A COLORIMETRIC METHOD FOR THE DETERMINATION OF CERTAIN KETO ACIDS

by

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INTRODUCTION

The colorimetric method for the determination of keto acids described in this paper is based on an observation made while studying the formation of hydroxamic acids from amino acids and hydroxylamine in the presence of extracts of microorganisms. The method of LIPMANN AND TUTTLE[1] had been modified to give greater sensitivity by the use of less acid conditions during color development with $FeCl_3$. Instead of the purple color usually obtained with hydroxamic acids, a bright yellow was formed after the incubation of L-serine with cell-free extracts. It was found that the unexpected color resulted from pyruvic acid which had been produced by the action of serine dehydrase. Similar results were obtained with L-threonine, presumably due to the formation of α-ketobutyric acid. With the acid reagents (final pH about 0.4) in the method of LIPMANN AND TUTTLE, no significant differences are visible between a reagent blank and samples containing keto acids. In fact, these authors were able to use large amounts of pyruvate, oxaloacetate and α-ketoglutarate in their studies on acylphosphate formation without interference by the keto acids in the colorimetric method for hydroxamic acids. Moreover, any slight differences in yellow color which might have existed in the presence of keto acids probably would not have been detected at 540 mμ, the wavelength used for measuring the purple color obtained with hydroxamic acids.

The procedure presented here is not so sensitive or specific as other methods for keto-acid determination[2-5], but adds a relatively simple auxiliary test to the existing ones. It may be used for the determination of hydroxamic acids, certain keto acids, and could probably be adapted to the estimation of other substances which produce colored complexes with $FeCl_3$.

MATERIALS AND METHODS

Colorimeter readings were made with a Klett-Summerson Photoelectric Colorimeter with a Klett-Summerson #42 (420 mμ) or #54 (540 mμ) filter. pH was measured with a Beckman, Model G, pH meter. Absorption spectra were plotted automatically by a Cary Recording Spectrophotometer.

A 6% solution (w/v) of trichloroacetic acid is stored in the cold to minimize decomposition. A solution of 20% (w/v) $FeCl_3 \cdot 6H_2O$ in 0.01 *M* HCl, and one of 1 *M* glycine in 1 *M* NaCl may be kept at room temperature. The $FeCl_3$ reagent is prepared by mixing 1 volume of the stock $FeCl_3$ with 0.5 volume of the glycine-NaCl solution and diluting to 10 volumes with distilled water. The pH should be about 2.0. The $FeCl_3$ reagent may be kept for a few days at room temperature, or for several weeks when refrigerated. After several days at about 25°, an opalescence appears in the reagent and eventually a precipitation will occur. It is best not to use the

References p. 128.

reagent after it becomes opalescent. A stock solution of 4 *M* $NH_2OH \cdot HCl$ may be stored for several weeks in the cold. It is diluted to 0.5 *M* with water just before use. Since the pH of color development is very important in the method, it is best to adjust all samples, including standards, to about the same concentrations of trichloroacetic acid and buffer, if a buffer is used in studying an enzymic reaction. For the data reported here, tris (hydroxymethyl) aminomethane buffer (Tris), pH 8.5 was used. Many other buffers of different pH may be substituted without exceeding the limits of the final pH range recommended for color development (pH 1.4–1.6). Some of these buffers are listed in the section on *Specificity*. Buffers such as citrate and tartrate are to be avoided since they form yellow chelates with $FeCl_3$. Inorganic pyrophosphate cannot be used since it forms a precipitate with $FeCl_3$. Orthophosphate is not suitable as a buffer since 50 μmoles reduce color production about 50%. The latter might be used in biochemical studies if the phosphate were removed as suggested by LIPMANN AND TUTTLE[1]. For the following studies, 0.01 *M* sodium pyruvate (reagent grade, Nutritional Biochemicals Corp.) was used.

Depending on the order of addition of the reagents and time of contact with hydroxylamine, different colors are obtained. When the $FeCl_3$ reagent is added to the acidified sample before or within 2 min after the addition of hydroxylamine, an amber color results. When the hydroxylamine is added to the pyruvate sample 5 min to 1 hour before the $FeCl_3$ reagent, a bright yellow color is obtained. The maximum absorption is about the same whether the hydroxylamine is in contact with the pyruvate at pH 6.5 or pH 1. It is, therefore, possible to have the hydroxylamine present as a trapping agent for keto acids during an enzymic reaction, or to omit it until after the addition of trichloroacetic acid. Whether the hydroxylamine is added before or after the $FeCl_3$, the absorption, as measured with a 420 mμ filter, is fairly stable for an hour or two after color development. There is usually no significant decrease in intensity for 1 hour, while at other times there may be a 10% loss in about 2 hours. The resulting amber or yellow solutions eventually take on a pinkish hue. These relationships are indicated in Figs. 1 and 2. In each case curve 1 represents the initial color, and curve 2 that obtained 24 hours later. Within 24 hours a marked decrease in absorption due to the decreased yellow color occurs at about 400 mμ, while an increase due to a pink component may be seen in the region of 500 mμ. The method in which hydroxylamine is added 5 to 10 min before the $FeCl_3$ results in somewhat higher readings at 420 mμ which become maximal within a minute after the addition of the $FeCl_3$. When the $FeCl_3$ is added before or shortly after the hydroxylamine, maximal absorption is delayed for about 10 min. The time of contact with hydroxylamine necessary for maximal color formation varies with the keto acid (see *Specificity*).

The following procedure was used for most of the results reported here. To 2 ml of a solution containing 0 to 4 μmoles of pyruvate, 0 to 100 μmoles of buffer and 3% trichloroacetic acid, is added 0.1 ml of 0.5 *M* $NH_2OH \cdot HCl$. After 5 to 10 min, 4 ml of the $FeCl_3$ reagent are added

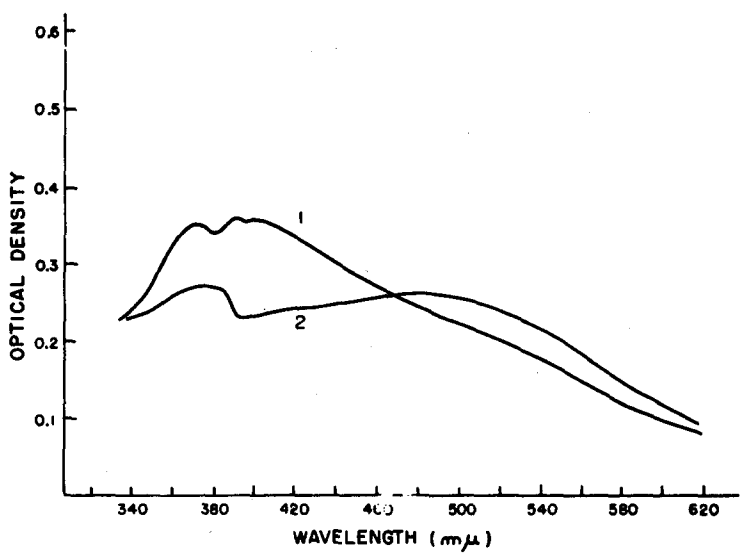

Fig. 1. Absorption spectra of color obtained with 2 μmoles of pyruvate. $FeCl_3$ reagent added just before the hydroxylamine. Curve 1, 30 min after the addition of hydroxylamine. Curve 2, 24 hours later.

References p. 128.

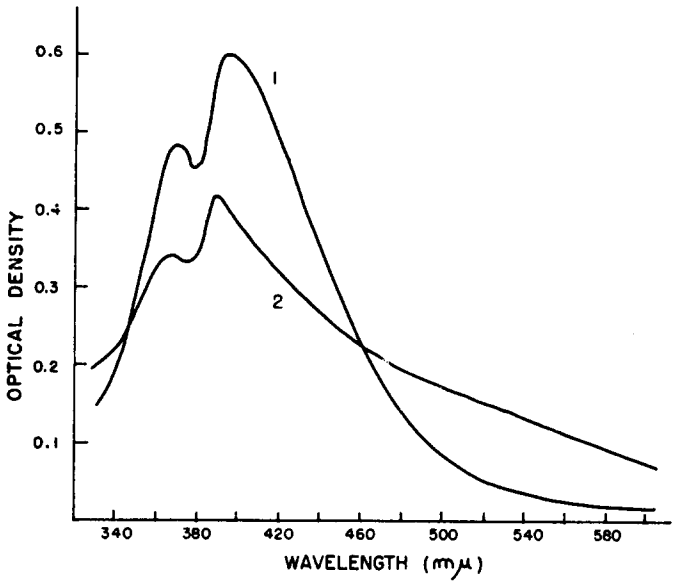

Fig. 2. Absorption spectra of color obtained with 2 μmoles of pyruvate. $FeCl_3$ reagent added 10 min after hydroxylamine. Curve 1, 40 min after the addition of $FeCl_3$. Curve 2, 24 hours later.

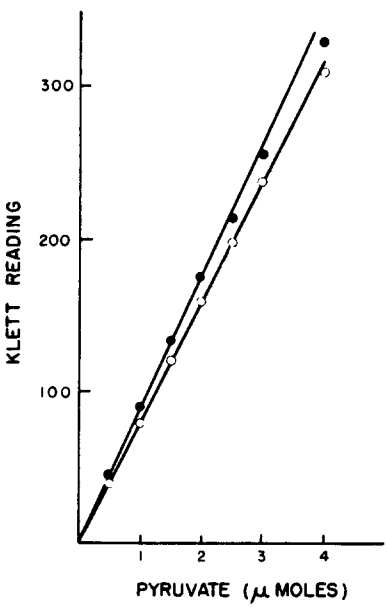

Fig. 3. Proportionality of color intensity with pyruvate concentration. Readings were made with a 420 mμ filter 30 min after the onset of color development. The reaction mixture included 50 μmoles of Tris buffer and the final pH was about 1.4. Upper line was obtained with samples treated with hydroxylamine for 10 min before the addition of $FeCl_3$. Lower line represents samples to which $FeCl_3$ was added before the addition of hydroxylamine.

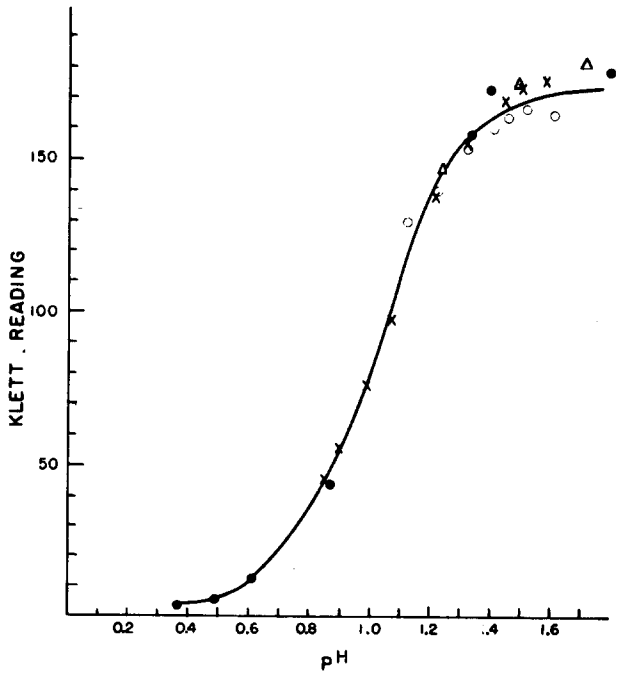

Fig. 4. Effect of pH on the color intensity obtained with 2 μmoles of pyruvate. Readings were made with a 420 mμ filter with reagent blanks at each pH as reference solutions. Each type of symbol represents a separate set of data obtained under slightly varied conditions.

References p. 128.

and the solutions are read with a 420 mμ filter against a reagent blank containing all components except pyruvate. With samples containing precipitated protein, an aliquot of the clear supernatant solutions may be used for assay, but it is not necessary to remove the protein until after color development. In the latter case, bubbles of nitrogenous gases resulting from the decomposition of hydroxylamine may tend to dislodge the pellet of protein after centrifugation. This is not likely to occur with the 50 μmoles of hydroxylamine recommended, but does occur with quantities of about 200 μmoles, especially when the room temperature is around 30–35°. This difficulty may be avoided by developing color with protein-free aliquots from the enzyme reaction, by decanting the developed supernatant fluids immediately after centrifugation, by cooling the tips of the centrifuge tubes containing the protein pellets in an ice bath before decanting into the colorimeter tube, or by de-gassing the solutions *in vacuo* before centrifuging. A de-gassing procedure was recommended by KAYE AND KENT for preventing interference from bubble formation in the colorimeter tubes under similar circumstances[6]. When the colorimeter tubes are clean, there is no difficulty from gas bubbles adhering to the sides of the tubes with the present method.

RESULTS

Proportionality

Fig. 3 shows the relationship between pyruvate concentration and color intensity as measured with a 420 mμ filter. The data represent Klett readings made 30 minutes after the onset of color development. One hour later the readings had decreased only 2 to 5%. For the upper line, hydroxylamine was allowed to react with the pyruvate for 10 minutes before the addition of $FeCl_3$. Deviation from linearity amounted to about 3% at 2 μmoles and 8% at 4 μmoles of pyruvate. For the lower line, $FeCl_3$ was added just before the addition of hydroxylamine. Deviation from linearity was only 3% at the level of 4 μmoles in the latter case.

Effect of pH

The influence of pH on the yellow color obtained with 2 μmoles of pyruvate is shown in Fig. 4. The data are taken from four experiments which were carried out under slightly varied conditions. Failure to obtain any visible color at the lower pH values is not merely due to an effect on the reactivity of pyruvate and hydroxylamine since no significant color was produced when treatment with hydroxylamine was carried out at pH 3.2 prior to acidification and addition of $FeCl_3$. Similarly, the purple color obtained with preformed succinohydroxamic acid is 50% more intense (540 mμ filter) when the pH is increased from about 0.4 in the method of LIPMANN AND TUTTLE[1] to a pH of 1.2 to 1.5 without decrease in stability. Others have also observed the effect of pH on the hydroxamic acid-$FeCl_3$ color reaction[6,7].

In the pH range of 1.4 to 1.6, the readings vary less than 10 Klett scale units for any given set of samples, and the working range could probably be extended upward to about pH 1.7. Glycine buffer was added to the $FeCl_3$ reagent in order to simplify the maintenance of the pH within this range. When the equivalent of 1 ml of 6% trichloroacetic acid is used per sample, the amount of buffer added from a buffered enzyme reaction and the amount of $NH_2OH \cdot HCl$ added may be varied from 0 to 100 μmoles each without exceeding the limits of pH 1.4 to 1.6. More than 4 mg of protein may be denatured by 1 ml of 6% trichloroacetic acid without affecting the readings obtained with added pyruvic acid. However, when aliquots of a sample contain less than the equivalent of 1 ml of 6% trichloroacetic acid, it is necessary to make up the deficiency with an appropriate amount of acid. The use of a pH less than 1.4 results in lower readings, but, so long as the pH values of the standard and unknown samples are the same, stability and proportionality will be maintained.

References p. 128.

In the absence of glycine, the reagent blanks have a decreasing absorption at 420 mμ with increasing pH up to about pH 1.6. In the presence of glycine, the blank absorption increases with increasing pH (see Table I) and becomes rather high for practical usage above pH 1.8.

When the reagent blanks and pyruvate samples are read at 420 mμ against water as the reference solution, it is evident that the colors are not stable. This instability increases with increasing pH and reaches a plateau at about pH 1.3. In spite of this instability, the *difference* in reading at 420 mμ between a reagent blank and pyruvate sample becomes maximal in about 10 to 15 minutes and is fairly stable for about an hour. These relationships are shown in Table I. The values for the reagent blanks were unusually high in this experiment, the readings ordinarily being about 200 to 250.

TABLE I

EFFECT OF pH ON THE INTENSITY AND STABILITY OF COLOR IN PYRUVATE SAMPLES (2 μmoles) AND REAGENT BLANKS

pH	Sample	Time of reading*						
		3	6	10	15	20	30	60
1.12	Pyruvate	382	387	385	382	376	370	356
	Blank	275	266	256	252	248	242	236
	Δ	107	121	129	130	128	128	120
1.22	Pyruvate	407	407	405	395	389	377	365
	Blank	288	274	266	255	251	243	234
	Δ	119	133	139	140	138	134	131
1.31	Pyruvate	435	435	430	420	411	400	385
	Blank	298	286	276	266	259	249	238
	Δ	137	149	154	154	152	151	147
1.45	Pyruvate	463	462	452	443	435	423	404
	Blank	310	300	290	279	273	263	248
	Δ	153	162	162	164	162	160	156
1.60	Pyruvate	500	493	480	470	463	453	439
	Blank	337	327	315	305	298	288	274
	Δ	163	166	165	165	165	165	165

* Times refer to minutes after the addition of the $FeCl_3$ reagent. Readings are Klett-Summerson colorimeter scale units obtained with a 420 mμ filter, with water as the reference solution. The reaction mixtures contained 40 μmoles hydroxylamine and varying amounts of trichloroacetic acid.

Effect of $FeCl_3$ concentration

Fig. 5 shows the effect of varying the concentration of $FeCl_3$ in a reaction mixture containing 2 μmoles of pyruvate. The pH varied only from 1.3 at the lowest concentration of $FeCl_3$ used, to 1.2 at the highest concentration, and, therefore, cannot account for the effects on color intensity. Maximal color production occurred when the $FeCl_3$ was 1.5% to 3% in the 4 ml of reagent used. The value of 2% was selected for the reagent.

References p. 128.

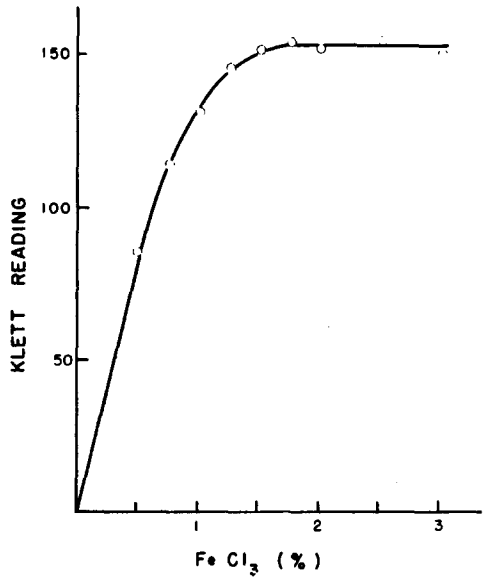

Fig. 5. Effect of concentration of $FeCl_3$ on the color intensity obtained with 2 μmoles of pyruvate. 4 ml of $FeCl_3$ reagent of the percentage indicated was used in a total volume of 6.1 ml containing 100 μmoles hydroxylamine and 1 ml of 6% trichloroacetic acid. An appropriate reagent blank was used at each concentration of $FeCl_3$.

Effect of hydroxylamine concentration

The effect of hydroxylamine concentration on the color intensity and stability obtained with 2 μmoles of pyruvate is given in Fig. 6. Maximum color formation occurs with about 20 to 40 μmoles of hydroxylamine. With 10 to 20 μmoles, the rate of color formation is somewhat delayed, while with more than 100 μmoles, the color becomes noticeably less stable. Since the pH varied only from 1.2 at the lowest concentration to 1.3 at the highest concentration of hydroxylamine, the effects were not due to pH differences. The reagent blank absorption at 420 mμ decreases with increasing concentration of hydroxylamine. With concentrations of hydroxylamine of about 200 μmoles or more per 6 ml of reaction mixture, the tendency to form bubbles increases.

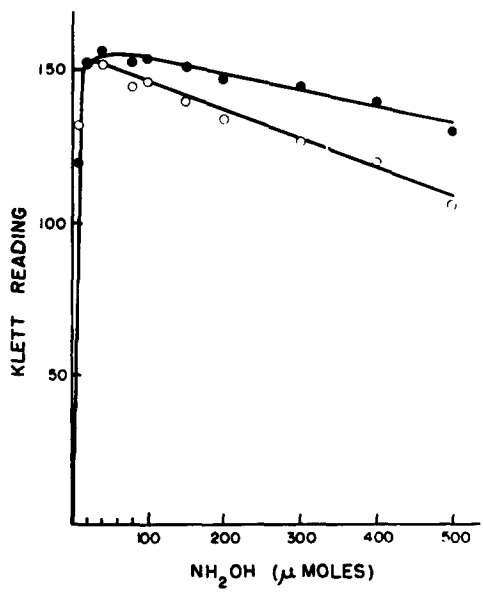

Fig. 6. Effect of hydroxylamine concentration on the color intensity obtained with 2 μmoles of pyruvate (420 mμ filter). The upper curve was determined about 10 min after the addition of $FeCl_3$ and the lower curve about 20 min later. The pH was about 1.2 with the lowest and about 1.3 with the highest concentration of hydroxylamine. Each sample was read against its appropriate reagent blank and the volumes were 6 ml.

References p. 128.

From a consideration of the above data, it was decided to use 50 μmoles of hydroxylamine for color development.

Specificity

The method is not specific. Oximes of the keto acids which react to give the yellow color would, of course, also produce the chromophores. Some phenolic and enolic substances, as well as hydroxamic acids, react with $FeCl_3$ to give colored chelates. Hydroxyacids often form yellow complexes with Fe^{+3}. Many of these substances are not usually present in biological preparations or may be present in amounts too small to be of significance. Some reactive substances produce colors other than yellow or form strongly colored complexes with $FeCl_3$ in the absence of hydroxylamine. Such substances may be distinguished from reactive α-keto acids. However, not all α-keto acids react under the conditions of this method to give significantly colored solutions.

Some substances which give no color when 10 μmoles, or more, are present, and also do not affect color production with pyruvate, include the following: glucose, fructose, acetone, Tris, pyridine, borate, veronal, maleate, glycylglycine, serine, and threonine. Other substances which produce no color at these levels of concentration, but have not been tested for their effect on pyruvate color development are: acetate, glutamate, aspartate, glutamine, asparagine, alanine, phenylalanine, arginine, leucine, fumarate, glutarate, succinate, methyl ethyl ketone, benzaldehyde, meta cresol, and levulinic acid (γ-ketovaleric acid). No color is obtained with 2 μmoles of formate.

Substances which have been tested and have been found to produce a chromophore in the present method are listed in Table II. The first six α-keto acids (1–6) show some absorption at 420 mμ in the absence of hydroxylamine, but produce a much more intense and relatively stable yellow color in its presence. In the absence of hydroxylamine, the colors obtained with phenylpyruvate and oxaloacetate are unstable, the former having an initial deep green color (reading of about 100) which rapidly fades to a pale yellow with the absorption decreasing to 20 within 1 hour. Oxaloacetate changes from a yellow-amber (reading = 40) to a pale yellow (reading = 14) in 1 hour. α-Ketobutyrate produces a gray coloration in the absence of hydroxylamine. The next 3 α-keto acids (7–9) form very little color in the presence or absence of hydroxylamine and their intensities are not affected by treatment with hydroxylamine for 10 to 30 min before the addition of the $FeCl_3$ reagent. Compounds 1, 4 and 6 give maximal absorption after treatment with hydroxylamine for 10 min, or less. α-Ketoglutarate requires about 20 min with hydroxylamine before $FeCl_3$ is added. Phenylpyruvate gives almost maximal color after 30 min of treatment with hydroxylamine, but the initial green coloration obtained upon addition of $FeCl_3$ does not occur and the resultant yellow is more stable when treatment with hydroxylamine is extended to 1 hour. In the case of the keto-analog of *iso*leucine (5), the same maximum reading (about 142) is attained whether the treatment with hydroxylamine is for 10 min or 1 hour before the addition of $FeCl_3$, but a longer time is required to reach maximal color development when the pretreatment with hydroxylamine is for 10 min.

Compounds No. 10, 11, 16, 17, 18 and 24 have relatively weak absorptions which are *lower* in the presence of hydroxylamine than in its absence. The color obtained with β-ketoglutarate (24) (acetone dicarboxylic acid) in the absence of hydroxylamine is quite unstable. When treated with hydroxylamine for 10 min before the addition

References p. 128.

TABLE II

SOME SUBSTANCES WHICH PRODUCE COLOR WITH $FeCl_3$ UNDER CONDITIONS OF THE METHOD

No.	Substance	μmoles	Klett reading (420 mμ) after 20 min color development	
			Without NH_4OH	With NH_4OH
1	Pyruvate	2	17	177
2	Phenylpyruvate	2	34	195
3	α-Ketoglutarate	2	22	174
4	α-Ketobutyrate	2	30	198
5	α-Keto-β-methylvalerate	2	7	132
6	Oxaloacetate	2	17	107
7	Acetopyruvate	2	11	10
8	Trimethylpyruvate	2	(32)*	17
9	Glyoxylate	2	(83)*	43
10	Acetoacetate**	10	98	52
11	Acetoacetic ester	10	23	11
12	Acetylacetone	1	67	14
13	Formaldehyde	1	1	206
14	Acetaldehyde	10	0	11
15	Glyceraldehyde	10	0	65
16	Glycolate	10	33	20
17	Malate	10	70	60
18	Lactate	10	105	70
19	Citrate	10	105	111
20	Tartrate	10	154	144
21	Resorcinol	10	11	19
22	Catechol	1	34	219
23	Succinohydroxamate	2	—***	105
24	β-Ketoglutarate	2	55	40

* Value obtained with 10 μmoles of substance.

** Prepared by saponification of a dilute solution of the ethyl ester for 24 hours at 25° with a slight excess of NaOH.

*** Succinohydroxamate prepared from succinic anhydride and an excess of hydroxylamine without isolating the product[1].

of $FeCl_3$ an amber color is formed. If the time of pretreatment with hydroxylamine is increased up to 1 hour, the color produced by the addition of $FeCl_3$ becomes purple. In any case, the colors are much less stable than that obtained with α-ketoglutarate. Acetylacetone also produces a pale color with $FeCl_3$ in the presence of hydroxylamine, but gives a fairly intense pink color (which absorbs even more significantly at about 500 mμ)[8] when hydroxylamine is omitted.

Acetaldehyde gives very little color in the method. Formaldehyde, however, produces a strong pinkish-amber color which absorbs very significantly with a 420 mμ filter and even more with a 540 mμ filter. A fresh solution of glyceraldehyde gives a rather weak yellow-amber color after 20 min which increases in intensity with time of reaction and becomes rather pink. The color production increases with increasing pH, and more than doubles during the first 30 minutes of color development when the pH is raised from 1.0 to 1.4. Solutions of glyceraldehyde which have been stored at about 5° for a week, or longer, produce more intense colors. It may be seen in Fig. 7 that the absorption spectra of succinohydroxamate and glyceraldehyde (after about 3 hours of color development with the latter) are very similar;

References p. 128.

they have higher values at 540 mμ than at 420 mμ, with maxima at 490–500 mμ.

Citrate and tartrate produce fairly strong and stable yellow colors which are about the same with or without hydroxylamine. The spectrum of the citrate complex in the absence of hydroxylamine is also given in Fig. 7. A very similar curve is obtained with stronger maxima in the presence of hydroxylamine. These spectra resemble that obtained with pyruvate (curve 1, Fig. 2) except that the latter has a larger molecular absorption.

Among phenolic substances, resorcinol gives very little color in the method. Catechol produces a strong pink-amber coloration which has less absorption at 540 mμ, however, than that given by an equivalent amount of formaldehyde.

The absorptions at 420 mμ produced by mixtures of 2 μmoles of pyruvate with 2 μmoles of succinohydroxamate, or with 10 μmoles of acetaldehyde or glyceraldehyde, are the same as the sums of the absorptions of the substances when tested alone.

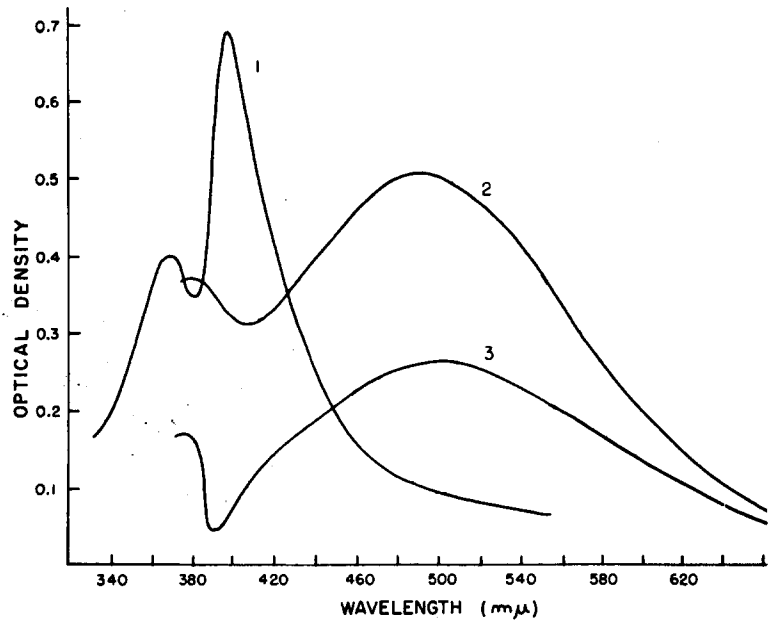

Fig. 7. Absorption spectra of colored solutions obtained with 10 μmoles of citrate (curve 1), 10 μmoles of D,L-glyceraldehyde (curve 2), and about 2 μmoles of succinohydroxamic acid (curve 3). Hydroxylamine was not present in the solution used for curve 1, but was present in the other two solutions. The spectra were recorded about 3 hours after the addition of $FeCl_3$.

DISCUSSION

It has been known for a long time that Fe^{+3} forms colored complexes with many substances such as phenols, enols, oximes and hydroxamic acids[9,10,11]. Such knowledge has been used in the characterization of organic substances such as aspergillic acid[12] and siderophilin[13]. Perhaps the widest application has been made in the quantitative determination of hydroxamic acids, or for the estimation of esters and lactones which react with hydroxylamine to produce hydroxamic acids[1,6,7,14–19].

References p. 128.

Colored complexes with $FeCl_3$ have been used in the location of choline esters[20] and oximes[21] on paper chromatograms. The determination of hydroxylamine has been carried out with Fe^{+3} after conversion to formohydroxamic acid[22].

In the present method for keto acids, the reaction with hydroxylamine probably gives rise to the corresponding oximes even though the pH is unfavorable for oximation[23]. The reaction of pyruvate with hydroxylamine under the specified conditions produces approximately the same final color and optical density whether the treatment is carried out for 10 min or 1 hour at pH 1 or pH 6.5. Chelation of the Fe^{+3} may occur between the oximino and adjacent carboxyl groups. The oximation process with $NH_2OH \cdot HCl$ and reaction with $FeCl_3$ would each result in the release of HCl[5,9]. This is in agreement with the fact that even in samples with only 2 μmoles of pyruvate, the pH was always lower by a few hundredths of a pH unit than in the corresponding reagent blanks. It is not clear why some of the substances tested do not give any significant color in the method.

The method is not specific for α-keto acids, but the yellow color produced by the reactive ones may be distinguished from many of the colors which develop when other substances are present. It should be possible to determine mixtures of several of these by differential spectrophotometry provided their spectral characteristics are known. The effect of the presence or absence of hydroxylamine on the color reaction with $FeCl_3$ would be useful in the characterization of chromophores.

The procedure works well when applied to enzymic systems in which the products are known and interfering substances are not present. It has been useful in a study of the kinetics of serine and threonine dehydrase with crude extracts of several microorganisms*. Since pyruvate was not metabolized further in the presence of these cell-free extracts, it was possible to omit hydroxylamine from the reaction mixtures after it was found that hydroxylamine inhibited serine dehydrase.

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SUMMARY

A method is reported for the colorimetric determination of several keto acids after reaction with hydroxylamine and $FeCl_3$. The effects of pH, concentration of $FeCl_3$ and hydroxylamine, and the order of addition of the latter on color development are described. A discussion is included of the colors given by several other substances which react under the conditions of the method.

* Unpublished experiments.

References p. 128.

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