The Sulfophosphovanillin Reaction for Serum Lipids: A Reappraisal

K. R. Johnson,¹ G. Ellis,² and C. Toothill³

New evidence is presented for the mechanism of this reaction, which is used in estimating lipids. The reaction occurs in two stages: (a) Stage 1 evidently involves an oxidation step, forming a specific type of carbonion called an alkyl cation of the general formula R—C+=C—R² (previous evidence had indicated that a carbonion of general formula R—C+=C—R² was formed by protonation only). (b) Stage 2 of the reaction was examined by nuclear magnetic resonance studies and by partition experiments, which have indicated that a vanillin phosphate ester is not formed as previously suggested. Comparative data from other acid–aldehyde reactions indicate that in this reaction 1,1-di(4-hydroxy-3-methoxyphenyl)ethene ion is formed, a product compatible with the formation of an alkyl cation at stage 1.

Additional Keyphrases: reaction between aldehydes in strong acids • reaction mechanisms • nuclear magnetic resonance

Routine analysis for individual lipids has not achieved the same stage of development and application in the investigation of disease as has amino acid analysis. We believe that this is partly due to lack of a simple analytical system similar to the ion-exchange chromatographic system used in amino acid analyzers, the success of which relies on the coupling of an established separation technique with a chemical method for which the reaction mechanism is well understood.

If the specificity of the sulfophosphovanillin (SPV) reaction were elucidated, its potential for lipid analysis would be comparable to that of the ninhydrin reaction for amino acids. The SPV reaction shows versatility in being applicable to both lipid extracts and lipoprotein solutions. The feasibility of this technique being used in already-established diagnostic lipid tests can be illustrated with reference to serum. Present recommendations are that lipids be analyzed in serum obtained after fasting, in which the main lipid components contain either cholesterol or an unsaturated fatty acid, both of which contain the chemical configuration necessary for SPV reactivity.

Materials and Methods

Chemicals

All chemicals of analytical grade were purchased from British Drug Houses Ltd.; cyclohexanol, cyclohexene, 1,3-cyclohexadiene, and 1,4-cyclohexadiene were obtained from R. N. Emanuel. Chromatography was on silica gel sheets (No. 6061; Eastman-Kodak Ltd.), in a solvent system recommended for the separation of lipid classes (1).

Reaction

The SPV reaction is done in two stages:

Stage 1 (chromogen formation): To 0.2 ml of concentrated sulfuric acid (980 g/liter) add 10 µl of the test solution (substance dissolved in chloroform or carbon tetrachloride), heat at 100 °C for 10 min, and cool to room temperature.

Stage 2 (chromophore production): Add 10 ml of freshly prepared phosphovanillin reagent [4 vol of H₃PO₄ (890 ml/liter) + 1 vol of aqueous vanillin, 6 g/liter], incubate at 37 °C for 15 min, cool, and measure the absorbance at 530 nm.

Effect of varying acid concentration; stage 1: This was investigated in the following ways:

(a) Pre-heating: 0.2-ml portions of sulfuric acid of several concentrations were substituted for the 0.2 ml of concentrated sulfuric acid.

(b) Post-heating: stage 1 of the reaction was carried out as described in a, and increasing amounts of water were added after cooling at stage 1. The resulting solutions were incubated at room temperature for 30 min before proceeding with stage 2 of the reaction. The absorbance at 530 nm was corrected for volume changes which resulted from dilution of the sulfuric acid.

Partition Experiments

Spectra of vanillin in water, ether, and phosphoric acid differ sufficiently to allow identification. Both phases resulting from the extraction into water of vanillin in ether (1 mg/ml) were examined spectrophotically. The ether phase of a biphasic system of a mixture

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Table 1. Compounds Examined for Positive Reaction (530 nm Absorbance) *

<table>
<thead>
<tr>
<th>A. Positive compounds</th>
<th>Cyclohexene</th>
<th>1,3-Cyclohexadiene (290, 300, 405)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleic acid (300)</td>
<td>1,4-Cyclohexadiene (320, 400)</td>
<td>2-Hydroxycyclohexanone</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>Cyclohexanone</td>
<td>Cyclohexen-1-ol</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>Dicyclopentadiene (300, 325)</td>
<td></td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol trioleate (300)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol (315, 410)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol stearate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol oleate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydrocholesterol (307, 410)</td>
<td>Propan-1-ol (300)</td>
<td>Propan-2-ol</td>
</tr>
<tr>
<td>Oct-1-ene (305)</td>
<td>Butan-1-ol</td>
<td>Butan-2-ol (300)</td>
</tr>
<tr>
<td>Oct-2-ene (305)</td>
<td>2-Methylpropan-1-ol (307)</td>
<td>2-Methylpropan-2-ol (300)</td>
</tr>
<tr>
<td>Octa-dec-1-ene (305)</td>
<td>Cyclohexanol</td>
<td>Cyclohexen-2-ol</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Negative compounds</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Stearic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>Cyclohexanone</td>
<td></td>
</tr>
<tr>
<td>Octanoic acid</td>
<td>Cyclopentanone</td>
<td></td>
</tr>
<tr>
<td>Glycerol tripalmitate</td>
<td>Octanoic lactone</td>
<td></td>
</tr>
<tr>
<td>Glycerol tristearate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL-α-Lecithin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(synthetic dipalmitoyl derivative)</td>
<td>Methanol</td>
<td>Ethanol</td>
</tr>
</tbody>
</table>

* Figures in parentheses are the wavelength of maximum absorption in sulfuric acid at stage 1.

of concentrated phosphoric acid (13.2 ml), ether (20 ml), and water (20 ml) was also examined for phosphate by the method of Robinson et al. (2).

Nuclear Magnetic Resonance (NMR)

We used this technique to examine the postulate of Knight et al. (3) that a prerequisite for stage 2 of the SPV reaction to occur was the initial formation of a vanillin phosphate ester. If this does occur, there will be a profound change in the proton NMR spectrum of vanillin.

The proton NMR spectra were recorded with a Varian 60A NMR spectrometer at 60 MHz and 38 °C probe temperature. All samples were contained in 5-mm (external diameter) glass tubes. Tetramethylsilane was added as internal reference to all samples except aqueous solutions. For aqueous samples the sodium salt of 2,2-dimethylsilapentone sulfonate was used as internal reference. Dioxan was used as a solvent for vanillin for most spectra because of the low solubility of vanillin in water. The 100-MHz spectrum of vanillin in dioxan was also recorded by the Science Research Council’s NMR service. A computer calculation by use of the spectral analysis program LAOCOON gave the chemical shifts and coupling constants, which were in agreement with the analysis of the 60-MHz spectrum done by noncomputerized calculation.

Results

Chemical Nature of the Chromogen (Stage 1)

Table 1 gives a list of substances investigated, classified according to their ability to yield a colored product with absorbance maximum of 530 nm. Many of the positive compounds were examined during the course of the reaction and were found to yield chromogens with maximum absorbance around 300 nm, after Stage 1 of the reaction; these compounds are also identified in Table 1.

The two common chemical groupings present throughout the extensive list of SPV-positive compounds are unsaturation or aliphatic alcohol, or both. This corroborates findings in other examinations of the reaction (3–5). It has been suggested by other workers that a common chromogen is formed in similar acid–aldehyde reactions during the first stage of the reaction. This formation was said to involve protonation only by Duke (4) and Knight et al. (3), working with alcohols and lipids respectively; Deno et al. (6, 7) in their investigation of alcohols and olefins by NMR analysis, suggested the presence of linear and cyclic alkenyl cations.

There is experimental evidence comparing the properties of the intermediate at stage 1 with previously
published data for (a) carbonium ions formed by simple protonation and (b) alkenyl cation formation.

The speed of formation, temperature dependence, and ultraviolet spectrum of the chromogen. Simple protonated carbonium ions have no ultraviolet spectrum at wavelengths longer than 210 nm when produced in the absence of air (8). The \( \lambda_{\text{max}} \) obtained near 300 nm after stage 1 with model lipids, oleic acid, and cholest-erol (Table 2) and other compounds (Table 1) suggests alkenyl cation formation (9–11). The formation of carbonium ions by simple protonation in an optimal environment is said to be instantaneous (8) and such ions are stable only at low temperatures (12).

With oleic acid, ultraviolet absorbance and color intensity were maximum after a 4-min heating. With cholesterol, the reaction was more complex, the \( \lambda_{\text{max}} \) shifting from 250 to 285 nm and the maximum being reached only after 10 min (Table 2).

Effects of concentration of sulfuric acid and alternative acids. For simple carbonium ion formation, reactive compounds require a large variation in acid strength and also in type to produce optimum protonation. These range from weak acids such as acetic through sulfuric acid to SbF\(_6\)/HF mixtures (13). We used various concentrations of sulfuric acid for six SPV-positive compounds. The concentrations of sulfuric acid that corresponded to maximum absorbance at 530 nm were: for dihydrocholesterol, 6% oleum; for cholesterol, oleic acid, and oleyl alcohol 98%; and for cyclohexene and cyclohexanol 88%—a relatively narrow range of acid concentrations. Of the various acids used at stage 1 in an attempt to produce chromogen, hydrochloric, perchloric, and acetic acids proved ineffective on the compounds tested, but phosphoric acid gave a positive reaction with three compounds. All showed a stronger reaction in sulfuric acid.

Reversibility on dilution of chromogen with water. Gold and Gruen (8) have shown that simple protonation of compounds in sulfuric acid is instantly reversed by adding water. This was examined in stage 1 of the reaction by diluting the reaction product to sulfuric acid concentrations over the range 20–98%. The model compounds used were cyclohexene and cyclohexanol and the presence of chromogen was monitored by color formation. The results (Figure 1) suggest that the intermediate formed at stage 1 is not destroyed in a manner similar to its formation.

Enhancement of stage 1 by oxidant. Leftin (14), using oct-1-ene, suggested that sulfuric acid caused oxidation and formation of alkenyl cation. Using oleic acid as the model substance, we found that addition of selenium dioxide yielded color formation at a normally unreactive concentration (72%) of sulfuric acid (Figure 2).

Stage 2 of the Reaction

Knight et al. (3) have suggested that in the SPV reaction a vanilllin phosphate ester is formed, which then reacts with the chromogen. An alternative view, that the acid in stage 2 merely acts as an optimal solvent environment in which aldehyde and chromogen can react, was advanced when estimating alcohols (4) and biotin (15). The possibility of vanilllin phosphate formation has
been examined by partition and NMR studies. The alternative proposal was studied by variation in the concentration of the components at stage 2.

Partition Experiments

These experiments were based on the assumption that the partition coefficient of vanillin and phosphoric acid between ether and water differed from that of the proposed ester (3). Substances of different chemical structure and polar groups exhibit readily detectable differences in partition.

Preliminary distribution between the two layers when ether containing vanillin (1 g/liter) was extracted with (a) water and (b) concentrated phosphoric acid in ether/water (followed by extraction of the ether phase by water) showed that vanillin was not partitioned into water and further that there was a low (0.1%) partition of phosphoric acid into ether.

Phosphovanillin reagent (13.3 ml) was mixed with diethyl ether (20 ml); after cooling, water was added until partition occurred and the separated ether phase was extracted with water (12 ml). The partition of phosphoric acid between ether–water phases was 0.1% and of vanillin between water–ether phases was zero; in addition, we detected no vanillin phosphate in the aqueous extract of the ether phase. These results are contrary to those to be expected if a phosphate ester of vanillin were produced in the phosphovanillin reagent.

Because of the excessive amount of water added to produce partition, the aqueous phase was tested for its ability to give SPV reaction and was found to be positive.

We prepared phosphovanillin reagent, using various concentrations of vanillin (12–1800 mg/liter), and repeated the extraction experiment. The range of phosphoric acid concentration detected in the ether phase was 1.8–4.8 mg. One problem of the partition experiment is that it gives no information on whether phosphate ester is formed as follows:

\[
\text{Vanillin} + \text{phosphoric acid} \xrightarrow{\text{fast}} \text{vanillin phosphate} \xrightarrow{\text{fast}} (<0.5\%)
\]

In an attempt to detect vanillin phosphate under these conditions, we added an ether extract of the chromogen from stage 1 to vanillin in ether. The resulting solutions were mixed with phosphoric acid of various concentration and were agitated continuously to ensure total mixing for complete reaction, especially in the tubes where the solution was biphasic. Theoretically, if the solution was biphasic, intimate contact of the layers would allow formation of phosphate ester in the more favorable layer; this could then react with the chromogen present to form chromophore, resulting in ester being removed. New phosphate ester would be formed and the reaction would continue to completion under the conditions of acidity prevailing in that particular tube. This reaction should be comparable to the observation in the wholly aqueous system. The results of such an experiment indicated that no reaction occurred until the mixture was monophasic, when there was total reaction.

**NMR with reference to vanillin.** In vanillin/dioxan solution single spectral lines (singlets) (Table 3) are observed for the methoxy, aldehyde, and hydroxyl protons. In aqueous solutions of vanillin, the hydroxy

**Table 3. Chemical Shift of Protons in the CHO and OCH₃ Groups of Vanillin Relative to Tetramethylsilane (TMS)**

<table>
<thead>
<tr>
<th>Composition of material for NMR</th>
<th>Chemical shifts (TMS) in Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dioxane</td>
<td>Water</td>
</tr>
<tr>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>80%</td>
<td>20%</td>
</tr>
<tr>
<td>60%</td>
<td>40%</td>
</tr>
<tr>
<td>40%</td>
<td>60%</td>
</tr>
<tr>
<td>20%</td>
<td>80%</td>
</tr>
<tr>
<td>Vanillin–dioxan (200 g/liter)</td>
<td>H₃PO₄ (88%)</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>4.9</td>
<td>0.1</td>
</tr>
<tr>
<td>4.6</td>
<td>0.4</td>
</tr>
<tr>
<td>4.5</td>
<td>0.5</td>
</tr>
<tr>
<td>4.2</td>
<td>0.8</td>
</tr>
<tr>
<td>4.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>
singlet disappears, owing to exchange with the water protons (or deuterons if $^3$H$_2$O is used). In the region characteristic of aromatics, the three ring protons of vanillin, because of interaction, give a complex second-order spectrum, which has to be analyzed by spectral analysis.

In vanillin the aromatic protons can be regarded approximately as an ABX system (see Figure 3). Here the coupling constant $J_{AB}$(Hz) is of the same order of magnitude as their chemical shift differences $\delta v_{AB}$(Hz). As the X nucleus has chemical shift differences $\delta v_{BX}$ and $\delta v_{AX}$ much greater than the coupling constants $J_{BX}$ and $J_{AX}$, respectively, one can use the "X approximation." This simplifies the calculation of the true chemical shifts and enables one to use sub-spectral analysis, i.e., the complex spectrum is a superimposition of a number of simple spectra (16-18). For an ABX system this is broken down into two a-b subspectra, each corresponding to a different spin value of the X nuclei (one nucleus spinning with the applied magnetic field, the other against it), i.e., this could be regarded as two different molecules where $X = +_2$ or $-_2$ (see Figure 4). Each subspectrum cannot be assigned to a particular proton.

Figure 5 shows a part of the spectrum attributable to A and B protons for X of opposite spins. Practically, there are fewer lines than theoretically calculated, due to overlap. The X part shows six lines; two pairs are so close together as to give broad single peaks (Figure 5). The center of the multiplet (a series of lines produced due to proton–proton interaction within the molecule) gives the chemical shift of X (i.e., $v_X$); calculation of the chemical shift for A and B protons gives:

1. $v_A = 447.3$ Hz  
2. $v_B = 443.2$ Hz  
3. $J_{AX} = 8.2$ (from 100 MHz calculation)  
4. $J_{AB} = 1.5$  
5. $v_x = 417.5$ Hz  
6. $J_{BX} = 0$

For comparative purposes, the lines in the multiplet can be assigned to each proton in vanillin as follows:

$447.3$ Hz = A  
$443.2$ Hz = B  
$417.5$ Hz = X

Study of phosphovanillin. An NMR study of vanillin–dioxan solutions with incremental additions of phosphoric acid (Figure 6) shows these comparative NMR spectra in the aromatic region over the range 0–20% phosphoric acid. These show gradual changes leading to a marked coalescence of the AB multiplet. The sub-spectrum derived from B(ab)+ collapses almost to a single line with line broadening, attributable to viscosity. The line broadening can be calculated by taking the width at half height ($\delta v_{1/2}$) of several lines in the spectra, such as tetramethylsilane reference, CH$_3$, or OCH$_3$. Tetramethylsilane shows no line broadening, whereas OCH$_3$ and CHO do; also, the aromatic protons are very much broadened. As tetramethylsilane is an isotropic nonpolar molecule, the effect of H$_3$PO$_4$ was much less on it than on other molecules. This points to the viscosity being a result of a strong hydrogen bonding.

![Diagram of ABX system](image)

**Fig. 3.** NMR nomenclature of vanillin, to show the ABX proton system

**Fig. 4.** Diagrammatic representation of building up a complex spectrum from single spectra, utilizing the principle of sub-spectral analysis

**Fig. 5.** NMR spectrum of vanillin–dioxan solution (200 g/liter), showing the AB and the X parts of the aromatic proton spectrum

**Fig. 6.** Composite spectrum showing AB part of spectrum of an ABX system due to two X nuclei: $+_2$ and $-_2$ (X component not shown)
However, it is possible to extract some features from these spectra and compare them with the spectrum of vanillin in dioxane

(1) $v_A = 443.4$ Hz
(2) $v_B = 440.0$ Hz
(3) $J_{AX} = 8.0$ Hz
(4) $v_X = 414.5$ Hz

The changes in the spectrum on the addition of $H_3PO_4$ are shown below:

<table>
<thead>
<tr>
<th>Spectrum</th>
<th>20% vanillin–dioxane, ml</th>
<th>$H_3PO_4$ (8%), ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>(b)</td>
<td>4.6</td>
<td>0.4</td>
</tr>
<tr>
<td>(c)</td>
<td>4.4</td>
<td>0.6</td>
</tr>
<tr>
<td>(d)</td>
<td>4.3</td>
<td>0.7</td>
</tr>
<tr>
<td>(e)</td>
<td>4.2</td>
<td>0.8</td>
</tr>
<tr>
<td>(f)</td>
<td>4.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Thus all three nuclei moved upfield by 3 to 4 Hz on addition of $H_3PO_4$, so that it appears to be a general susceptibility effect (i.e., a solvent effect). On gradual addition of water to vanillin–dioxane solutions they exhibit a similar collapse of the multiplet.

A variable-temperature study of the dioxane–vanillin solution containing phosphoric acid/water (20/80 by vol) was undertaken. This concentration of vanillin phosphoric acid produced SPV positivity at stage 2. The temperature range used was 40–100 °C. The results (Figure 7) exhibit a reversal of the phenomenon seen on addition of increments of phosphoric acid. This observation was also consistent with a solvent effect. An association between vanillin and phosphoric acid is most likely to occur by hydrogen bonding and would involve mainly the OH and OCH$_3$ groups and possible the carbonyl group. Chemical shifts of OCH$_3$ and CHO have been measured relative to tetramethylsilane (Table 3), and it can be seen that little change occurs in either group (OH is not observable due to exchange with H$^+$).

The Nature of the Chromophore (Stage 2 of the Reaction)

Theories have been proposed on the nature of the final colored product when reacting aromatic aldehyde in strong acid with different types of SPV-positive compounds (3–5, 15, 19–21). Most documented methods use a one-step technique, the acid used for dissolving the aldehyde also producing the chromogen at stage 1. Analogous vanillins have been used in this reaction, especially in attempts to isolate the chromophore produced.

Comparison of the ultraviolet spectra at stage 1 and the visible spectra in Table 2 shows a direct relationship between rate of development of an ultraviolet spectral peak and color at 530 nm, indicating a direct link between chromogen and chromophore formation.

Evidence from aldehyde variation. (a) A possible reaction mechanism postulated was the production of a catalyst at stage 1, which initiated the condensation of two molecules of aldehyde to produce chromophore. To test this theory we incorporated two aromatic aldehydes (benzaldehyde + vanillin) in the stage 2 reagent. Theoretically, if condensation is catalyzed, three spectral peaks should be observed. Only two were observed, corresponding to the benzaldehyde chromophore and the vanillin chromophore.

(b) Various authors had reported differing $\lambda_{max}$ for different aldehydes and also different $\lambda_{max}$ for the same aldehyde, depending on their analytical conditions, e.g., Zollner and Kirsch (5), examining lipids, showed $p$-hydroxybenzaldehyde to give a $\lambda_{max}$ at 510 nm, whereas Cookson et al. (22), when isolating the derivative, pro-
duced when reacting p-hydroxybenzaldehyde with an olefin gave a colored product with a maximum 665 nm. Examining different aldehydes under our optimal analytical conditions gave results for p-hydroxybenzaldehyde which agreed with Zollner and Kirsch (5). However, when in the reaction we used differing concentrations of the same aldehyde (vanillin), not only rates of color development were altered with increasing concentration but a second spectral peak, at 679 nm, developed slowly, indicating a second reaction step (Figure 8). When p-methoxybenzaldehyde was substituted for vanillin, a slower reaction was also observed with a maximum at 645 nm.

Alterations from changes in acid. Alterations in phosphoric acid concentration affected the rate of color development and its sensitivity and stability. Color development and stability were maximal at 70.4% (704 g/liter) phosphoric acid. At higher concentrations, rate of development slowed but the color was more stable (Figure 9). With vanillin, substitution of other acids for phosphoric acid—perchloric, hydrochloric, sulfuric, and acetic acids—showed that only sulfuric acid at a concentration of 750 ml/liter produced color, which was at the same \( \lambda_{\text{max}} \) of 530 nm but only 25% as sensitive.

Discussion

With the possible use of the SPV technique for quantitation of lipid fractions the influence of interfering compounds increases. A detailed knowledge of the reaction mechanism is necessary to be able to predict reactive lipids and interfering compounds.

The Chemical Nature of the Chromogen (stage 1)

Comparing the evidence between the two major possibilities shows the following: the ultraviolet spectrum at about 300 nm, the need for heat to produce this spectrum (Table 2), the irreversible nature of the reaction (Figure 1), the narrow range of acid concentration over which the chromogen is formed, and the influence of an oxidizing agent on reactivity (Figure 2). All these weight the evidence strongly in favor of an alkenyl cation being formed.

Other evidence from the literature for alkenyl cations being formed in sulfuric acid reactions shows similar properties to our observations. Deno et al. (6), utilizing alcohols, have shown the reaction products with sulfuric acid to be equal amounts of a mixture of acyclic, highly branched, saturated hydrocarbons (insoluble in sulfuric acid) and a mixture of cyclopentenyl cations, which are soluble in the sulfuric acid layer; the latter components absorbed strongly in the 300-nm region.

Cyclopentenyl cations are one example of a larger group of cations termed alkenyl cations, most of which absorb intensely between 275 nm and 310 nm. Nuclear magnetic resonance has permitted the positive identification of alkenyl cations by relating them to electronic spectra. Deno et al (6, 7) showed that, in 96% sulfuric acid, butan-1-ol, butan-2-ol, 2-methylpropan-2-ol, and 2,2,4-trimethylpent-1-ene reacted to give the same products. In all cases the insoluble organic layer formed contained the products of reduction, mainly C4-C18 alkanes, while the sulfuric acid layer contained the oxidation products, which were a number of alkenyl cations. These workers (7) also showed that dimethylcyclohexene in the presence of acid undergoes disproportionation into cyclopentenyl cations and cyclo alkanes. In contrast, in 75% H2SO4 the products were dimers and trimers of C4H8. Deno et al. (23) have identified a series of cyclic and linear alkenyl ions by NMR spectroscopy. The properties observed in this
work are similar to those reported for those alkenyl cations that have been characterized.

The suggested general formula for chromogen formed is:

\[ + \quad \text{R} - \text{C} - \text{C} = \text{C} - \text{R} \]

Because of the complexity of reaction in sulfuric acid at stage 1 and the numerous products of reaction indicated by other workers (6, 7), it is essential to demonstrate a positive link between chromogen and chromophore. This was done by showing, with oleic acid and cholesterol, the simultaneous development of ultraviolet spectra for stage 1 and the absorbance at 530 nm for stage 2 (Table 2).

Role of Acid at Stage 2: Reagent or Solvent?

In their investigation of lipids with the SPV reaction, Knight et al. (3) suggested that the phosphoric acid played a much more positive role in reactivity by forming a phosphate ester with vanillin. Our experiments involving partition and NMR studies of vanillin and vanillin in phosphoric acid do not support this suggestion. Vanillin and phosphoric acid show no significant difference in partition between ether and aqueous solutions, either when added singly or as a prepared phosphovanillin reagent. The NMR data show that phosphoric acid has a general susceptibility effect on vanillin in solution. This indicates a general solvent effect affecting all the vanillin protons. Observed changes in substituents in the aromatic ring of vanillin produced profound changes in the observed \( \lambda_{\text{max}} \). This corroborates the findings of Zollner and Kirsch (5). A change from phosphoric acid to sulfuric acid would be anticipated to produce similar changes if the ester were formed, but such was not observed, and this substantiates the proposal that no ester was formed under the SPV reaction conditions. Duke (4) showed that the acid in stage 2 provides protons, enabling the product at stage 2 to exhibit tautomerism, yielding a colored quinonoid structure. McCormick and Roth (15) also indicated that proton concentration was critical for maximal color production from biotin, high concentrations being detrimental to color production. Our experiments substantiate the original suggestions (4, 15) that the acid at stage 2 provides a protonic environment, which is favorable for maximal chromophore production. These are different for the various reactive compounds. A time-based study of the effect of phosphoric acid (Figure 9) on chromophore formation demonstrated the proton dependence of stage 2. Substitution for phosphoric acid of 75% sulfuric acid gave a spectrum with the same \( \lambda_{\text{max}} \) but with only 25% of the initial absorbance; this demonstrates the essential nature of the lower proton concentration in phosphoric acid while water availability remains low, a unique property of phosphoric acid.

The Nature of the Chromophore

The constant \( \lambda_{\text{max}} \) observed with any one aldehyde could indicate that chromophore formation involves the dimerization of vanillin initiated by a catalyst formed at stage 1. The experiment in which two aldehydes were used simultaneously showed this not to be the case. Variation of the vanillin concentrations yielded a second absorption peak, \( \lambda_{\text{max}} \) 679 nm, with prolonged reaction (Figure 8). A similar finding had previously been described by Schaltegger (19) in his quantitative method for estimation of vitamin D. The development of the peak was paralleled by a decrease in the 530-nm peak (Figure 8), suggesting a second and much slower stage in the reaction. This observation has proved useful in linking the observation of Cookson et al. (22) on aryl polyene formation from olefins when reacted with aldehyde in strong acid.

An examination of the reaction mechanisms suggested from the various areas of analytical chemistry has led to the emergence of a general hypothesis for the reaction between aldehydes in strong acids and the wide range of reactive compounds already mentioned, namely, the reaction between a “three-atom unit” and the corresponding aldehyde, which may be diagrammatically represented as follows:

\[
\begin{align*}
(1) \quad \text{aldehyde} & \quad \overset{\text{C}}{=} \overset{\text{C}}{=} \overset{\text{C}}{=} \quad \text{aldehyde} \\
\text{aldehyde} & \quad \overset{\text{C}}{=} \overset{\text{C}}{=} \quad \text{aldehyde} \\
\text{aldehyde} & \quad \overset{\text{C}}{=} \quad \text{aldehyde}
\end{align*}
\]

This is compatible with the proposed alkenyl cation skeleton for the chromogen formed at stage 1 of the reaction \( \text{R} - \text{C}^+ - \text{C} = \text{C} - \text{R} \). This structure is corroborated by the following theoretical consideration. Diaryl olefins are known to dissolve in weakly acidic media such as acetic acid—sulfuric acid mixtures to give highly colored species (24). Cookson et al. (22) showed that the olefin reacted with solvent to form an aryl polyene, which was then readily protonated to give the colored ion. The system studied was 1,1-di(p-methoxyphenyl)-ethylene in formic acid, which rapidly acquires an intense blue color \( \lambda_{\text{max}} \) 665 nm). The blue color was isolated as a perchlorate and shows a similar structure to that of the proposed chromophore for the slower second reaction. Grace and Symons (25) have attributed the color reaction of other species in weak acids to similar phenomena. The former shows a conjugated 5-carbon chain linking four aromatic systems. In the case of the 1,1-di(p-methoxyphenyl)ethylene ion, if the structure were formed from an aldehyde/strong acid reaction then the aldehyde involved in the formation of this cation would be \( p \)-methoxybenzaldehyde. This aldehyde in 72% phosphoric acid exhibits an absorption maximum at 370 nm. The structure proposed by Cookson et al. (22) would have been formed by the condensation of 4 moles of aldehyde with the reactive species from stage 1 of the reaction. This has a \( \lambda_{\text{max}} \) of 665 nm. The proposed mechanism of color formation (Figure 10) suggests the use of 2 moles of aldehyde only, which with vanillin yields a compound with a \( \lambda_{\text{max}} \) of 530 nm and
with p-methoxybenzaldehyde a $\lambda_{\text{max}}$ at 510 nm. As the wavelength of absorption is mainly related to the degree of conjugation existing in the system, the following comparison can be made:

<table>
<thead>
<tr>
<th>Number of conjugated double bonds</th>
<th>Practical $\lambda_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Methoxybenzaldehyde in 72% phosphoric acid</td>
<td>3</td>
</tr>
<tr>
<td>Proposed chromophore in 72% phosphoric acid (with p-methoxybenzaldehyde used as aldehyde)</td>
<td>8</td>
</tr>
<tr>
<td>Slower developing peak (24 h) in 72% phosphoric acid, with p-methoxybenzaldehyde</td>
<td>14</td>
</tr>
<tr>
<td>1,1-Di(p-methoxyphenyl)ethylene cation in formic acid</td>
<td>14</td>
</tr>
</tbody>
</table>

The difference between the aldehyde and the ethylene cation is 11 double bonds and 275 nm. The proposed reaction product contains seven conjugated double bonds. The difference between the proposed reaction product and p-hydroxybenzaldehyde is five conjugated double bonds. If equal sharing of the shift in $\lambda_{\text{max}}$ is assigned to each double bond, then 5/11 $\times$ 275 + 370 nm would be the theoretical $\lambda_{\text{max}}$ of our proposed product. This gives a theoretical maximum of 495 nm, compared with a measured $\lambda_{\text{max}}$ of 510 nm when p-hydroxybenzaldehyde is used in place of vanillin in the SPV reaction. From a comparison of the properties of other reactions with those of the SPV and from the practical and theoretical considerations put forward here, the proposed intermediate at stage 1, the structure of the chromophore at stage 2 for the sulfophosphovanillin reaction, and the general reaction sequence are given in Figure 10. It is also evident that due to the complex nature of reactions in sulfuric acid, the optimal conditions for reaction with various compounds will be different. Similarly, the aldehyde condensation step at stage 2 will have differing acid requirements for optimal reaction.

Aldehyde/strong acid reagent has been used in many applications as a one-step reaction. Because of this, maximum sensitivity will not have been achieved. Possible interferences must be recognized and steps taken to eliminate them when attempting to estimate lipid fractions. These processes are substantially aided by an understanding of the reaction mechanism and the knowledge of optimal conditions for individual lipid reaction by SPV.

References


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