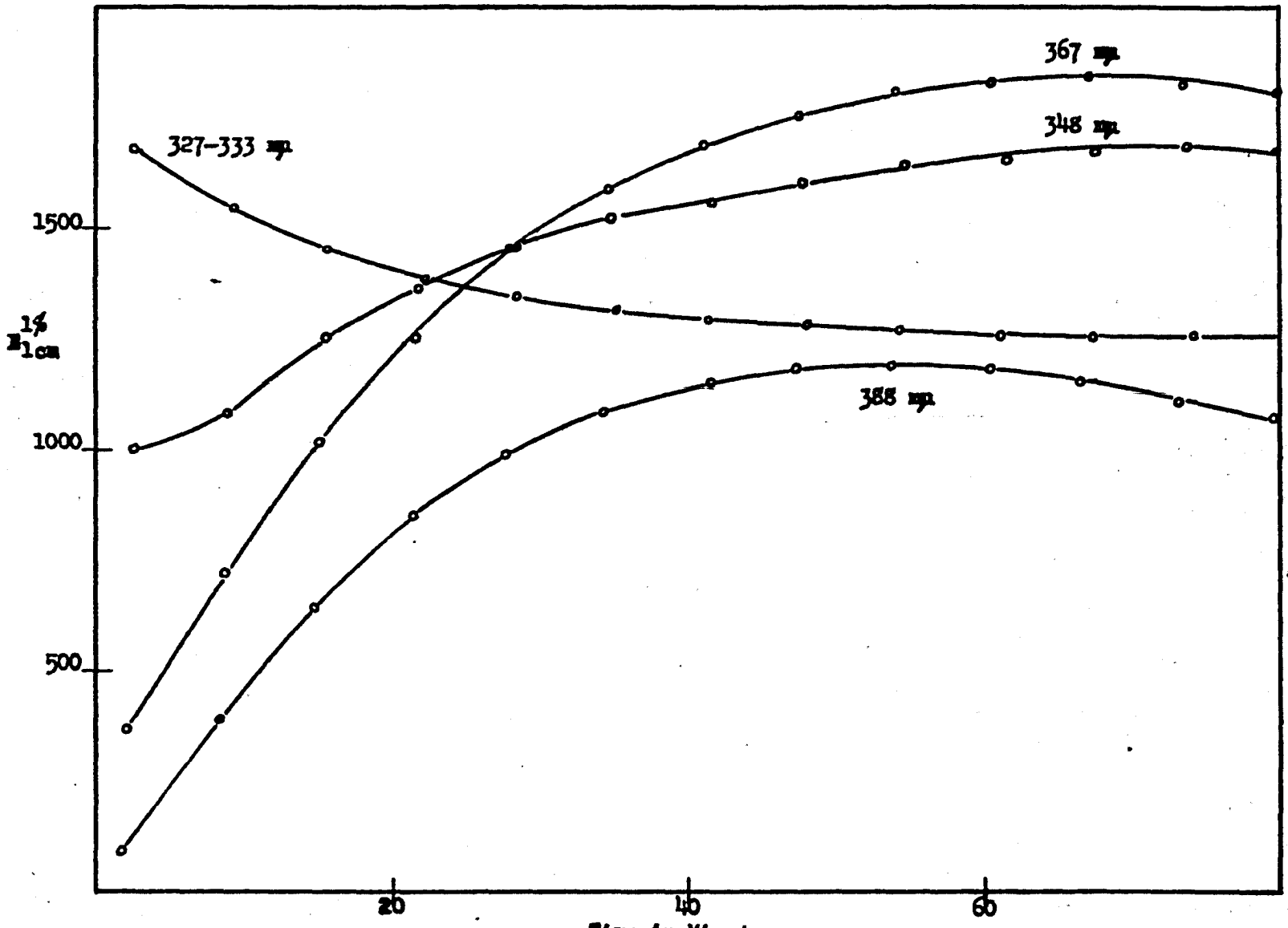


Figure 35. Changes in the Extinction Coefficients with Time at the Absorption Maxima Resulting during the Reaction of Vitamin A with Alcoholic HCl.

containing 25.6 micrograms of vitamin A was mixed with 4 ml. of Shohan GDH. After standing for 2 minutes, 1 drop of n-butylamine was added and mixed with the colored solution. The violet color immediately disappeared. Part of the clear solution was poured into a 10 mm. absorption cell and the absorption spectrum determined in the spectrophotometer. Absorption maxima were observed at 308, 286 and 274 mμ, a deep minimum at 265 mμ and increasing absorption at wavelengths below 260 mμ. The peculiar shape of this absorption spectrum brought up the question of possible reaction between glycerol dichlorohydrin with the n-butylamine to give some product which contributed to the resulting absorption curve. To check this possibility, a mixture of 1 ml. chloroform, 4 ml. of GDH and 1 drop of n-butylamine was prepared and poured into a 10 mm. absorption cell. The zero point was set at a value of 0.5 on the instrument chart in case the reaction mixture absorbed less light than the blank solution (1 ml. chloroform plus 4 ml. GDH). The absorption slowly increased as the tracing proceeded from 400 to 310 mμ. Beyond this point the curve dropped well below the original zero line until a minimum occurred at about 270 mμ and then it rose quite abruptly to the point at which the tracing was completed (240 mμ). The shape of this curve makes it quite obvious that there had been a reaction between the amine and GDH which accounted for the odd shape of the ab-

sorption curve of the product following the quenching of the vitamin A-GDH color reaction.

Since 95% ethanol does not react with GDH, this reagent was chosen to quench the GDH-vitamin A reaction. In a preliminary experiment in which the color reaction was arrested by the addition of 2 ml. of ethanol to the reaction mixture, absorption maxima were observed at 282, 271 and 261 mμ. An experiment was then set up to determine the absorption spectra of the products of the GDH-vitamin A reaction when the colors were quenched at various time intervals after vitamin A and GDH were mixed.

Each color reaction was initiated by mixing 1 ml. of a chloroform solution containing 51.22 micrograms of vitamin A with 4 ml. of Shohan GDH. The colors were discharged by the addition of 2 ml. of 95% ethanol at 15, 20, and 40 seconds and 1, 2, 4 and 8 minutes after the color reactions were begun. The ultraviolet absorption spectra were determined over the range of 240 to 400 mμ. Figure 36 shows the results of these absorption spectral studies. A curve made by mixing 1 ml. of the vitamin A solution with a solution containing 4 ml. of Shohan GDH and 2 ml. of ethanol was included to indicate the absorption spectrum of vitamin A that had not undergone any color reaction. The small absorption maximum at about 390 mμ was observed which probably indicates the beginning of a dehydration reaction

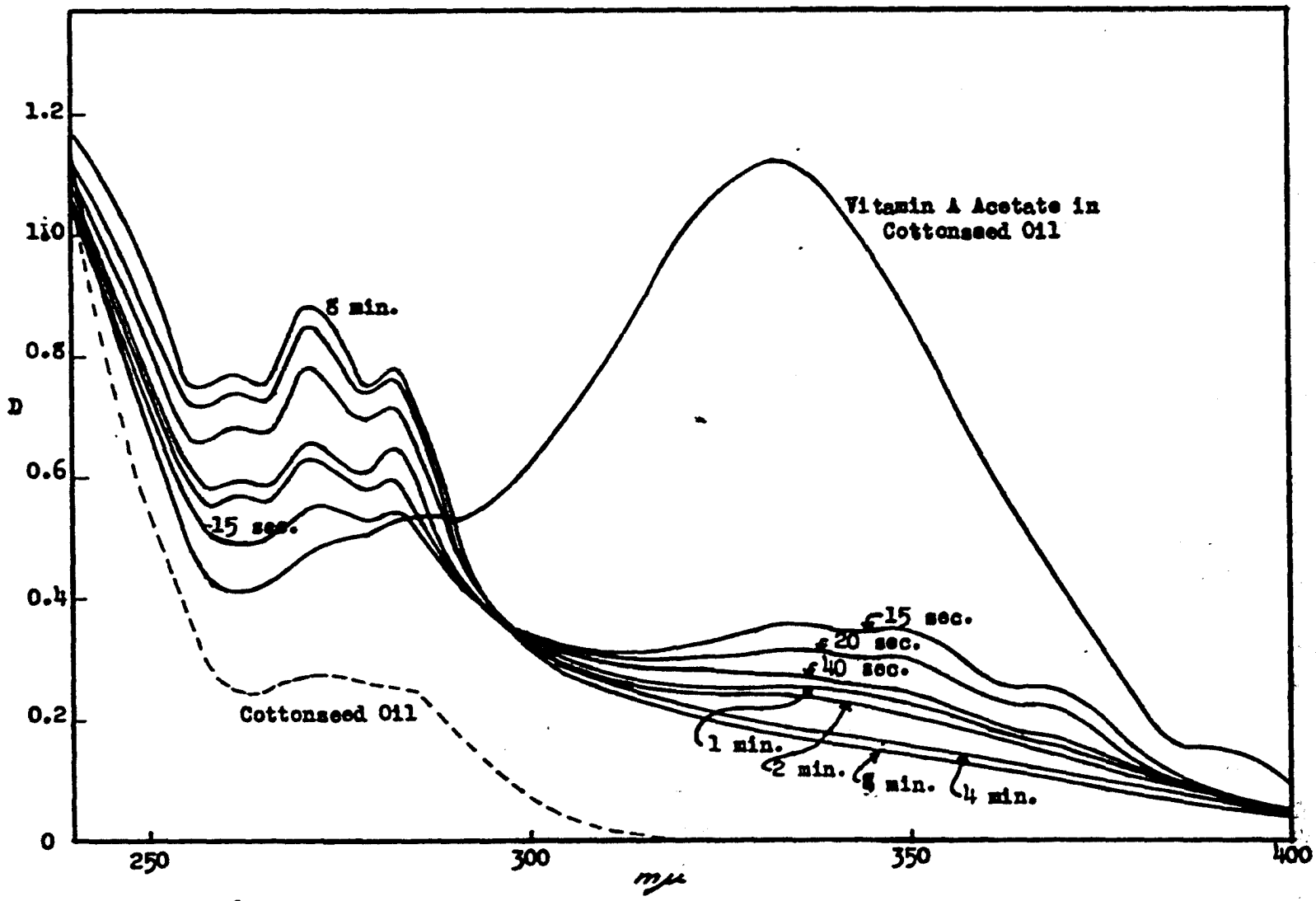


Figure 36. Ultraviolet Absorption Spectra of Cottonseed Oil, Vitamin A Acetate in Cottonseed Oil and the Products that Resulted from Reactions of Vitamin A with GDH (Reactions Quenched with Ethanol at Various Time Intervals).

due probably to the presence of free HCl in the GDH. A curve showing the absorption of a mixture of 1 ml. of a chloroform solution of cottonseed oil, 4 ml. of GDH and 2 ml. of ethanol was also included.

The absorption spectra of the reaction mixtures which had been quenched at less than 1 minute after color formation had maxima at 335, 349 and 369 mμ in addition to three others at 261, 272 and 282 mμ. Reaction mixtures which were allowed to stand over one minute before quenching with alcohol had no absorption maxima at wave lengths greater than 300 mμ but did absorb to greater extents at the lower absorption maxima. As the time of reaction increased the absorption at the lower wavelengths increased while that at the longer wavelength maxima decreased.

In another experiment the reaction of GDH plus alcohol on cottonseed oil was determined. There was essentially no change in the ultraviolet absorption spectrum over the range of 260 to 400 mμ as the mixture aged.

Ultraviolet Absorption Spectra as the GDH-Vitamin A Color Reaction Aged

1:5 Solvent-reagent ratio. Multipot adjustments were made over the range of 250 to 400 mμ with a blank composed of one part chloroform solution of cottonseed oil (0.451 gram per 25 ml.) and five parts of Shohan GDH. One ml. of chloroform solution containing 52.92 micrograms vitamin A (crystalline vitamin A acetate in cottonseed oil) was mixed

with 4 ml. of Shohan GDH. The colored solution was poured into a 10 mm. cell and the absorption spectrum determined with the Cary recording spectrophotometer. An absorption spectral tracing was made every 6 minutes over a 50-minute period. Curves 1 and 2 in Figure 37 are the first and last spectral absorption curves obtained. Absorption maxima occurred at 262, 272, 283, 299, 312 and 356 mμ. Curve 3 is the absorption spectrum of 52.92 micrograms of vitamin A per 6 ml. of chloroform run against a blank containing a quantity of cottonseed oil equivalent to that present in the vitamin A solution.

5:1 Solvent-reagent ratio. Five ml. of a chloroform solution containing a total of 52.92 micrograms of vitamin A were mixed with 1 ml. of GDH and the ultraviolet absorption spectrum of the solution obtained with the recording spectrophotometer. The blank consisted of 1 ml. of GDH and a mixture of 5 ml. of chloroform containing a quantity of cottonseed oil equivalent to that present in the vitamin A solution. A series of twelve absorption curves were traced over an 80-minute period. Several of these are shown in Figure 37. Curve 4 was traced, starting at the 400 mμ mark, 1.5 minutes after the reaction was initiated. Curves 5, 6, 7 and 8 were started at 7.5, 19.5, 43.5 and 80 minutes. As the reaction progressed the absorption at the shorter wavelengths increased and that between 300 and 400 mμ decreased. Ab-

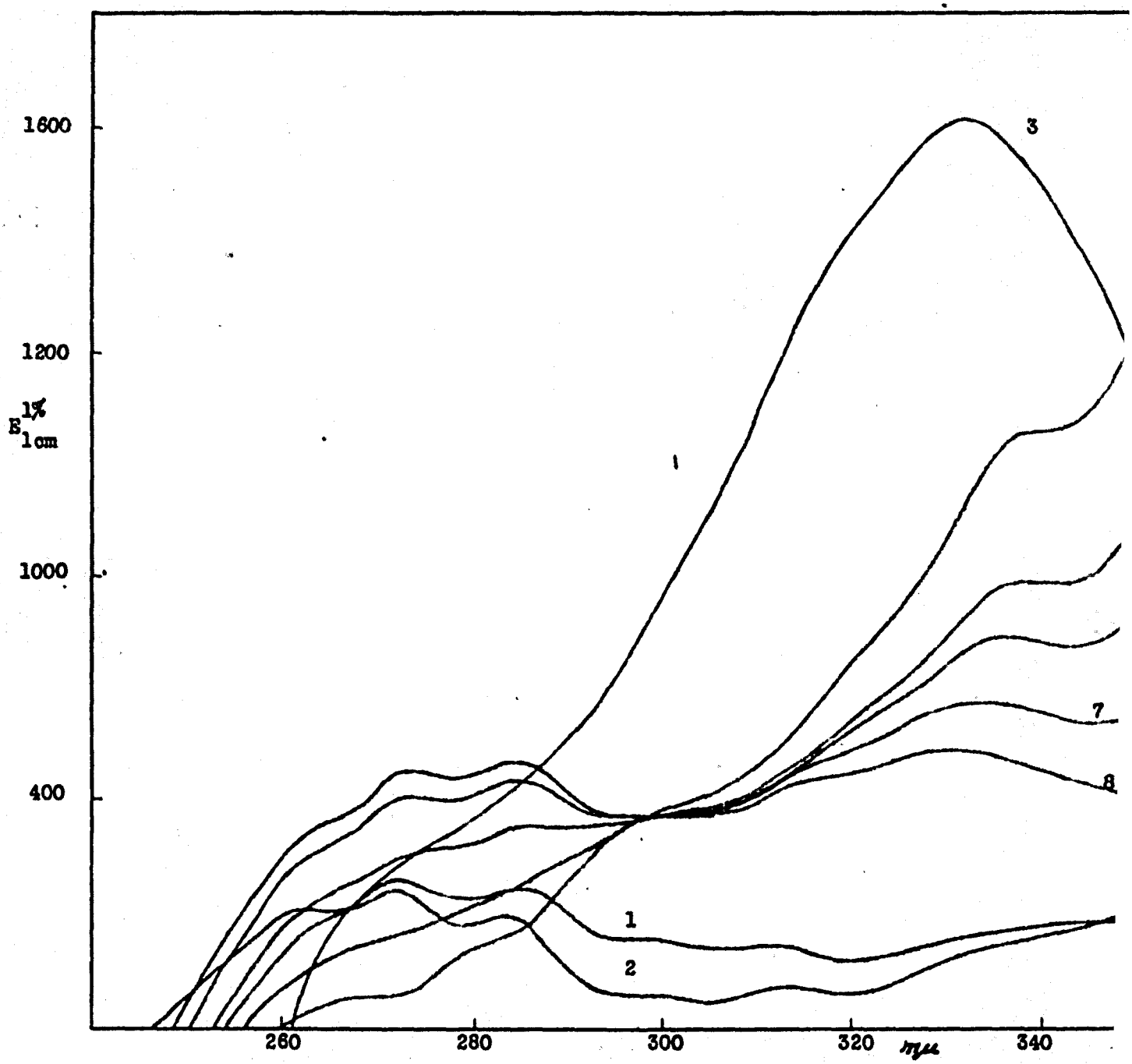
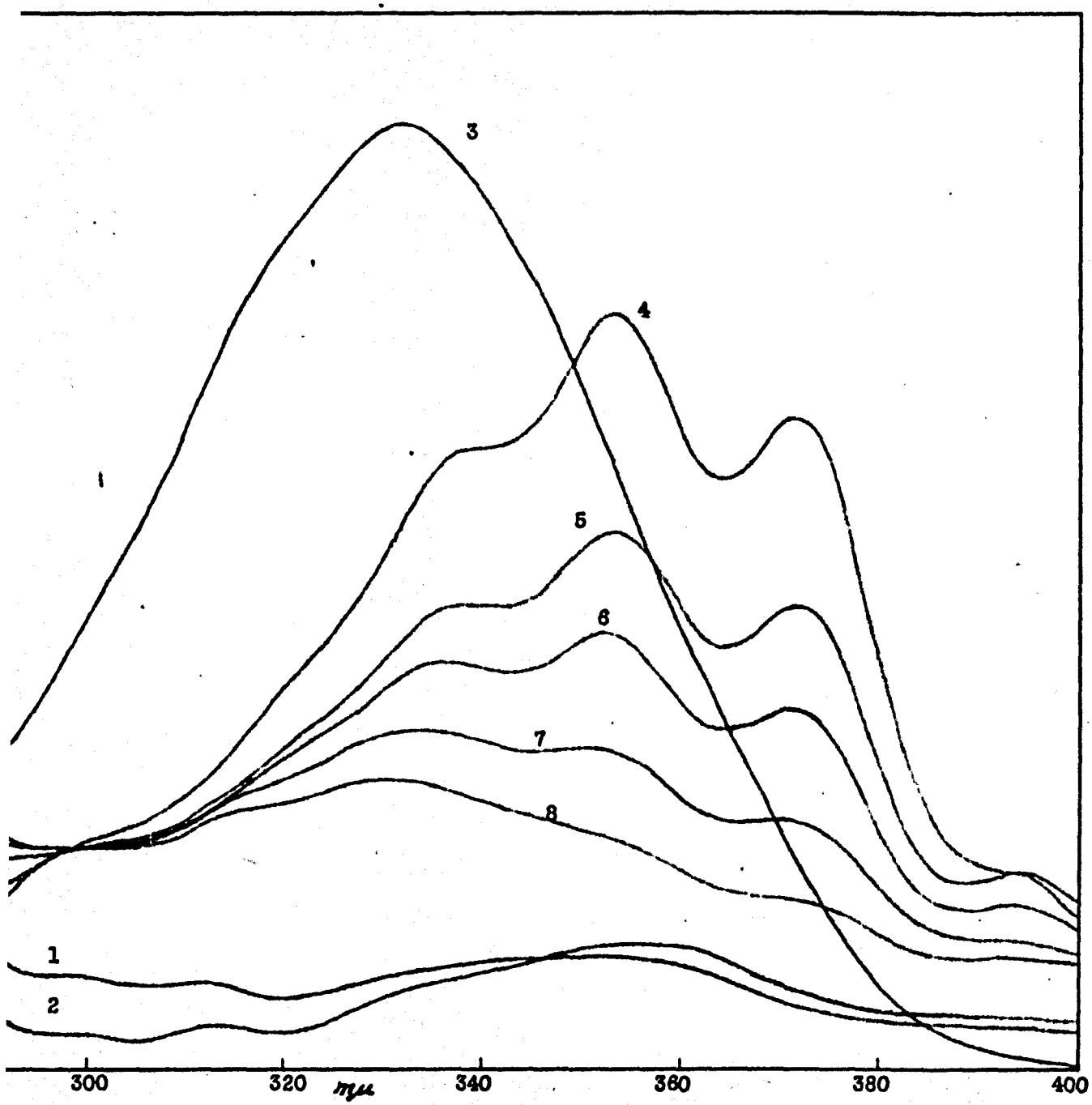


Figure 37. Ultraviolet Absorption Spectra of the Products of the React
Curves 1 and 2 were Traced 2 and 60 Minutes after 1 Part of
was Mixed with 5 Parts Shohan GDH. Curves 4, 5, 6, 7 and 8
and 80 Minutes after 5 Parts of Chloroform Solution of Vita
GDH. Curve 3 is the Absorption Spectrum of Vitamin A Aceta



ıorption Spectra of the Products of the Reactions of Vitamin A with Shohan GDH. were Traced 2 and 60 Minutes after 1 Part of Chloroform Solution of Vitamin A 5 Parts Shohan GDH. Curves 4, 5, 6, 7 and 8 were Traced 1.5, 6.7, 19.5, 43.5 after 5 Parts of Chloroform Solution of Vitamin A was Mixed with 1 Part Shohan .s the Absorption Spectrum of Vitamin A Acetate.

sorption maxima occurred at 262, 272 and 283 mμ which were the same as those observed when the 1:5 solvent-reagent ratio was employed.

DISCUSSION

It was found that glycerol dichlorohydrin, either the 1,3- or a mixture of 2,3- and 1,3-dichlorohydrins, could be activated with a number of compounds and reagents. By activation is meant the ability of the GDH to react with a chloroform solution containing vitamin A to produce a color. Certain inorganic acids such as concentrated hydrochloric, concentrated sulfuric, chlorosulfonic and 60% perchloric acids caused good activity. Moderate activity was produced by addition of small amounts of aqueous hydrobromic (40%) and aqueous hydrofluoric (52%) acids. When the above activated glycerol dichlorohydrins were tested with vitamin A, a blue color which changed to violet resulted in each case except the hydrofluoric acid-activated GDH which resulted in a faint blue color which faded quite rapidly. Aqueous hydriodic acid (47%) resulted in essentially no activation while a completely negative result was obtained when concentrated nitric acid was added.

The following carboxylic acids were tested and found to produce no activity: acetic, monochloroacetic, trichloroacetic and citric acids. Penketh (179) suggested that hydrochloric acid, or perhaps hydrogen ions, is the activating agent. The fact that concentrated nitric, acetic and trichloroacetic acids failed to produce activity appears to

indicate that hydrogen ions per se do not necessarily cause activation. The anion may be just as important as the hydrogen ion.

The organic sulfonic acids tested were found to activate GDH. The color formed with vitamin A was blue which slowly changed to violet. A closely related compound, methyl sulfate, was a good activating agent. Activity in this case may be the result of reaction between GDH and methyl sulfate to produce some sulfuric acid along with an equivalent amount of methyl ether of GDH. The sulfuric acid may be the agent that actually causes the activation in this case.

Benzoyl and acetyl chlorides caused fairly good activation. This may possibly be explained on the basis of reaction between the hydroxyl group of GDH with the acid chloride to produce a small amount of ester along with an equivalent amount of HCl, the latter being responsible for the activation.

Certain inorganic halides were found to produce good activity when added in small amounts. These included phosphorus trichloride, phosphorus oxychloride, arsenic trichloride and aluminum trichloride. Here again the activity was probably due to the presence of HCl formed by reaction with GDH. The formation of HCl by the reaction of anhydrous aluminum trichloride with GDH in a carbon disulfide medium has been demonstrated by Claus and Mercklin (31).

The organic derivative was said to be $CH_2ClCHOAlCl_2CH_2Cl$.

Sulfur dioxide dissolved in GDH failed to cause activation. As a matter of fact, this reagent when mixed with active GDH inhibited normal color formation.

Quantitative activation studies were made with hydrochloric acid, sulfuric acid and chlorosulfonic acid. Figure 38 includes the absorption spectra of the colors resulting from reactions of 0.1% HCl-activated GDH, 0.046% $ClSO_3H$-activated GDH and 1.0% H_2SO_4-activated GDH with vitamin A. The curves for the HCl- and $ClSO_3H$-activated reagents were very similar in shape, both having absorption maxima at 553 and 358 mμ. The H_2SO_4-activated reagent had absorption maxima at 452 and 545 mμ after the reaction had progressed for 5 to 6 minutes. The shift to 545 mμ and the prominence of the 452 mμ band indicates the formation of a complex between vitamin A and GDH that is different than that formed with HCl- or $ClSO_3H$-activated GDH. Perhaps the most interesting observation was the excellent stability of the 545 mμ maximum of the H_2SO_4-activated GDH-vitamin A color. The reasons for this stability and the lack of good stability in the case of the HCl-activated reagent are not clear. The stability of the color produced by the $ClSO_3H$-activated reagent was better than that of the HCl-activated GDH. It appears therefore that the SO_3H group aids to some extent in stabilizing the color produced by reaction of GDH with

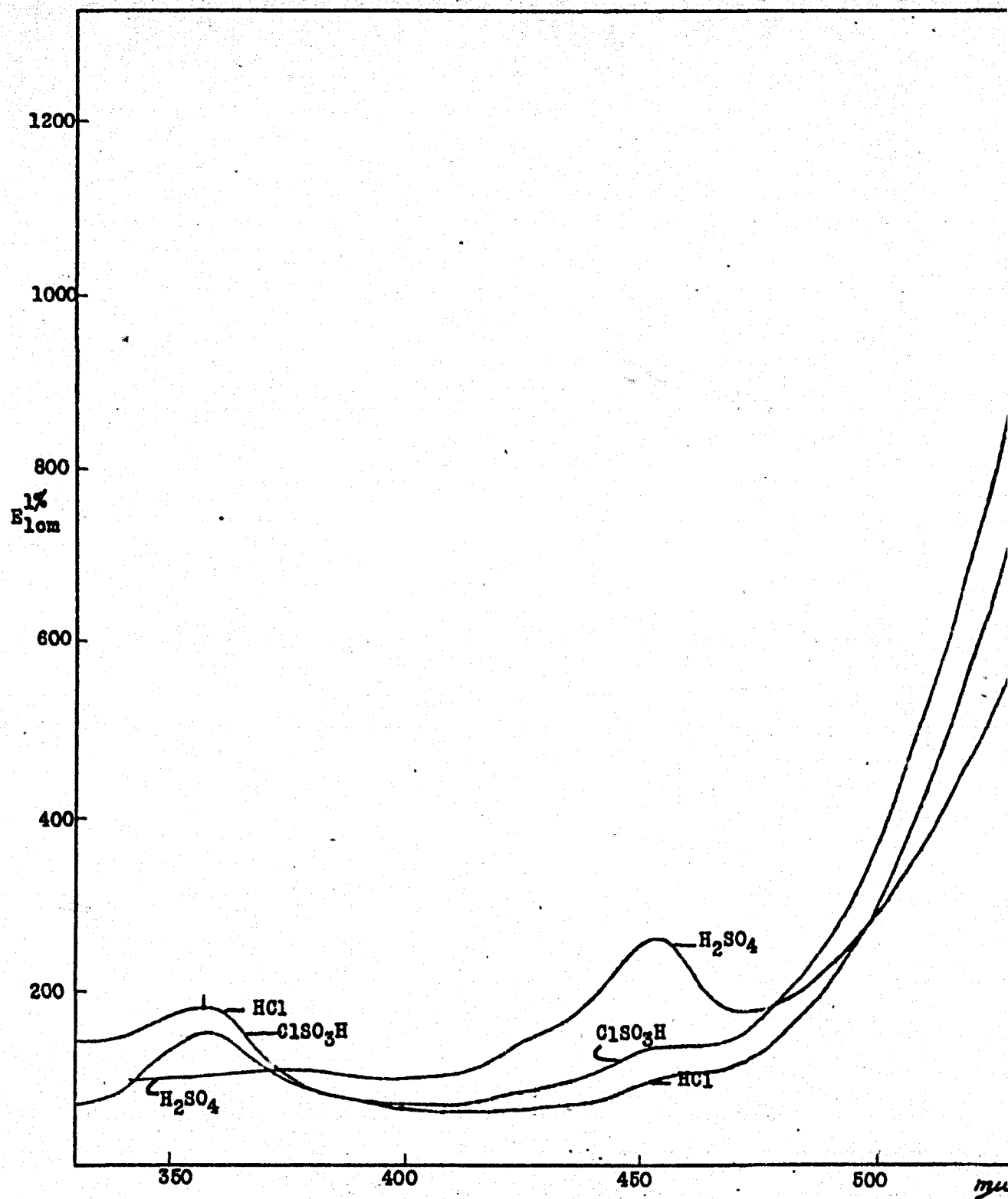
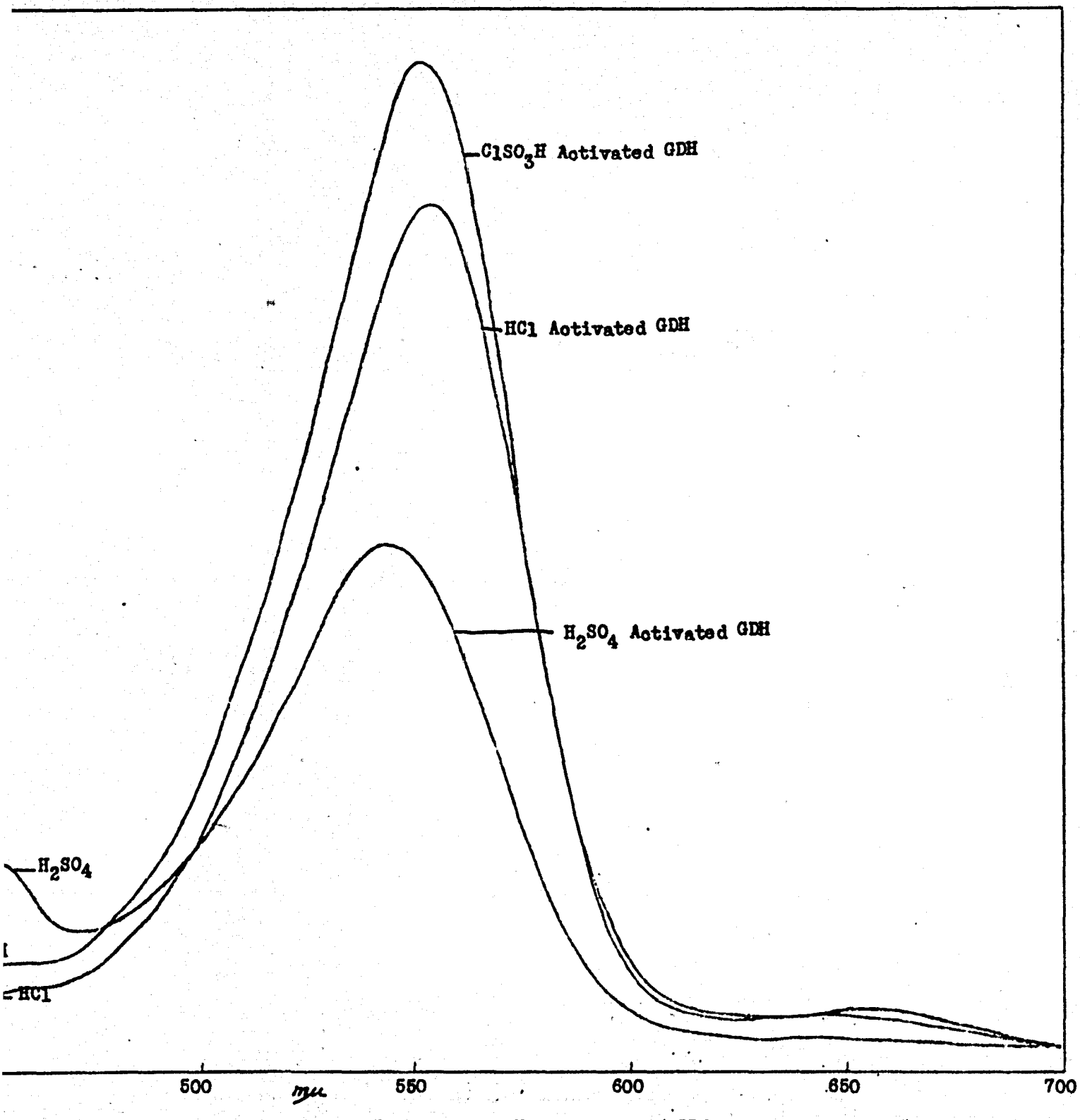


Figure 38. Absorption Spectra of the Colors Produced by Concentrated Hydrochloric Acid, Concentrated



tra of the Colors Produced by the Reactions of Vitamin A with GDH Activated with drochloric Acid, Concentrated Sulfuric Acid and Chlorosulfonic Acid.

vitamin A. Chlorosulfonic acid may possibly react with GDH to produce HCl and a substituted sulfuric acid. The latter, even though present in small amounts, may be responsible for the increased stability of the violet color.

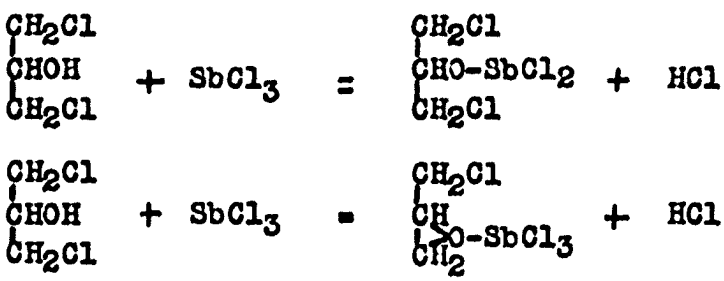
In another series of experiments the quantities of anhydrous HCl and of chlorosulfonic acid required to produce maximum activation of GDH were investigated. Figures 4 and 9 show the concentrations of each acid required to produce maximum activation. In each case there was quite a wide concentration range that resulted in good activation. After several of these acid-activated reagents had been stored for several weeks in clear, glass-stoppered bottles the activity was rechecked. Reagents containing relatively large quantities of acid were found to either increase in activity or decrease only to a slight extent. Reagents which had optimum activity when freshly prepared were found to decrease in activity. The reagents containing only traces of activating acids and having low initial activity were observed to increase in activity on standing in the laboratory. This activity, however, began to decrease on continued storage. These findings point to the probability of light decomposition of GDH to produce at least two products, one which increases activity and another which has an inhibitory effect on color formation when GDH reacts with vitamin A. High concentrations of acid seem to slow down the formation of

the inhibitor whereas reagents originally containing optimum levels of acid begin to decrease in activity because of inhibitor formation. Reagents containing little acid and having low activity became more active because of the formation of an activating substance which is probably HCl. On further storage the accumulation of color inhibitor finally overcame the effect of the activator and resulted in diminished activity.

Activation of GDH was accomplished by simply heating with antimony trichloride. A sample containing about 0.1% antimony trichloride by weight was subjected to a temperature of 85°C. for periods ranging from 1 to 4 hours. After cooling, the ultraviolet absorption spectra of these reagents was determined. The absorption spectrum was different for each reagent, showing increased absorption in the range of 230 to 300 mμ as the reagent was heated for longer periods (see Figure 12). These data are presented as possible evidence for the formation of some sort of complex between antimony trichloride and GDH. Apparently there was no reaction when antimony trichloride was dissolved in GDH at room temperature. This was demonstrated by determining the absorption spectra of GDH alone, antimony trichloride alone and GDH plus antimony trichloride using methanol as the solvent in each case. The absorption spectra of the latter was the summation of the other two.

The activity of the reagents prepared by heating GDH with antimony trichloride increased with increased heating time. Although entirely unsuspected, the negative control containing no antimony trichloride was found to develop good activity by the action of heat alone. The stability of the color produced by reaction of vitamin A with the antimony trichloride-activated reagent was much better than with the reagent activated solely by heating for 4 hours. The former decreased by 26% and the latter by 50% over a 6-minute period.

It seems likely that some sort of complex formed by heating antimony trichloride with GDH which resulted in a reagent of good activity capable of producing a stable color with vitamin A. The following equations are postulated as possible mechanisms for the antimony trichloride activation:



HCl could then be the principal activation agent and the antimony complex could be partially responsible for increased stability of the GDH-vitamin A color. These possible reaction mechanisms are based largely on the known reaction of

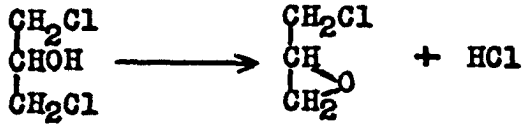
aluminum chloride with GDH (31).

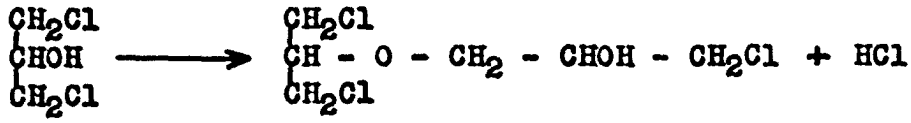
Penketh (179) claimed that HCl was the activating principle when activated GDH was prepared by vacuum distillation with antimony trichloride. The present investigation confirms this idea, at least in part. A reagent prepared by vacuum distillation of GDH with 1% antimony trichloride was found to have good activity and the stability of the GDH-vitamin A color was quite good. Redistillation of this reagent resulted, as indicated by the ultraviolet absorption spectrum, in a diminished antimony trichloride content but the reagent had increased activity and the stability of color was improved. Another redistillation of the GDH produced a reagent which contained practically no antimony trichloride and had diminished activity and stability of color. Activation in the latter case was probably due to the presence of HCl in the reagent. When approximately 0.02% antimony trichloride was added to the reagent containing no antimony, the activity was decreased and the course of color development was influenced; maximum color formation was not attained until about 3 minutes after initiation of the color reaction. Earlier it was shown (see Figure 3) that addition of 0.013% antimony trichloride *per se* to a reagent activated by 0.5% hydrochloric acid caused an improvement in stability of the GDH-vitamin A color but reduced the activity of the

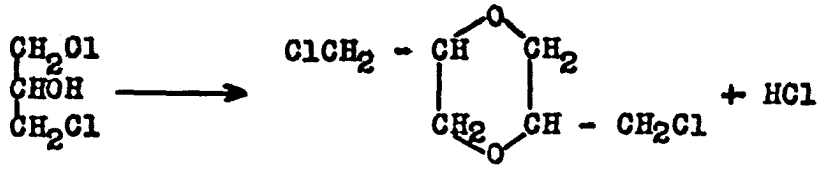
reagent to a slight extent. All these experiments indicate quite definitely that activity is not dependent upon the presence of antimony in the reagent, but that its introduction into the reagent by way of codistillation with GDH under vacuum or by heating a mixture of GDH and antimony trichloride results in a reagent with better activity and improved stability of the GDH-vitamin A color. It seems quite probable therefore that antimony is, at least in part, associated with GDH in some way that results in improved stability of the GDH-vitamin A color.

Glycerol dichlorohydrin in an inactive form was found to become active gradually upon exposure to laboratory light. This was observed with all brands of GDH tested. The rate of activation by light was diminished when the GDH was stored in brown bottles. Heating was also found to cause activation of Shell GDH but not freshly deactivated Shohan GDH. However, upon standing for several weeks in a clear, glass-stoppered bottle even the latter type of GDH became activated. Inactive GDH gave no test for free halogen ion with alcoholic silver nitrate, but after activation by heat or light it gave a strongly positive halogen ion test. The mechanism of this decomposition of GDH by heat and light is, as far as could be ascertained, now known. The following

possible mechanisms are suggested:

1. 

2. 

3. 

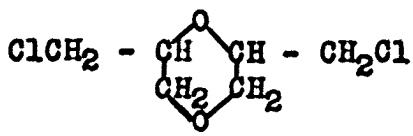
The first equation does not appear too likely since epichlorohydrin is known to react with HCl quite readily and thus the tendency for reaction would most likely be in the reverse direction. Also it was observed that epichlorohydrin was very effective in inhibiting the GDH-vitamin A color. If this was the decomposition reaction, the epichlorohydrin would probably cause inhibition to about the same extent as the HCl would cause activation and the net result would be no activation.

The second equation may take place with the formation of an ether linkage. If this is the correct mechanism, it would seem possible that continued decomposition reaction could occur to give long-chain compounds resulting from splitting out HCl and the formation of ether linkages. The over-all equation would then be

$$(X+2)\quad \begin{matrix}CH_2Cl\\ CHOH\\ CH_2Cl\end{matrix} \longrightarrow \begin{matrix}CH_2Cl\\ CH\\ CH_2Cl\end{matrix} - O - CH_2 - \begin{matrix}CH\\ CH_2Cl\end{matrix} - O - CH_2 - \begin{matrix}CHOH\\ CH_2Cl\end{matrix} + (X+1)\ HCl$$

The third equation indicates the possible formation of a dioxane derivative along with HCl. The accumulation of this derivative could result in a dimunition of the GDH-vitamin A color intensity since it has been shown that dioxane itself caused some inhibition. The formation of this cyclic ether from glycerol dichlorohydrin appears to be quite possible since Faucounier and Sanson (61) prepared it by reaction of HCl with dry glycerol at 180-200°C., a reaction which will produce glycerol dichlorohydrin. Stoeht (222) found it as a by-product in the distillation of glycerol with ammonium phosphate and ammonium chloride. Battegay and associates (6) were successful in synthesizing it by fusing diglycerol with phosphorus trichloride. The pure compound is crystalline with a melting point of 112-113°C. and a boiling point of 232-233°C.

In the case of a mixture of glycerol 1,3-dichlorohydrin and glycerol 2,3-dichlorohydrin, the proposed dioxane derivative could be



Aside from the positive test for the chloride ion no further investigation of the nature of the GDH-decomposition products was made.

Meunier and Vinet (138) reported that compounds which donate electrons caused discharge of the blue color resulting when vitamin A was adsorbed on an acid clay. They found pyridine to be very effective and indicated that alcohols, acetone, ether and amines also caused discharge of the blue color. The introduction of a solvent which donates electrons also stops the antimony trichloride reaction with vitamin A (133). Addition of several types of compounds to activated GDH was found to decrease the activity of the colorimetric reagent. The most powerful compounds tested were pyridine, aniline, n-butylamine and epichlorohydrin. The nitrogen-containing compounds had essentially the same inhibitory influence, concentrations of approximate 0.01% by weight caused complete inhibition. Epichlorohydrin was somewhat less effective in the prevention of the violet GDH-vitamin A color. Aqueous ammonia in all concentrations tested completely inhibited the color formation. Aqueous KOH was quite effective, while dioxane, alcohol and water had lesser effects.

It appears that compounds having an affinity for HCl cause inhibition of the GDH-vitamin A color. The reason for this probably lies in the combination with free HCl which is probably the activating principle in the antimony trichlor-

ide-activated GDH. In addition there may be a possibility of reaction of the amines with glycerol dichlorohydrin to form amino derivatives followed by the formation of the hydrochlorides. Dioxane, water and alcohol could possibly have caused some inhibition by the formation of oxonium salts with HCl. Even though traces of moisture caused very little inhibition of the GDH-vitamin A color, it became evident from this study that anhydrous conditions are to be preferred in any vitamin A analysis procedure.

Other glycerol derivatives were tested in an effort to ascertain the structural requirements required for the formation of good color with vitamin A. A number of qualitative activation tests were made with glycerol α-monochlorohydrin and 1,2,3-trichloropropane. Practically all compounds or solutions that activated the glycerol dichlorohydrins caused weak activation of glycerol α-monochlorohydrin. These included concentrated hydrochloric, concentrated sulfuric, chlorosulfonic and 60% perchloric acids, methyl sulfate, phosphorus trichloride, phosphorus oxychloride, arsenic trichloride and aluminum trichloride. The color first formed in each case was a light blue which changed to a faint violet within a minute or so. As in the case of the GDH activation, nitric and trichloracetic acids gave completely negative results.

Of all the substances tested only two, concentrated

sulfuric acid and 60% perchloric acid, caused activation of 1,2,3-trichloropropane. Activation is probably an incorrect term to use in this case since chloroform solutions of vitamin A gave color reactions when these acids were added.

Activation of glycerol α-monochlorohydrin and 1,2,3-trichloropropane by codistillation with 1% antimony trichloride under vacuum was attempted. The former was found to be somewhat active and the absorption spectrum of the color produced by reaction with vitamin A was not unlike that of the GDH-vitamin A color. The absorption spectrum differed mainly in the magnitude of absorptions at the 553 and 373 mμ bands. Table 27 shows the effect of the solvent-reagent ratios on the extinction coefficients at the 553 and 373 mμ bands when activated GDH and glycerol α-monochlorohydrin were tested with vitamin A. The GDH values at the 555 mμ band were considerably higher than those obtained with glycerol monochlorohydrin. However, at the 373 mμ band the extinction coefficients were approximately the same at each solvent-reagent ratio. These data appear to discredit the possibility that glycerol α-monochlorohydrin was contaminated with glycerol dichlorohydrin. The 1,2,3-trichloropropane that was vacuum distilled with antimony trichloride failed to give a color test with vitamin A.

The evidence thus far accumulated appears to indicate that a hydroxyl group and two chlorine atoms on different

carbon atoms in a glycerol derivative are required for good color development when reacted with vitamin A. Sobel (207) indicated that ethylene chlorohydrin, trimethylene chlorohydrin, propylene chlorohydrin, propylene glycol, acetylene tetrachloride, trichlorohydrin, 1-chloro-2,3-epoxypropane

Table 27

Comparison of the Extinction Coefficients of the Colors Produced by the Reaction of Vitamin A with Shohan GDH and $SbCl_3$-Activated Glycerol Monochlorohydrin

mμ	Solvent-Reagent Ratio	GMH[a]	GDH
		$E^{1\%}_{1cm}$	
553	1:5	434	1178
	1:1	342	919
	5:1	18	150
373	1:5	222	180
	1:1	336	217
	5:1	934	901

[a] Activated glycerol α-monochlorohydrin

and glycerol α-monochlorohydrin were tested with essentially negative results. No compound closely related to glycerol has yet been shown to have activity as great as glycerol dichlorohydrin.

A rather extensive study was made of the effect of the

solvent-reagent ratio on the absorption spectrum of the GDH-vitamin A color. Ratios of 1:9, 1:7, 1:5, 1:3, 1:1, 3:1, 5:1, 7:1 and 9:1 were employed with redistilled U.S.P. chloroform as the solvent for the vitamin A and Shohan GDH as the reagent. Table 13 summarizes the extinction coefficients at the absorption maxima observed at various time intervals. When low solvent-reagent ratios were used the principal absorption band was at 555 mμ, whereas the most intensive absorption maximum was at 353 mμ when high solvent-reagent ratios were employed.

Figure 39 indicates the effect of the solvent-reagent ratio on the absorption at 555 and 353 mμ at 2.72 and 4.37 minutes, respectively, after initiation of the reactions. Apparently the extinction coefficient at 555 mμ was not changed to any significant extent when an excess of reagent was added, i.e., the absorption at 555 mμ was essentially the same when ratios of 1:3, 1:5, 1:7 and 1:9 were used. When these same reaction mixtures were used, the absorption at 353 mμ was low and essentially constant. The selection of a 1:4 or 1:5 solvent-reagent ratio is quite acceptable for measurement of the violet color developed by reaction of vitamin A with activated Shohan GDH.

As the quantity of solvent in the reaction mixture increased, the absorption at 353 mμ increased, while the 555 mμ absorption decreased to practically zero. The reason for

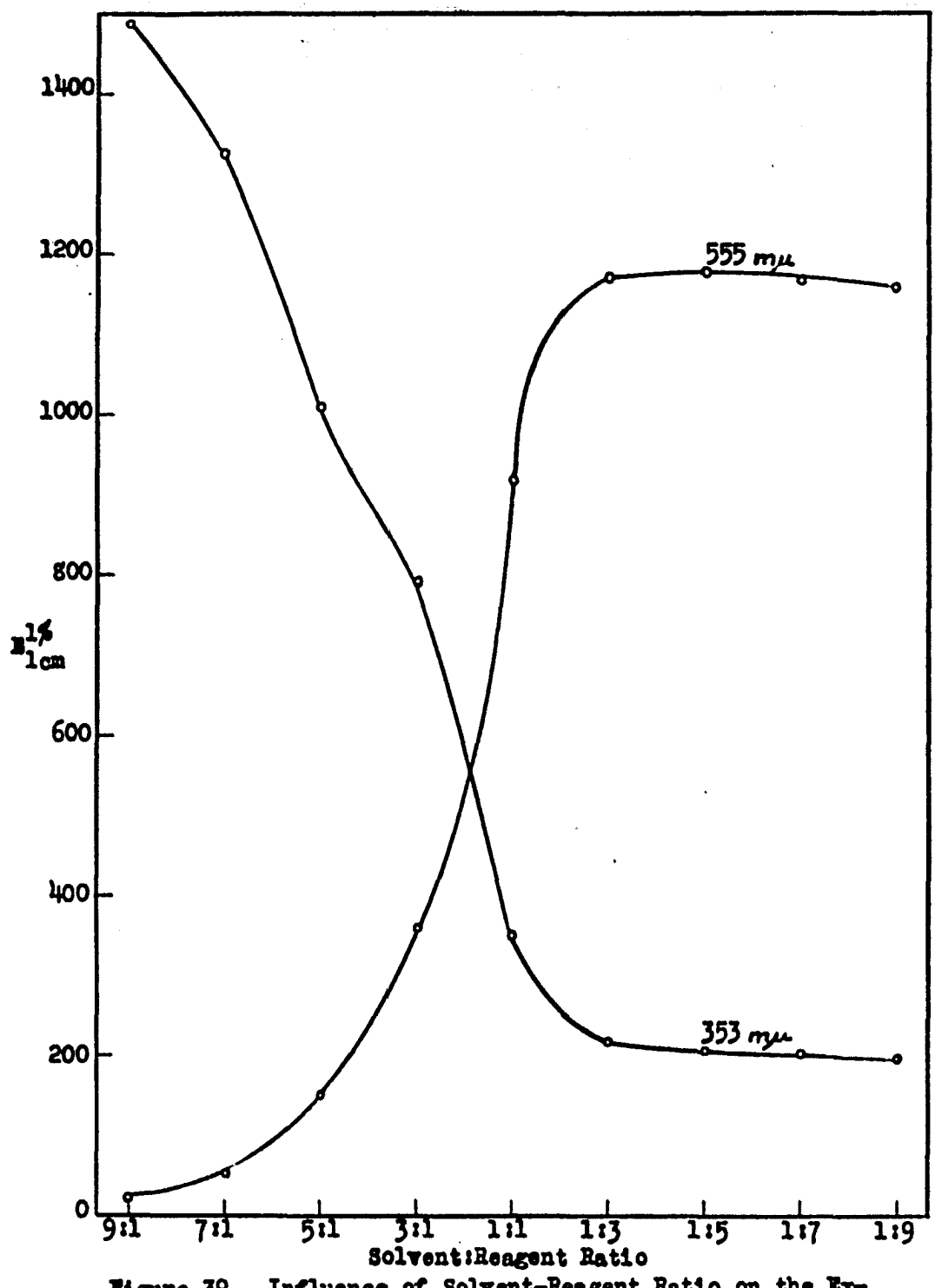


Figure 39. Influence of Solvent-Reagent Ratio on the Extinction Coefficients at 353 and 555 mμ Resulting from the Reaction of Vitamin A with Shohan GDH.

the high 353 mμ extinction coefficient is not known, but it may possibly be related to (1) the purity of the activated GDH or (2) the HCl concentration in the reagent. The shape of the absorption spectral curves of these GDH-vitamin A reaction products resembles that of the product resulting from dehydration of vitamin A with anhydrous HCl.

The effect of 1:4, 2:3, 3:2 and 4:1 solvent-reagent ratios on the absorption spectra of GDH-vitamin reaction products was investigated by using a batch of recovered, reactivated, once-used Shohan GDH. The shape of these absorption curves was the same as those obtained with fresh Shohan GDH, but the magnitude of the absorption at the lower wave lengths was quite different. Considerably higher extinction coefficients were observed at 353 mμ when the recovered product was used at a 4:1 solvent-reagent ratio. The stability of this absorption maximum, and also of the 555 mμ band, was better than that observed with the original Shohan GDH. The reason for these differences in reagents is not definitely known, but it appears that the differences may be due to the HCl and antimony trichloride contents in the reagents.

If the 4:1 ratio reaction involves a dehydration, the high extinction coefficient at 353 mμ could be explained on the basis of the HCl content in the reagent. It was shown by other workers (48, 205) that the rate of formation of

anhydrovitamin A was dependent upon the concentration of HCl in alcohol and the temperature of the reaction mixture. It may not be entirely unreasonable to postulate that the reaction involved only the HCl content when high solvent-reagent ratios were used. In fact, it may be possible to estimate the approximate free HCl content in a batch of GDH by measuring the absorption at 353 mμ when a high solvent-reagent ratio is used. This point seems worthy of further investigation.

Activated GDH reacts with β-carotene to produce a product which absorbs weakly at 500 and 625 mμ and shows increasing absorption between 750 and 800 mμ (213). It was found that the absorption increased with the wavelength up to 1000 mμ and appeared to reach a maximum near 1030 mμ. The readings above 1000 mμ may not be entirely acceptable since the use of the Beckman spectrophotometer is not recommended at wavelengths longer than 1000 mμ. Stability of the extinction coefficients decreased as the wavelength was increased above 900 mμ. Because of the relatively high and fair stability of the extinction coefficient at 950 mμ, the measurements of carotene in calf blood plasmas are being made at this wavelength. Measurements are made exactly at 4 minutes after mixing the GDH with the chloroform solutions of the plasma extracts. This method permits a more sensitive estimation of carotene than the usual method of measuring it

at 440 mμ in the petroleum ether extract.

The activities of several brands of activated GDH were tested. Shohan GDH was used without further treatment, while the other brands were activated by vacuum distillation with 1% antimony trichloride. There appeared to be little difference in the activities of the various reagents. The Shohan GDH-vitamin A color was not quite as stable as the colors produced by activated Eastman glycerol (75% α,β-; 25% α,γ-) dichlorohydrin, Shell glycerol dichlorohydrin, and Paragon glycerol α,γ-dichlorohydrin. The reason for this difference in color stability may possibly involve the time interval between activation and actual use in the color reaction. All reagents activated in the laboratory were tested within two weeks after activation. Storage between activation and the time of testing was in brown-glass bottles or in clear-glass bottles kept inside light-tight cartons. The activation date of Shohan GDH was not known.

Several experiments were performed in an attempt to determine the mechanism of the GDH-vitamin A color. It was found that the color reaction could be readily quenched by the addition of ethanol. The ultraviolet absorption spectrum of the solution then gave an indication of any change in the vitamin A molecule resulting from the reaction with activated GDH. Absorption maxima were observed at 335, 349

and 369 mμ in addition to three others at 261, 272 and 282 mμ when the color reaction was allowed to proceed up to 1 minute before quenching (see Figure 36). The compound responsible for the absorption at the longer wavelengths may be isoanhydrovitamin A.

Shantz, Cawley and Embree (205) reported the formation of isoanhydrovitamin A (absorption maxima at 330, 350 and 370 mμ) by the prolonged reaction of vitamin A with alcoholic HCl or by treating anhydrovitamin A with HCl or antimony trichloride. Isler and associates (89) prepared this compound by treating vitamin A with alcoholic HCl (0.033 N) for 12 hours at 20°C. After extraction with petroleum ether and chromatographing through alumina, a compound having absorption maxima at 330, 347 and 367 mμ was obtained. The formation of isoanhydrovitamin A has also been reported by other workers (198, 236).

The absorption spectra of the reaction mixtures quenched within 1 minute after the reactions were initiated also resembled those of vitamin A that was irradiated for short periods. Sobotka, et al. (217) reported that there was spectroscopic similarity of the irradiated product with isoanhydrovitamin A, which indicated structural similarity of the two products. These compounds were, however, not the same since the isoanhydrovitamin A was non-fluorescent and the irradiation product, in contrast to the isoanhydrovitamin A,

did not pass through an alumina column. The work of Chevallier and Duboulos (28, 29) also indicated the formation of a substance absorbing at about the same wavelengths by irradiation of vitamin A by a mercury arc.

Certain compounds related to vitamin A have been found to absorb at about the same wavelengths as the product of the GDH-vitamin A reaction. Shantz (203) prepared a compound which lacked only the terminal carbinol group of vitamin A and had absorption bands at 330, 348 and 367 mμ. Milas and associates (142) synthesized a compound that they called allo-vitamin A ethyl ether. This had the same absorption bands as the hydrocarbon prepared by Shantz. Karrer and Benz (95) synthesized axerophten which has the same structure as anhydrovitamin A except for hydrogenation of the terminal double bond. This compound exhibited absorption maxima at 331, 346 and 364 mμ. Karrer and Jucker (96) prepared a compound, $C_{20}H_{30}O_2$, which absorbed at 333.5, 350 and 367.5 mμ by treating vitamin A epoxide with chloroform containing a small amount of HCl. Embree and Shantz (52) found that subvitamin A formed an anhydro derivative which had ultraviolet absorption maxima at 332, 348 and 367 mμ.

An examination of Figure 36 shows that some substance resulting from the reaction of vitamin A with GDH for periods over 1 minute exhibits absorption maxima at 261, 272 and 282 mμ. The absorption at these bands increased as

the color reaction time increased, at least up to 8 minutes. The chemical nature of the substance responsible for these absorption bands is not known, but the substance may be similar to or identical with certain products reported by other workers. A decomposition product of vitamin A which absorbed at 275-285 mμ was reported by Morton and Heilbron (148). Karrer, et al. (101) separated a highly concentrated vitamin A into two fractions, the main one absorbing at 328 mμ and the other at 270 mμ. Pritchard and associates (181) found a biologically active material in a vitamin A concentrate which was insoluble in 83% methanol and possessed an absorption band at 285 to 290 mμ. Treatment of a vitamin A concentrate with ozonized oxygen rapidly destroyed vitamin A but compounds with absorption maxima at 290 and 272 mμ were detected in the early stages (48). LePage and Pett (109) found a substance, absorbing at 275 mμ, in blood and feces of human subjects after ingestion of large doses of vitamin A. This substance was related to an oxidation product of vitamin A obtained by peroxide oxidation of vitamin A in the nonsaponifiable fraction from fish liver oils.

Karrer and Jucker (96) synthesized vitamin A epoxide (absorption maximum at 275 mμ) by the reaction of monoperphthalic acid with pure vitamin A. A second oxidation product which absorbed at 339 mμ was also formed in the reaction. Troitskiĭ (230) proposed a structure for vitamin A

epoxide based on deductions made from the absorption maxima of known vitamin A members by considering the nature and number of double bonds and the position of the methyl hydroxyl and other groups. Vinet and Meunier (235) prepared a water-soluble compound by the oxidation of vitamin A with perphthalic acid. The ultraviolet absorption spectrum included maxima at 282 and 235 mμ. They pointed out that the compound probably contained a secondary hydroxyl group in the number ten position which divides the conjugated system into two unequal parts.

Embree and Shantz (52) reported a substance called subvitamin A which had an absorption maximum at 290 mμ. Apparently it is an oxygenated derivative of vitamin A occasionally found in the nonsaponifiable fraction from shark liver oil. Hawkins and Hunter (79) suggested that subvitamin A is probably the primary oxidation product of vitamin A where oxygen presumably attacks the double bond in the β-ionone ring.

Ultraviolet irradiation of vitamin A causes certain changes in the molecule that results in the formation of a product or products absorbing at wavelengths below 300 mμ. Sobotka and associates (217) reported the formation of a substance which absorbed at 275 mμ when vitamin A acetate was irradiated for 40 minutes. The photochemical destruction of the primary irradiation product, absorbing at 325,

346 and 364 mμ, was probably an oxidation reaction resulting in an oxidative degradation of the side chain to yield a substance with three conjugated double bonds with absorption around 275 mμ. Halpern (77) also described the formation of a substance absorbing at 270 mμ by destructive irradiation of vitamin A.

The formation of a product or products with principal absorption bands at 262, 272 and 283 mμ was observed spectrophotometrically as the GDH-vitamin A color reaction was in progress (see Figure 37). Solvent-reagent ratios of 1:5 and 5:1 were used in this study. The formation of the three maxima was more rapid in the first reaction mixture, and did not change to any marked extent over a 50-minute period. These maxima were slow in developing when the 5:1 ratio was employed, and were found to increase with decreasing absorption at the longer wavelengths.

As far as could be ascertained, no report has been made regarding the formation of a compound related to vitamin A with an absorption spectrum like the one indicated above. It is, however, conceivable that the peaks on either side of the 272 mμ band could have been missed in some of the studies of oxidation products of vitamin A.

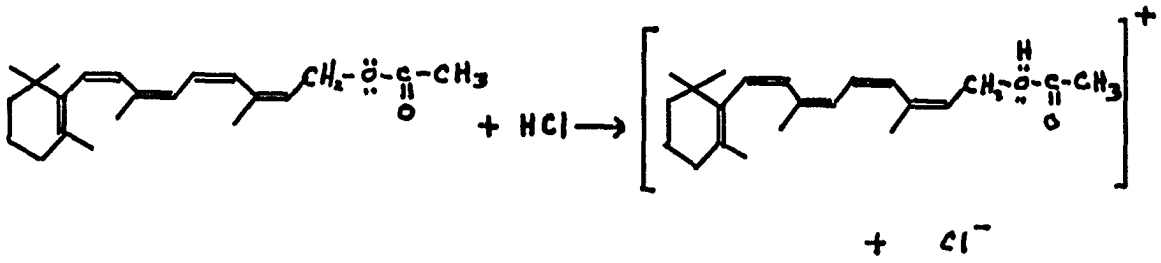
On the basis of the spectrophotometric findings and comparison of the data with the ultraviolet absorption spectra of certain compounds reported in the literature, the

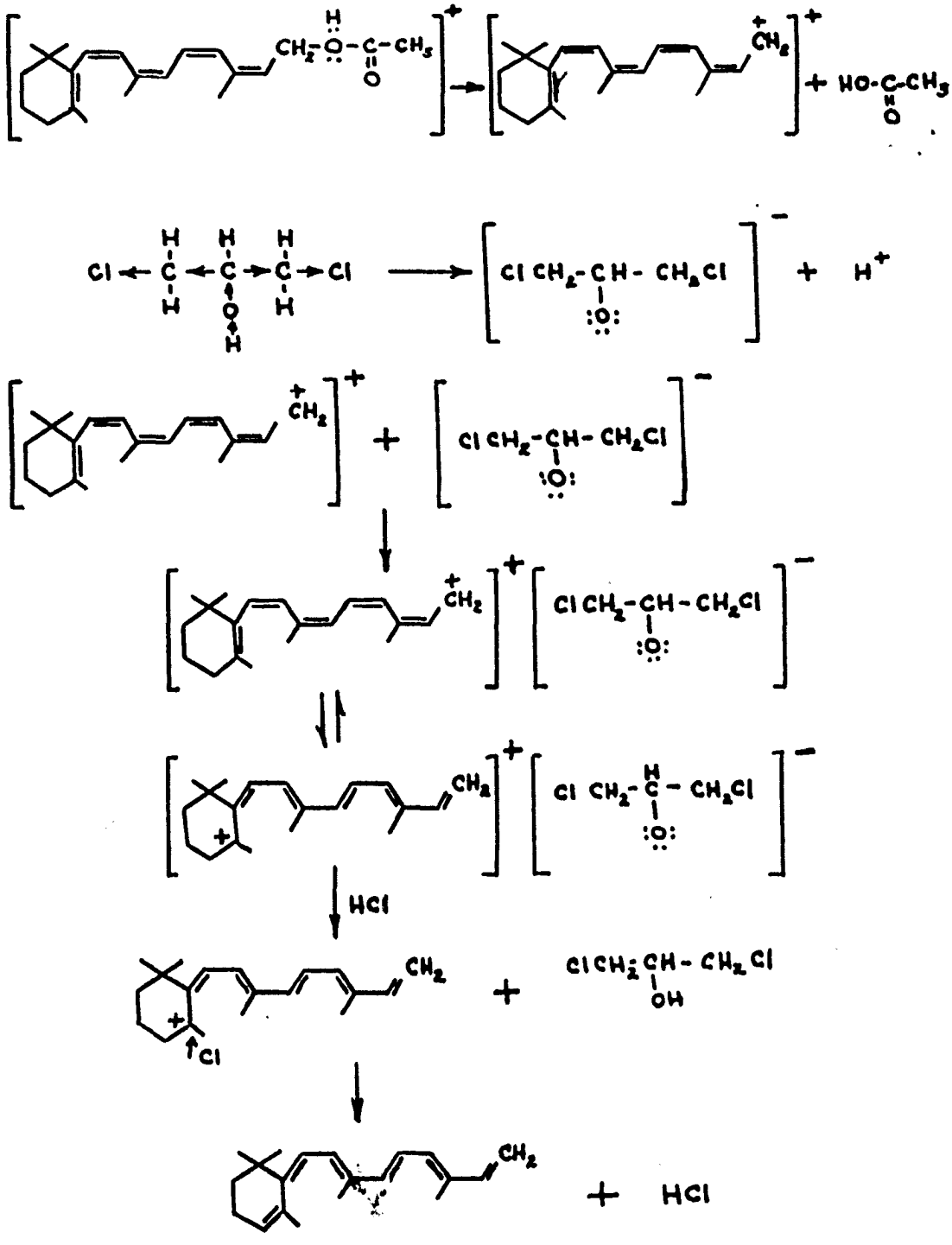
mechanism of the GDH-vitamin A reaction appears to involve a dehydration followed by oxidative degradation of the vitamin A side chain. As pointed out above, the initial step in the reaction appears to involve dehydration with the probable formation of anhydrovitamin A or at least something closely akin to it. As the reaction progresses the formation of isoanhydrovitamin A seems likely. On standing for longer periods of time, there appears to be some sort of oxidative degradation to form a compound with absorption maxima at 262, 272 and 283 mμ. The following scheme is suggested:

Vitamin A acetate ⟶ anhydrovitamin A ⟶ isoanhydrovitamin A ⟶ oxidative degradation products

The color probably results from a loose combination of GDH with anhydro- or isoanhydrovitamin A. As the color fades the formation of degradation products undoubtedly takes place.

The following mechanism which may possibly account for the color formation is suggested:





Some evidence for part of these steps has been published. Luder and Zuffanti (114) described the formation of acidic ions when esters react with H-acids. The work of Meunier (129) also indicates the formation of the vitamin A cation. The tendency for glycerol dichlorohydrin to ionize to some extent appears to be due to the inductive effect of the two chlorine atoms. The relative proton-escaping tendency of the link RO-H increases as the relative electron-attraction of the R group increases (93). The GDH anion would then be attracted by the vitamin A cation to form a colored complex. Further action of HCl could possibly regenerate GDH and finally result in the formation of anhydrovitamin A. Meunier and associates (134) have proposed a mechanism for the formation of anhydrovitamin A by the action of HCl. The structure of isoanhydrovitamin A has not yet been established. The nature of the final product of the GDH-vitamin A reaction is quite obscure but appears to be a product resulting from degradation of the vitamin A side chain.

Although the data reported here shed some light on the mechanism of the color reaction, further investigation will be required in order to completely establish the identity of the several products of the GDH-vitamin A reaction.

SUMMARY AND CONCLUSIONS

Pure glycerol dichlorohydrin gave no color test with vitamin A, but was activated by the addition of small amounts of certain inorganic acids, metallic and non-metallic halides, acyl halides and several other compounds. Compounds or solutions which were good activating agents included concentrated hydrochloric acid, concentrated sulfuric acid, chlorosulfonic acid, 60% perchloric acid, phosphorus trichloride, phosphorus oxychloride, aluminum chloride, arsenic trichloride and methyl sulfate. The hydrogen ion *per se* is not the sole activating agent since addition of nitric acid, acetic acid, monochloroacetic acid or trichloroacetic acid did not result in an activated reagent.

Good activation of GDH was produced by concentrations of anhydrous hydrogen chloride ranging from 0.002 to 0.08 N, with optimum activity at 0.01 N hydrogen chloride.

Good activation was produced by concentrations of chlorosulfonic acid ranging from 0.02 to 0.5% acid, with optimum activity at 0.1% chlorosulfonic acid.

The activity of GDH activated with either anhydrous HCl or chlorosulfonic acid changed on standing in the laboratory. Reagents originally showing optimum activity became less active, while those having excess or mere traces of acid became somewhat more active on standing at room temperature

and on exposure to laboratory light.

GDH activated by the addition of 1% concentrated sulfuric acid produced a blue color when first mixed with a chloroform solution of vitamin A. Within 5 or 6 minutes the blue changed to a fairly stable but not very intense red-violet color with maximum absorption at 545 mμ and a smaller absorption band at 452 mμ.

GDH was activated by heating up to 4 hours at 85°C. with concentrations of antimony trichloride ranging from 0.3 to 1%. Activity was greatest with the higher levels and longer heating periods. The ultraviolet absorption spectrum of GDH activated by heating with 0.3% antimony trichloride indicated a change in the reagent due to a reaction of antimony trichloride with GDH to produce a derivative containing antimony or a chemical bonding between the reactants.

Good activity was produced by vacuum distilling several brands of GDH with 1% antimony trichloride. No significant difference between brands was observed when solvent reagent ratios of 1:5 and 5:1 were employed. The activity of these reagents was equal to or better than GDH activated with either HCl or $ClSO_3H$. Apparently antimony trichloride reacted with GDH during the heating and distillation to produce HCl along with a distillate which contained antimony.

Glycerol dichlorohydrin activated by the addition of concentrated hydrochloric acid, chlorosulfonic acid, or by vacuum distillation with antimony trichloride, when present in excess, reacted with vitamin A acetate to produce a violet color which had a principal absorption maximum at 555 mμ and a small absorption band at 358 mμ. The stability of the color was determined by measuring the absorption spectrum at various time intervals after initiation of the color reaction. In each case the extinction coefficient at the 555 mμ maximum decreased on standing.

Antimony trichloride-activated GDH was deactivated by heating with activated charcoal, filtering, addition of a small quantity of aqueous KOH and finally vacuum distilling to give a clear, colorless reagent. On standing in a clear, glass-stoppered bottle for several weeks, this product became active due to decomposition, a process which was hastened by heating. Free chloride ion was found in this active reagent. It was postulated that hydrogen chloride was one of the decomposition products responsible for the activation of the GDH.

Glycerol α-monochlorohydrin gave weak color tests when activated by the same agents which activated GDH. The absorption spectrum of the color produced when vitamin A reacted with antimony trichloride-activated glycerol monochlorohydrin was not unlike that of the GDH-vitamin A color

except in magnitude. The monochlorohydrin color was only about half as intense as that produced by the GDH-vitamin A reaction when a 1:5 solvent-reagent ratio was used. At the 5:1 ratio the absorption at 373 mμ was nearly the same with both reagents. Vacuum distillation of 1,2,3-trichloropropane with 1% antimony trichloride failed to produce an active reagent. It is concluded that no glycerol derivative thus far tested is as effective as a vitamin A colorimetric reagent as glycerol dichlorohydrin. This evidence supports the view that a hydroxyl group and two chlorine atoms, each on separate carbon atoms of a propane skeleton are required for maximum color formation with vitamin A.

Solvent-reagent ratios of 1:9, 1:7, 1:5, 1:3, 1:1, 3:1, 5:1, 7:1, and 9:1 were tested. The extinction coefficients at 555 mμ were essentially the same when the 1:9, 1:7, 1:5 and 1:3 ratios were employed. As the ratios were increased above 1:3, the absorption at 555 mμ decreased while the absorption at 338, 353, 373, 397 and 422 mμ increased. These maxima in and near the ultraviolet possibly indicate the formation of anhydrovitamin A.

Pyridine, aniline, n-butylamine, aqueous ammonia and epichlorohydrin inhibit the GDH-vitamin A color reaction. Aqueous KOH, ethanol, water and dioxane had some inhibitory action. Apparently compounds that react readily with HCl prevent the GDH-vitamin A color reaction.

Activated GDH reacted with β-carotene to form a product which absorbed strongly at and apparently above 1000 mμ. Above 880 mμ the stability of the extinction coefficients decreased with increasing wavelength at which measurements were made.

A spectrophotometric study was made of the conversion of vitamin A acetate into anhydrovitamin A by the action of alcoholic hydrogen chloride. Absorption maxima occurred at 348, 367 and 388 mμ and an inflection at 333 mμ. The 367 mμ absorption band was the highest of the three principal bands and was the last to reach a maximum.

The GDH-vitamin A color reaction was readily quenched by adding ethanol. The ultraviolet absorption spectrum of the clear solution resulting when the reaction was quenched within 1 minute indicated the presence of a substance which absorbed at 335, 349 and 369 mμ. This absorption spectrum closely resembled that of isoanhydrovitamin A. When the color reaction was permitted to stand for several minutes before quenching, the ultraviolet absorption showed the formation of some sort of degradation product with absorption bands at 261, 272 and 282 mμ.

LITERATURE CITED

(1) Allen, R. S., Wise, G. H. and Jacobson, N. L. Occurrence in blood plasma from certain dairy calves of factors that interfere with the color reactions of activated glycerol dichlorohydrin with vitamin A and carotenoids. J. Dairy Sci., 32, 688 (1949).

(2) Antoniani, C., Federico, L. and Artom, A. Use of glycerol 1,3-dichlorohydrin as a reagent in the determination of vitamin A in oil concentrates. Olearia, 1948, 405. (Original not available for examination; abstracted in C. A., 42, 8244 (1948).

(3) Association of Vitamin Chemists, Inc. Methods of vitamin assay. New York, Interscience Publishers, Inc. 1947.

(4) Awapara, J., Mattson, F. H., Mehl, J. W. and Deuel, H. J. A new spectrophotometric method for the determination of vitamin A. Science, 104, 602 (1946).

(5) Basu, U. P. and Gupta, S. K. S. On the fortification of edible fat with vitamin A. J. Am. Chem. Soc., 70, 413 (1948).

(6) Battegay, M., Buser, H. and Schlager, E. Sur une acétine et un diglicide cristallisés. Compt. rend., 188, 796 (1929).

(7) Baumann, C. A. and Steenbock, H. The vaginal smear method of determining vitamin A. Science, 76, 417 (1932).

(8) Baxter, J. G. and Robeson, C. D. Crystalline vitamin A palmitate and vitamin A alcohol. Science, 92, 203 (1940).

(9) Baxter, J. G. and Robeson, C. D. Crystalline aliphatic esters of vitamin A. J. Am. Chem. Soc., 64, 2407 (1942).

(10) Baxter, J. G. and Robeson, C. D. Crystalline vitamin A. J. Am. Chem. Soc., 64, 2411 (1942).

(11) Benham, G. H. The antimony trichloride method for the determination of vitamin A. Can. J. Research, B22, 21 (1944).

(12) Bessey, O. A., Lowry, O. H., Brock, M. J. and Lopez, J. A. The determination of vitamin A and carotene in small quantities of blood serum. J. Biol. Chem., 166, 177 (1946).

(13) Bessey, O. A. and Wolback, S. B. Vitamin A, physiology and pathology. In The vitamins. p. 27-54. Chicago, American Medical Association. 1939.

(14) Boekestein, P. T. Antimony pentachloride as a reagent for vitamin A. Acta Brevia Neerland. Physiol., Pharmacol., Microbiol., 12, 89 (1942).

(15) Bolomey, R. Oxidative decomposition of vitamin A. I. Stability of vitamin A towards oxidation and irradiation. J. Biol. Chem., 169, 323 (1947).

(16) Boyer, P. D., Phillips, P. H., and Smith, J. K. The separation of vitamin A for the determination of vitamin A in blood plasma. J. Biol. Chem., 152, 445 (1944).

(17) Boyer, P. D., Spitzer, R., Jensen, C. and Phillips, P. H. Determination of vitamin A and carotene in milk. Ind. Eng. Chem., Anal. Ed., 16, 101 (1944).

(18) Brew, W. and Scott, M. B. Chemical determination of vitamin A in mixed feeds and feedstuffs. Ind. Eng. Chem., Anal. Ed., 18, 46 (1946).

(19) Brode, W. R. and Magill, M. A. A critical study of the antimony trichloride test for vitamin A. J. Biol. Chem., 92, 87 (1931).

(20) Caldwell, M. J. and Hughes, J. S. Changes in the absorption spectra due to aging of the Carr-Price reaction mixture with vitamin A and the common carotenoid pigments. J. Biol. Chem., 166, 565 (1946).

(21) Caldwell, M. J. and Hughes, J. S. Effect of temperature on fading of the Carr-Price colors of vitamin A and common carotenoid pigments. J. Biol. Chem., 170, 97 (1947).

(22) Caldwell, M. J. and Parrish, D. B. The effect of light on the stability of the Carr-Price color in the determination of vitamin A. J. Biol. Chem., 158, 181 (1945).

(23) Caldwell, M. J., Parrish, D. B. and Schrenk, W. G. The response of different photometers to the color produced by vitamin A and carotene with antimony trichloride. Trans. Kansas Acad. Sci., 49, 197 (1946).

(24) Campbell, J. A. Modified glycerol dichlorohydrin reaction for vitamin D_3. Anal. Chem., 20, 766 (1948).

(25) Carr, F. H. and Price, E. A. Colour reactions attributed to vitamin A. Biochem. J., 20, 497 (1926).

(26) Carreyett, R. A. Color reaction for vitamin A. Chemist and Druggist, 151, 44 (1949). (Original not available for examination; abstracted in Chem. Abstr. 43, 4978 (1948).)

(27) Castle, D. C., Gillam, A. E., Heilbron, I. M. and Thompson, H. W. Absorption experiments with vitamin A concentrates. Biochem. J., 28, 1702 (1934).

(28) Chevallier, A. and Dubouloz, P. Etude de la destruction photochemique de la vitamine A en milieu alcoolique. I. Réactions primaires. Bull. soc. chim. biol., 18, 703 (1936).

(29) Chevallier, A. and Dubouloz, P. Etude de la destruction photochimique de la vitamine A en milieu alcoolique. II. Réaction secondaire. Bull. soc. chim. biol., 18, 1115 (1936).

(30) Chilcote, M. E., Guerrant, N. B. and Ellenberger, H. A. Stability of vitamin A acetate under laboratory conditions. Anal. Chem. 21, 960 (1949).

(31) Claus, A. and Mercklin, H. Ueber die Unsetzung von Aluminumchloride mit Hydroxylverbindungen. Ber., 18, 2932 (1885).

(32) Cocking, T. T. and Price, E. A. The search for color reactions of vitamin A. Chemist and Druggist, 105, 246, 279 (1926). (Original not available for examination; abstracted in Chem. Abstr. 21, 121 (1927).

(33) Color tests for vitamin A. Nutrition Rev., 4, 107 (1946).

(34) Cooley, M. L., Christiansen, J. B. and Koehn, R. C. Vitamin A in mixed feeds. Anal. Chem., 21, 593 (1949).

(35) Cooley, M. L., Christiansen, J. B. and Schroeder, C. H. Chromatographic estimation of vitamin A in mixed feeds. Ind. Eng. Chem., Anal. Ed., 17, 689 (1945).

(36) Corbet, R. E., Geisinger, H. H. and Holmes, H. N. Substances which interfere with the antimony trichloride test for vitamin A. J. Biol. Chem., 100, 657 (1933).

(37) Coward, K. H. The relation of the growth response to dosage of vitamin A. Confirmation of a curve relating response to dose of vitamin A given. Biochem. J., 28, 865 (1934).

(38) Coward, K. H. The biological standardisation of the vitamins. 2d ed. Baltimore, The Williams and Wilkins Co. 1947.

(39) Coward, K. H., Cambden, M. R. and Lee, E. M. The determination of vitamin A by means of its influence on the vaginal contents of the rat. Biochem. J., 29, 2736 (1935).

(40) Coward, K. H., Dyer, F. J., Morton, R. A. and Gaddum, J. H. Determination of vitamin A in cod liver oils (a) biologically, (b) chemically, (c) physically, with a statistical examination of the results. Biochem. J., 25, 1102 (1931).

(41) Coward, K. H., Key, K. M., Dyer, F. J. and Morgan, B. G. E. The determination of vitamin A. Biochem. J., 24, 1952 (1930).

(42) Crews, S. K. and Cox, S. J. The relationship between the Carr-Price value and the 328 mμ absorption coefficient of preparations containing vitamin A. Analyst, 59, 85 (1934).

(43) Dann, W. J. Physico-chemical methods for the estimation of vitamin A. Biol. Symposia, 12, 13 (1947).

(44) Dann, W. J. and Evelyn, K. A. The determination of vitamin A with the photoelectric colorimeter. Biochem. J., 32, 1008 (1938).

(45) Drummond, J. C. The nomenclature of the so-called accessory food factors (vitamins). Biochem. J., 14, 660 (1920).

(46) Drummond, J. C. and Coward, K. H. Researches on the fat-soluble accessory factor (vitamin A): VI. Effect of heat and oxygen on the nutritive value of butter. Biochem. J., 14, 734 (1920).

(47) Drummond, J. C. and Morton, R. A. Observations on the assay of vitamin A. Biochem. J., 23, 785 (1929).

(48) Edisbury, J. R., Gillam, A. E., Heilbron, I. M. and Morton, R. A. Absorption spectra of substances derived from vitamin A. Biochem. J., 26, 1164 (1932).

(49) Ellenberger, H. A., Guerrant, N. B. and Chilcote, M. E. The new vitamin A reference standard and its use in evaluating the vitamin A potency of fish oils. J. Nutrition, 37, 185 (1949).

(50) Embree, N. D. The occurrence of cyclized vitamin A in fish liver oils. J. Biol. Chem., 128, 187 (1939).

(51) Embree, N. D. Physicochemical assay of vitamin A. Ind. Eng. Chem., Anal. Ed., 13, 144 (1941).

(52) Embree, N. D. and Shantz, E. M. A possible new member of the vitamins A_1 and A_2 group. J. Am. Chem. Soc., 65, 906 (1943).

(53) Embree, N. D. and Shantz, E. M. Kitol, a new provitamin A. J. Am. Chem. Soc., 65, 910 (1943).

(54) Emmerie, A. An inhibitor of the antimony trichloride test for vitamin A in cod liver oil. Nature, 101, 364 (1933).

(55) Emmerie, A., Eekelen, M. and Wolff, L. K. Vitamin A and the antimony chloride reaction. Nature, 128, 405 (1931).

(56) Ender, F. The reaction of fish-liver oils with antimony trichloride. Biochem. J., 26, 1118 (1932).

(57) Esme, A. Le contrôle chemique des bentonites par test coloré de la montmorillonite. Ann. chim. anal., 28, 31 (1946).

(58) Ettinger, R. and Sobel, A. E. Estimation of ergosterol with activated glycerol dichlorohydrin. Federation Proc., 8, 196 (1949).

(59) Evans, H. M. and Bishop, K. S. On the existence of a hitherto unrecognized dietary factor essential for reproduction. Science, 56, 650 (1922).

(60) Ewing, D. T., Vandenbelt, J. M., Emmett, A. D. and Bird, O. D. Spectrophotometric determination of vitamin A. Critical study of applicability to fish liver oils. Ind. Eng. Chem., Anal. Ed., 12, 639 (1940).

(61) Faucounier, A. and Sanson, J. Sur quelques produits engendrés par l'action du gaz chlorhydrique sur la glycerine. Bull. soc. chim. France, 48, 236 (1887).

(62) Fearon, W. R. A study of some biochemical color tests. III. Color tests associated with vitamin A. Biochem. J., 19, 888 (1925).

(63) Feinstein, L. Colorimetric determination of vitamin A. J. Biol. Chem., 159, 569 (1945).

(64) Foy, J. R. and Morgareide, K. Biological assay of vitamin A-liver storage test of Guggenheim and Kock. Anal. Chem., 20, 304 (1948).

(65) Gallup, W. D. and Hoefer, J. A. Determination of vitamin A in liver. Ind. Eng. Chem., Anal. Ed., 18, 288 (1946).

(66) Gibson, G. P. and Taylor, R. J. Dynamic method for observing the antimony chloride reaction with vitamin A and related substances. Analyst, 70, 449 (1945).

(67) Gillam, A. E. A modified spectrophotometric method for the assay of carotene and vitamin A in butter. Biochem. J., 28, 79 (1934).

(68) Gray, E. L. and Cawley, J. D. The state of vitamin A in the liver of the rat. II. The effect of feeding the vitamin over extended periods. J. Nutrition, 23, 301 (1942).

(69) Gray, E. L., Hickman, K. C. D. and Brown, E. F. The state of vitamin A in the liver of the rat after feeding various forms of the vitamin. J. Nutrition, 19, 39 (1940).

(70) Gridgeman, N. T. The estimation of vitamin A. London, Lever Brothers & Unilever Limited. 1944.

(71) Gridgeman, N. T. Recent work on the chemistry and estimation of vitamin A. I. Chemistry & Industry, 1947, 555.

(72) Gridgeman, N. T. Recent work on the chemistry and estimation of vitamin A. II. Chemistry & Industry, 1947, 574.

(73) Guerrant, N. B. Influence of age and of vitamin A intake on the storage of vitamin A in the liver of the rat. J. Nutrition, 37, 37 (1949).

(74) Guerrant, N. B., Chilcote, M. E., Ellenberger, H. A. and Dutcher, R. A. Vitamin A acetate as a vitamin A standard. Anal. Chem., 20, 465 (1948).

(75) Guggenheim, K. and Kock, W. A liver storage test for the assessment of vitamin A. Biochem. J., 38, 256, (1944).

(76) Gutzeit, G. Sur une réaction colorimetrique des vitastérines. Arch. sci. phys. et nat., 9, 155 (1927).

(77) Halpern, G. R. Spectrophotometric changes during oxidation of vitamin A oils. Ind. Eng. Chem., Anal. Ed., 18, 621 (1946).

(78) Hanze, A. R., Conger, T. W., Wise, E. C. and Weisblat, D. I. Crystalline vitamin A methyl ether. J. Am. Chem. Soc., 70, 1253 (1948).

(79) Hawkins, E. G. E. and Hunter, R. F. The 'cyclization' of vitamin A and allied compounds. Biochem. J., 38, 34 (1944).

(80) Heilbron, I. M. Recent developments in the vitamin A field. J. Chem. Soc., 1948, 386.

(81) Heilbron, I. M., Gillam, A. E. and Morton, R. A. Specificity in tests for vitamin A. A new conception of the chromogenic constituents of fresh and aged liver oils. Biochem. J., 25, 1352 (1931).

(82) Heilbron, I. M., Heslop, R. N., Morton, R. A., Rea, J. L. and Drummond, J. C. Characteristics of highly active vitamin A preparations. Biochem. J., 26, 1178 (1932).

(83) Heilbron, I. M., Jones, W. E. and Bacharach, A. L. The chemistry and physiology of vitamin A. Vitamins and Hormones, 2, 155 (1944).

(84) Heilbron, I. M., Morton, R. A. and Webster, E. T. The structure of vitamin A. Biochem. J., 26, 1194 (1932).

(85) Hickman, K. C. D. Molecular distillation. II. The state of the vitamins in certain fish liver oils. Ind. Eng. Chem., 29, 1107 (1937).

(86) Hoch, H. Micromethod for estimating vitamin A by the Carr-Price reaction. Biochem. J., 37, 425 (1943).

(87) Hoch, H. and Hoch, R. The state of vitamin A in human serum. Brit. J. Exp. Path., 27, 316 (1946).

(88) Holmes, H. N. and Corbet, R. E. The isolation of crystalline vitamin A. J. Am. Chem. Soc., 59, 2042 (1937).

(89) Isler, O., Huber, W., Ronco, A. and Kofler, M. Synthesis des Vitamin A. Helv. Chim. Acta, 30, 1911 (1947).

(90) Isler, O., Ronco, A., Guex, W., Hindley, N. E., Huber, W., Dialer, K. and Kofler, M. Über die Ester und Äther des synthetischen Vitamins A. Helv. Chim. Acta, 32, 489 (1949).

(91) Javillier, M. and Meunier, P. De récents progrès de la biochimie inspirés par la théorie électronique de la valence. Représentation et langage. Bull. soc. chim. biol., 29, 16 (1947).

(92) Johnson, A. W. Recent advances in science: organic chemistry. The synthesis of vitamin A and derived products. Science Progress, 36, 496 (1948).

(93) Johnson, J. R. Modern electronic concepts of valence. In Gilman, H. Organic chemistry, an advanced treatise. p. 1894. New York, N.Y., John Wiley & Sons, Inc. 1945.

(94) Jones, G. I., Sanford, F. B., McKee, L. G. and Miyauchi, D. T. Adaptation of the Beckman quartz spectrophotometer for measurement of vitamin A by the Carr-Price reaction. Anal. Chem., 19, 142 (1947).

(95) Karrer, P. and Benz, J. Zur Synthese des Axerophtens. II. Mitteilung. Helv. Chim. Acta, 32, 232 (1949).

(96) Karrer, P. and Jucker, E. Über Vitamin A-Epoxyd (Hepaxanthin) II. Helv. Chim. Acta, 30, 559 (1947).

(97) Karrer, P., Karanth, K. P. and Benz, J. Synthese des Iso-axerophtens. Helv. Chim. Acta, 32, 436 (1949).

(98) Karrer, P., Morf, R. and Schöpp, K. Zur Kenntnis des Vitamins-A aus Fischtranen. II. Helv. Chim. Acta, 14, 1431 (1931).

(99) Karrer, P., Morf, R. and Schöpp, K. Synthesis des Perhydrovitamins-A. Helv. Chim. Acta, 16, 557 (1933).

(100) Karrer, P. and Schwyzer, R. Überführung von Vitamin A in Anhydrovitamin A und ein Carotinoid, wahrscheinlich identisch mit β-carotin. Helv. Chim. Acta, 31, 1055 (1948).

(101) Karrer, R., Walker, O., Schopp, K. and Morf, R. Isomeric forms of carotene and the further purification of vitamin A. Nature, 132, 26 (1933).

(102) Kaser, M. and Stekol, J. A. A critical study of the Carr-Price reaction for the determination of β-carotene and vitamin A in biological materials. J. Lab. Clin. Med., 28, 904 (1943).

(103) Kimble, M. S. The photocolorimetric determination of vitamin A and carotene in human plasma. J. Lab. Clin. Med., 24, 1055 (1939).

(104) Kobayashi, K. and Yamamoto, K. The color reaction of the Japanese clays upon liver oils and vitamin A on the market. J. Soc. Chem. Ind., Japan, 27, 1060 (1924).

(105) Koch, W. and Kaplan, D. Simultaneous Carr-Price reaction for the determination of vitamin A. J. Biol. Chem., 173, 363 (1948).

(106) Kreider, H. R. Reaction of vitamin A with superfiltrol. Science, 101, 377 (1945).

(107) Lathbury, K. C. Vitamin A determination: Relation between the biological, chemical and physical methods of test. Biochem. J., 28, 2254 (1934).

(108) League of Nations, Health Organization: Report of the permanent commission on biological standardisation. p. 68-72. 3rd Session. Geneva, (1931).

(109) LePage, G. A. and Pett, L. B. Absorption experiments with vitamin A. J. Biol. Chem., 141, 747 (1941).

(110) Lesné, E. and Vagliano, M. Différenciation de la vitamine A et du facteur antirachitique. Compt. rend., 177, 711 (1923).

(111) Little, R. W. Destructive irradiation technique of spectrophotometric vitamin A assay. Ind. Eng. Chem., Anal. Ed., 16, 288 (1944).

(112) Lowman, A. A new reagent for vitamin A. Science, 101, 183 (1945).

(113) Lowry, O. H. and Bessey, O. A. The adaptation of the Beckman spectrophotometer to measurements on minute quantities of biological materials. J. Biol. Chem., 163, 633 (1946).

(114) Luder, W. F. and Zuffanti, S. The electronic theory of acids and bases. p. 120. New York, John Wiley & Sons, Inc. 1946.

(115) MacWalter, R. J. Some factors which affect the assay of vitamin A by the spectrographic method. Biochem. J., 28, 472 (1934).

(116) Mayer, G. G. and Sobotka, H. The color reaction of vitamin A on acid earth. Science, 101, 158 (1945).

(117) McCollum, E. V. and Davis, M. Necessity of certain lipins in the diet during growth. J. Biol. Chem., 15, 167 (1913).

(118) McCollum, E. V. and Davis, M. Observations on the isolation of the subatance in butterfat which exerts a stimulating influence on growth. J. Biol. Chem., 19, 245 (1914).

(119) McCollum, E. V. and Davis, M. Nutrition with purified food substances. J. Biol. Chem., 20, 641 (1915).

(120) McCollum, E. V. and Davis, M. The influence of certain vegetable fats on growth. J. Biol. Chem., 21, 179 (1915).

(121) McCollum, E. V. and Davis, M. The nature of dietary deficiencies of rice. J. Biol. Chem., 23, 181 (1915).

(122) McCollum, E. V. and Davis, M. The essential factors in the diet during growth. J. Biol. Chem., 23, 231 (1915).

(123) McCollum, E. V., Simmonds, N., Becker, J. E. and Shipley, P. G. Studies on experimental rickets. XXI. An experimental demonstration of the existence of a vitamin which promotes calcium deposition. J. Biol. Chem., 53, 293 (1922).

(124) McFarlan, R. L., Bates, P. K. and Merrill, E. C. Spectrographic characteristics of vitamin A materials. Ind. Eng. Chem., Anal. Ed., 12, 645 (1940).

(125) Medical Research Council. Vitamins: A survey of present knowledge. London, His majesty's Stationary Office. 1932.

(126) Mellanby, E. A further demonstration of the part played by accessory food factors in the aetiology of rickets. J. Physiol. (London), 52, 1iii (1919).

(127) Meunier, P. De l'action des argiles montmorillonites sur la vitamine A et les phénomènes de mésomérie dans le groupe des caroténoïdes. Compt. rend., 215, 470 (1942).

(128) Meunier, P. Chromatographie et mésomérie. Les rapports de la couleur d'halochromie avec la constitution dans le groupe des caroténoïdes et de la vitamine D. Compt. rend., 221, 64 (1945).

(129) Meunier, P. Chromatographie et mésomérie dans le groupe des caroténoïds; I. Méchanisme de la réaction de la vitamine A et du β-carotène au trichlorure d'antimoine. Bull. soc. chim., 13, 73 (1946).

(130) Meunier, P. Chromatographie et mésomérie dans le groupe des caroténoïdes; II. Relations entre la couleur d'halochromie et la constitution dans cette série chimique. Bull. soc. chim., 13, 77 (1946).

(131) Meunier, P. La configuration des caroténoïdes et l'intensité de leurs reactions d'halochromie. Compt. rend., 222, 1528 (1946).

(132) Meunier, P. Sur la véritable formule de l'axérophtène. Compt. rend., 227, 206 (1948).

(133) Meunier, P., Dulou, R. and Vinet, A. Sur la constitution de la vitamine A, dite cyclisée. Compt. rend., 216, 907 (1943).

(134) Meunier, P., Dulou, R. and Vinet, A. Sur les conditions de formation et la constitution de la vitamine A dite «cyclisée». Bull. soc. chim. biol., 25, 371 (1943).

(135) Meunier, P. and Raoul, Y. Étude cinétique de la reaction de Carr et Price. Applications à la recherche qualitative et quantitative des vitamines A. Bull. soc. chim. biol., 20, 861 (1938).

(136) Meunier, P. and Rauol, Y. Vitamines A et caroténoïdes. Étude cinétique de la reaction de Carr et Price. Compt. rend., 206, 1148 (1938).

(137) Meunier, P. and Rauol, Y. Le dosage comparé de la vitamine A par la réaction de Carr et Price et la spectrographie dans l'ultra-violet. Bull. soc. chim. biol., 25, 173 (1943).

(138) Meunier, P. and Vinet, A. L'argile montmorillonite, réactif de la vitamine A. Bull. soc. chim. biol., 25, 327 (1943).

(139) Meunier, P. and Vinet, A. Sur un nouveau pigment derive de l'axérophtol, l'éther biaxerophtylique. Compt. rend., 219, 141 (1944).

(140) Meunier, P. and Vinet, A. Sur la constitution de pigment obtenu par chromatographie de l'axérophtol (vitamine A). Bull. soc. chim. biol., 27, 186 (1945).

(141) Milas, N. A. The synthesis of vitamin A and related products. Vitamins and Hormones, 5, 1 (1947).

(142) Milas, N. A., Sakal, E., Plati, J. T., Rivers, J. T., Gladding, J. K., Grossi, F. X., Weiss, Z., Campbell, M. A. and Wright, H. F. Synthesis of products related to vitamin A. VI. The synthesis of biologically active vitamin A ethers. J. Am. Chem. Soc., 70, 1597 (1948).

(143) Moll, T., Dalmer, O., Dobeneck, P., Domagh, G. and Laquer, F. Über das Vitamin A-Konzentrate „Vogan" zugleich ein Beitrag zur Wertbestimmung von Vitamin A. Arch. exp. Path. Pharmakol., 170, 176 (1933).

(144) Moore, T. Vitamin A and carotene. V. The absence of the liver-oil vitamin A from carotene. VI. The conversion of carotene to vitamin A in vivo. Biochem. J., 24, 692 (1930).

(145) Morton, R. A. The use of 7-methylindole in the antimony trichloride colour test for vitamin A. Biochem. J., 26, 1197 (1932).

(146) Morton, R. A. The application of absorption spectra to the study of vitamins, hormones and coenzymes. 2d ed. London, Adams Hilger, Limited. 1942.

(147) Morton, R. A. and Heilbron, I. M. The absorption spectrum of vitamin A. Biochem. J., 22, 987 (1928).

(148) Morton, R. A. and Heilbron, I. M. The absorption spectrum of vitamin A. Nature, 122, 10 (1928).

(149) Morton, R. A. and Stubbs, A. L. A re-examination of halibut-liver oil. Relation between biological potency and ultraviolet absorption due to vitamin A. Biochem. J., 41, 525 (1947).

(150) Morton, R. A. and Stubbs, A. L. Studies in vitamin A. IV. Spectrophotometric determination of vitamin A in liver oils. Correction for irrelevant absorption. Biochem. J., 42, 195 (1948).

(151) Müller, P. B. Chromatographische Trennung von Vitamin A-Alkohol, Vitamin A-Ester und β-Carotin und ihre spektrophotometrische bzw. stufenphotometrische Bestimmung. Helv. Chim. Acta, 27, 443 (1944).

(152) Müller, P. B. Zur kolorimetrischen Bistimmung von Vitamin A, Vitamin D und β-Carotin unter besonderer Berücksichtigung des Vitamin D. Helv. Chim. Acta, 30, 1172 (1947).

(153) Munsell, H. E. Vitamin A, methods of assay and sources in food. In The vitamins. p. 87-109. Chicago, American Medical Association. 1939.

(154) Murley, W. R., Jacobson, N. L., Wise, G. H. and Allen, R. S. Filled milks for dairy calves. II. Comparative effects of various types of soybean oils and of butter oil on health, growth and certain blood constituents. J. Dairy Sci., 32, 609 (1949).

(155) Narod, M. and Verhagen, D. Preformed vitamin A in mixed feeds. Chemical estimation by chromatographic methods. Anal. Chem., 20, 627 (1948).

(156) Nassi. L. Una nuova reazione della vitamina A. Bull. Soc. Ital. Biol. sperim., 15, 1175 (1940).

(157) Neal, R. H., Haurand, C. H. and Luckmann, F. H. Determination of total vitamin A content of dairy butters. Ind. Eng. Chem., Anal. Ed., 13, 150 (1941).

(158) Neal, R. H. and Luckmann, F. H. Determination of vitamin A content of margarine. Spectrophotometric method. Ind. Eng. Chem., Anal. Ed., 16, 358 (1944).

(159) Neff, A. W., Parrish, D. B., Hughes, J. S. and Payne, L. F. The state of vitamin A in eggs. Arch. Biochem., 21, 315 (1949).

(160) Nelson, E. M. and DeWitt, J. B. Biological assay for vitamin A. Biol. Symposia, 12, 1 (1947).

(161) Nelson, E. M., Walker, R. and Jones, D. B. Determination of vitamin A by a preventative method. J. Biol. Chem., 92, vi (1931).

(162) Norris, E. R. and Church, A. E. A study of the antimony trichloride reaction for vitamin A. J. Biol. Chem., 85, 477 (1929-30).

(163) Norris, E. R. and Church, A. E. A study of the antimony trichloride reaction for vitamin A: II. The dilution curve of codliver oil with antimony trichloride reagent. J. Biol. Chem., 87, 139 (1930).

(164) Norris, E. R. and Danielson, I. S. Comparison of biological and colorimetric assays for vitamin A as applied to fish oils. J. Biol. Chem., 83, 469 (1929).

(165) Notevarp, O. and Weedon, H. W. Spectrographic studies on the antimony trichloride reaction for vitamin A. II. Influence of oxidizing agents on the reaction. Biochem. J., 32, 1054 (1938).

(166) Notevarp, O. and Weedon, H. W. Spectrographic studies on the antimony trichloride reaction for vitamin A. III. The relation of the spectral absorption of the blue solutions of oils to that of their concentrates. Biochem. J., 32, 1668 (1938).

(167) Osborne, T. B. and Mendel, L. B. The relation of growth to the chemical constituents of the diet. J. Biol. Chem., 15, 311 (1913).

(168) Osborne, T. B. and Mendel, L. B. The influence of cod liver oil and some other fats on growth. J. Biol. Chem., 17, 401 (1914).

(169) Osborne, T. B. and Mendel, L. B. Further observations of the influence of natural fats upon growth. J. Biol. Chem., 20, 379 (1915).

(170) Osborne, T. B. and Mendel, L. B. Nutritive factors in plant tissues: IV. Fat-soluble vitamine. J. Biol. Chem., 41, 549 (1920).

(171) Oser, B. L. Simplified form of Morton and Stubbs correction of vitamin A absorption curves. Anal. Chem., 21, 529 (1949).

(172) Oser, B. L., Melnick, D. and Pader, M. Chemical and physical determinations of vitamin A in fish liver oils. Ind. Eng. Chem., Anal. Ed., 15, 717 (1943).

(173) Oser, B. L., Melnick, D. and Pader, M. Estimation of vitamin A in food products. Ind. Eng. Chem., Anal. Ed., 15, 724 (1943).

(174) Oser, B. L., Melnick, D., Pader, M., Roth, R. and Oser, M. Determination of vitamin A. Correlation of improved spectrophotometric and colorimetric methods with multi-level bioassays. Ind. Eng. Chem., Anal. Ed., 17, 559 (1945).

(175) Pacini, A. E. and Taras, M. H. An improved color test for vitamin A. J. Am. Pharm. Assoc., 26, 721 (1937).

(176) Parrish, D. B., Wise, G. H. and Hughes, J. S. The state of vitamin A in colostrum and in milk. J. Biol. Chem., 167, 673 (1947).

(177) Parrish, D. B., Wise, G. H. and Hughes, J. S. Effect of vitamin A supplements upon the state of vitamin A in blood serum of the dairy cow and in blood serum and liver of its neonatal calf. J. Biol. Chem., 172, 355 (1948).

(178) Parrish, D. B., Wise, G. H. and Hughes, J. S. Vitamin A and carotenoids in the blood serum of dairy cattle. Anal. Chem., 20, 230 (1948).

(179) Penketh, D. D. Glycerol dichlorohydrin and vitamin A. Nature, 161, 893 (1948).

(180) Pharmacopoeia of the United States. 13th revision. p. 719. Easton, Pa., Mack Printing Company. 1947.

(181) Pritchard, H., Wilkenson, H., Edisbury, J. R. and Morton, R. A. A discrepancy between biological assays and other methods of determining vitamin A. II. Biochem. J., 31, 258 (1937).

(182) Pugsley, L. I., Wells, G. and Crandall, W. A. The biological assay of vitamin A by means of its influence on the cellular contents of the vagina of rats. J. Nutrition, 28, 365 (1944).

(183) Reed, G., Wise, E. E. and Frundt, R. J. L. Quantitative separation of alcohol and ester forms of vitamin A. Ind. Eng. Chem., Anal. Ed., 16, 509 (1944).

(184) Reti, L. Sur l'état de combinaision de la vitamine A dans les huiles de foie. Compt. rend. soc. biol., 120, 577 (1935).

(185) Robertson, E. C. Recent work on the tissue changes in vitamin A deficiency. Am. J. Med. Sci., 192, 409 (1936).

(186) Robin, E. D. Reaction of vitamin A with Liebermann-Burchard reagent. Science, 102, 17 (1945).

(187) Rosenberg, H. R. Chemistry and physiology of the vitamins. Revised reprint. New York, Interscience Publishers, Inc. 1945.

(188) Rosenheim, O. and Drummond, J. C. On the relation of the lipochrome pigments to the fat-soluble accessory food factor. Lancet, 1920, I. 862.

(189) Rosenheim, O. and Drummond, J. C. A delicate colour reaction for the presence of vitamin A. Biochem. J., 19, 753 (1925).

(190) Rosenheim, O. and Schuster, E. A new colorimeter based on the Lovibond color system, and its application to the testing of cod-liver oil, and other purposes. Biochem. J., 21, 1329 (1927).

(191) Rosenheim, O. and Webster, T. A. A critical study of color tests suggested for vitamin A. Lancet, 1926, II, 806.

(192) Rosenheim, O. and Webster, T. A. The nature of Fearon's colour reaction and its non-specificity for vitamin A. Biochem. J., 20, 1342 (1926).

(193) Rosenthal, E. and Erdélyi, J. A new color test for the determination of vitamin A. Biochem. J., 28, 41 (1934).

(194) Rosenthal, E. and Erdélyi, J. Weitere Untersuchungen über die neue Farbenreaktion des Vitamins A. Biochem. Z., 271, 414 (1934).

(195) Rosenthal, E. and Szilárd, C. A new method of determining the vitamin A content of blood. Biochem. J., 29, 1039 (1935).

(196) Rosenthal, J. and Weltner, M. Spectrophotometric investigation of a new vitamin A color test. Biochem. J., 29, 1036 (1935).

(197) Schaltegger, H. Sterine als ionoide Systeme. I. Über eine Vitamine D-Bestimmungsmethode auf Carbeniumsalzbasis. Helv. Chim. Acta, 29, 285 (1946).

(198) Schwarzkopf, O., Cahnmann, H. F., Lewis, A. D., Swidinsky, J. and Wüest, H. M. Zur Syntheses des Vitamins A. 1. Milleilung. Ein neue Methode zur direkten Darstellung von vitamin A von hoher biologischer Wirksamkeit. Helv. Chim. Acta, 32, 443 (1949).

(199) Servigne, M., Pinta, M. and Montgareuil, P. G. Sur le dosage de l'axérophtol dans les huiles. Bull. soc. chim. biol., 30, 454 (1948). (Original not available for examination; abstracted in C. A., 43, 3055 (1949).)

(200) Sherman, H. C. and Cammack, M. L. A quantitative study of the storage of vitamin A. J. Biol. Chem., 68, 69 (1926).

(201) Sherman, H. C. and Smith, S. L. The vitamins. 2d ed. New York, The Chemical Catalogue Company, Inc. 1931.

(202) Sherman, H. C. and Todhunter, E. N. The determination of vitamin A values by a method of single feedings. J. Nutrition, 8, 347 (1934).

(203) Shantz, E. M. Synthesis of compounds related to vitamin A from hydroxymethylene beta-ionone. J. Am. Chem. Soc., 68, 2553 (1946).

(204) Shantz, E. M. Isolation of pure vitamin A_2. Science, 108, 417 (1948).

(205) Shantz, E. M., Cawley, J. D. and Embree, N. D. Anhydro ("cyclized") vitamin A. J. Am. Chem. Soc., 65, 901 (1943).

(206) Smith, E. L. and Hazley, V. The reaction of antimony trichloride with cod-liver oil and its unsaponifiable fraction. Biochem. J., 24, 1942 (1930).

(207) Sobel, A. E. The Jewish Hospital of Brooklyn. (Personal Communication.) February 13, 1949.

(208) Sobel, A. E., Mayer, A. M. and Kramer, B. New colorimetric reaction of vitamins D_2 and D_3 and their provitamins. Ind. Eng. Chem., Anal. Ed., 17, 160 (1945).

(209) Sobel, A. E., Owades, P. S. and Owades, J. L. Sulfate esters as intermediates in the formation of 7-dehydrocholesterol and dicholesteryl ether. J. Am. Chem. Soc., 71, 1487 (1949).

(210) Sobel, A. E., Sherman, M., Lichtblau, J., Snow, S. and Kramer, B. Comparison of vitamin A liver storage following administration of vitamin A in oily and aqueous media. J. Nutrition, 35, 225 (1948).

(211) Sobel, A. E. and Snow, S. D. The estimation of serum vitamin A with activated glycerol dichlorohydrin. J. Biol. Chem., 171, 617 (1947).

(212) Sobel, A. E. and Werbin, H. Spectrophotometric study of a new colorimetric reaction of vitamin A. J. Biol. Chem., 159, 681 (1945).

(213) Sobel, A. E. and Werbin, H. Activated glycerol dichlorohydrin. A new colorimetric reagent for vitamin A. Ind. Eng. Chem., Anal. Ed., 18, 570 (1946).

(214) Sobel, A. E. and Werbin, H. Determination of vitamin A in fish liver oils with activated glycerol dichlorohydrin. Anal. Chem., 19, 107 (1947).

(215) Sobotka, H., Kann, S. and Loewenstein, E. The fluorescence of vitamin A. J. Am. Chem. Soc., 65, 1959 (1943).

(216) Sobotka, H., Kann, S. and Winternitz, W. Fluorophotometric analysis of vitamin A esters. J. Biol. Chem., 152, 635 (1944).

(217) Sobotka, H., Kann, S., Winternitz, W. and Brand, E. The fluorescence of vitamin A. II. Ultraviolet absorption of irradiated vitamin A. J. Am. Chem. Soc., 66, 1162 (1944).

(218) Societe des Nations. Rapport de la seconde conférence pour la standardisation des vitamins. I. Vitamine A. Bull. Trimestriel de L'Organisation D'Hygiene, 3, 453 (1934).

(219) Squibb, R. L. Effect of soybeans and soybean products on carotene and vitamin A utilization by dairy cows. Unpublished Ph.D. Thesis. Ames, Iowa, Iowa State College Library. 1948.

(220) Squibb, R. L., Cannon, C. Y. and Allen, R. S. Effect of raw soybeans and of soybean oil on plasma carotene and on vitamin A as measured by activated glycerol dichlorohydrin. J. Dairy Sci., 31, 421 (1948).

(221) Squibb, R. L., Cannon, C. Y. and Allen, R. S. Effect of raw soybeans and soybean oil meal on the vitamin A and carotene concentrations in blood plasma and milk of lactating cows. J. Dairy Sci., 32, 565 (1949).

(222) Stoehr, C. Ueber cyklische Aether mehrwerthiger alkohole. J. prakt. Chem., 55, 78 (1897).

(223) Sycheff, V. M. Extraction of oil and vitamin A in shark liver analysis. The xylene-centrifuge method. Ind. Eng. Chem., Anal. Ed., 16, 126 (1944).

(224) Takahashi, K. and Kawakami, K. Chemistry of vitamin A. I. Separation of the effective constituent of liver oil and its properties. Preliminary report. J. Chem. Soc. Japan, 44, 950 (1923). (Original not available for examination; abstracted in C. A., 20, 1653 (1926).)

(225) Takahashi, K., Nakamiya, Z., Kawakami, K. and Kitasato, T. On the physical and chemical properties of biosterin (a name given to fat-soluble A) and on its physiological significance. Sci. Papers Inst. Phys. Chem. Research (Tokyo), 3, 81 (1925).

(226) Takeda, K. On the color reaction of vitamin A. Trans. Tottori Soc. Agr. Sci., 2, 38 (1930),

(227) Tastaldi, H. Détermination photométrique de la vitamine A. Bull. soc. chim. biol., 29, 734, 742, 748 (1947).

(228) Tompkins, P. C. and Bolomey, R. A. Methylene chloride in the extraction and determination of vitamin A and oil in soupfin shark livers. Ind. Eng. Chem., Anal. Ed., 15, 437 (1943).

(229) Troitskiĭ, G. V. Spectroscopic study of the color reaction with antimony pentachloride on vitamin A and related compounds. Biokhimiya, 6, 3 (1941). (Original not available for examination; abstracted in C. A., 35, 6186 (1941).)

(230) Troitskiĭ, G. V. Relationship between structure and absorption spectra of members of the vitamin A group. Biokhimiya, 13, 7 (1948). (Original not available for examination; abstracted in C. A., 42, 8169 (1948).)

(231) Ueno, S., Ota, Y. and Ueda, Z. On the color reaction of vitamin A, vitamin D, vitamin E and some sterols. J. Soc. Chem. Ind. Japan, 38, Suppl. Binding 742b (1935).

(232) United States Pharmacopoeia Vitamin Advisory Board. U.S.P. vitamin A reference standard. Instructions for use. Letter 177, p. 547. 1947. (original not available for examination; cited in Ellenberger, H. A., Guerrant, N.B. and Chilcote, M. E. J. Nutrition, 37, 185 (1949).)

(233) Urban, F., Milder, B. and Carruthers, C. Microdetermination of vitamin A and carotenes. Biochem. J., 37, 295 (1943).

(234) Vecher, A. S. Colorimetric determination of vitamin A by adsorption on bentonite. Biokhimiya, 13, 501 (1948). (Original not available for examination: abstracted in C. A., 43, 3056 (1949).)

(235) Vinet, A. and Meunier, P. Oxydation et solubilisation dans l'eau de la vitamine A. Bull. chim. soc. biol., 29, 25 (1947).

(236) Vinet, A. G., Meunier, P., Jouanneteau, J. and Gourevitch, M. Sur les corps obtenus par action progressive de l'acide chlorhydrique sur la vitamine A et leur activé biologique. Compt. rend., 226, 128 (1948).

(237) Wall, M. E. and Kelley, E. G. Determination of vitamin A ester in fortified poultry mashes with activated glycerol dichlorohydrin. Anal. Chem., 20, 757 (1948).

(238) Wilkie, J. B. Report on vitamin A. The present status of the chromatographic spectrophotometric procedure for the estimation of vitamin A in margarine. J. Assoc. Offic. Agr. Chemists, 30, 382 (1947).

(239) Willimott, S. G. and Moore, T. Fearon's "pyrogallol" test as a possible basis for the estimation of vitamin A. Biochem. J., 20, 869 (1926).

(240) Willimott, S. G., Moore, T. and Wokes, F. Effects of various agents on colour tests for vitamin A. Biochem. J., 20, 1292 (1926).

(241) Willimott, S. G. and Wokes, F. Color tests for vitamin A. Their application to naturally occurring products. Lancet, 1927, II, 8.

(242) Wise, E. C. and Heyl, F. W. Vitamin A colorimetric and biological assay. J. Am. Pharm. Assoc., 21, 1142 (1932).

(243) Wokes, F. and Willimott, S. G. A study of antimony trichloride as a possible quantitative reagent for vitamin A. Analyst, 52, 515 (1927).

(244) Yang, S. P. Absorption of vitamin A and carotene by dairy calves. II. Effect of dispersion. Unpublished M. S. Thesis, Ames, Iowa, Iowa State College Library. 1949.

(245) Zechmeister, L. and Sandoval, A. The coloration given by vitamin A and other polyenes on acid earths. Science, 101, 585 (1945).

(246) Zscheile, F. P. and Henry, R. L. Ultraviolet absorption of vitamin A in various solvents. Ind. Eng. Chem., Anal. Ed., 14, 422 (1942).

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