The Analyst

Determination of carbonyl compounds by the oxidative chemiluminescence of 2,4-dinitrophenylhydrazine

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The oxidative chemiluminescence of phenylhydrazines can be applied to the determination of carbonyl compounds by utilising the attenuation of the signal which occurs on conversion to the corresponding phenylhydrazone. Using an assay for 2,4-dinitrophenylhydrazine, off-line derivatisation for a period of 2 h was found to be optimised for a temperature of 30 °C and for a sulfuric acid concentration of 0.05 M. The optimum conditions for 2,4-dinitrophenylhydrazone formation can be understood in terms of a stepwise mechanism of carbinolamine formation *via* a zwitterion intermediate. The log–log calibration for the combined derivatisation/assay of hexanal in optimised conditions is linear (r = 0.9931, n = 5) from 1×10^{-6} to 2×10^{-5} M. The limit of detection calculated as above was 1.7×10^{-7} M (1.7 pmol of hexanal). Applications to the oxidative deterioration of linoleic acid and to the effect of lipoxygenase on linoleic acid are described.

Keywords: *Flow injection; chemiluminescence; permanganate oxidation; carbonyl compounds; phenylhydrazines; lipid peroxidation; lipoxygenase*

The phenylhydrazines have a long history of application in the analysis of carbonyl compounds.[1] 2,4-Dinitrophenylhydrazine derivatives, having a high absorptivity, are particularly useful for spectrophotometric detection and Esterbauer and Zollner[2] have reported their use in this context in the study of the aldehydic products of lipid peroxidation. Industrial applications of carbonyl compound determination by 2,4-dinitrophenylhydrazine include atmospheric monitoring,[3] leak detection, analysis of smoke stack emissions[4] and quality control of a variety of products.[5]

More recent approaches to the analysis of carbonyl compounds include the use of chemiluminescent detection, which often offers considerable advantages in detectivity and selectivity. Vogin *et al.*[6] reported a procedure in which aldehydes were determined by their inhibition of the chemiluminescent luminol/hydrogen peroxide reaction in the presence of hexacyanoferrate(III). Other workers[7,8] have reported the use of chemiluminescent detection of aldehydes in assays employing an oxidoreductase and bacterial luciferase. Some carbonyl compounds give more direct chemiluminescence, *e.g.*, formaldehyde on treatment with an alkaline hydrogen peroxide/gallic acid reagent (the Trautz–Schoringin reaction), which has been successfully applied to gas-phase samples.[9]

We have already reported the development of an assay of 2,4-dinitrophenylhydrazine based on its chemiluminescence when it is oxidised by permanganate.[10,11] The present study investigates the attenuation of the chemiluminescence when the 2,4-dinitrophenylhydrazine is converted to corresponding hydrazones and describes the application of this phenomenon to the determination of carbonyl compounds. The method is calibrated using hexanal standards and used to investigate the oxidative deterioration of linoleic acid and the effect of lipoxygenase on linoleic acid.

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Experimental

Apparatus

This consists of a two-channel flow injection manifold and has been described previously.[10] The sample is injected into a carrier solution, which is merged with the oxidant at a T-junction before passing through a luminometer, which outputs to a chart recorder; peak heights were measured for each signal and expressed as the voltage output of the photomultiplier tube.

Materials

Water used in the experiments described was deionised by reverse osmosis to a resistivity of 10 MΩ cm. All chemicals were analytical-reagent grade and were used as supplied unless otherwise stated. They were obtained from the following sources:

From Aldrich (Gillingham, Dorset, UK); 2,4-dinitrophenylhydrazine (this was bought moist and before use dried at 100 °C, recrystallised from butan-1-ol, washed first in mother liquor and then in a little butan-1-ol, dried at 95–100 °C and stored in a dessicator).

From BDH-Merck (Poole, Dorset, UK); potassium permanganate, formic acid (98–100%), sulfuric acid (sp. gr. = 1.84), butan-1-ol, boric acid, potassium hydroxide.

From Rhone-Poulenc (Manchester, UK); propan-2-ol.

From Sigma (Poole, Dorset, UK); sodium hydroxide, hexanal, linoleic acid (*cis*-9-*cis*-12-octadecadienoic acid, 99%), lipoxygenase (EC 1.13.11.12) (type I-S from soybean, 61 000 U mg^{-1} solid; 1 U in 3.0 ml volume with 1 cm path-length increases A_{234} of linoleic acid by .001 min^{-1}), kept as a stock solution of 100 mg lipoxygenase dissolved in 100 ml ice-cold 0.2 M potassium borate buffer, pH 9.0, stored frozen at −20 °C. For use, it was thawed completely, mixed well and placed in an ice-water bath. Repeated freezing and thawing did not seem to affect the enzyme activity.

Procedure

2,4-Dinitrophenylhydrazine assay

The optimisation of the assay has been previously described.[11] Samples dissolved in 22.0% aqueous propan-2-ol were injected into a carrier of 0.75 M formic acid, which merged with an oxidant of 5.0×10^{-5} M potassium permanganate in 0.041 M sulfuric acid, the combined flow-rate being 3.25 ml min^{-1}, all solutions being maintained at 40 °C.

The results in most of the experiments are expressed as the ratio of the signals net of blank, $(R - B)/(A - B)$, where A is the mean signal for the analyte sample, B for the blank and R for the reagent blank. Because of a log–log relationship existing between the net signals and the determination concentration,[11] $(R - B)/(A - B)$ can be shown algebraically to be equal to the ratio of 2,4-dinitrophenylhydrazine concentrations before and

after derivatisation, raised to a power equal to the slope (which is close to unity) of the regression line expressing the log–log relationship. So the $(R - B)/(A - B)$ measures the extent of derivatisation and is thus the most rational criterion. It is also a sensitive criterion; its relationships to both the extent of derivatisation and the slope of the 2,4-dinitrophenylhydrazine calibration line contribute to distinguishing determinand concentrations; this is crucial to an assay based on signal attenuation.

Optimising derivatisation temperature

Samples were aqueous 22.0% propan-2-ol solutions of 5.0×10^{-6} M 2,4-dinitrophenylhydrazine containing 1.0×10^{-5} M hexanal and 0.05 M sulfuric acid. 2,4-Dinitrophenylhydrazine (5.0×10^{-6} M) in aqueous 22.0% propan-2-ol containing 0.05 M sulfuric acid was the reagent blank. The blank was aqueous 22.0% propan-2-ol containing 0.05 M sulfuric acid. Samples and blanks were incubated at the stated temperature for 2 h and 10 μl injections made into the flow injection manifold for the 2,4-dinitrophenylhydrazine assay.

Optimising acid concentration for derivatisation

Samples were aqueous 22.0% propan-2-ol solutions of 5.0×10^{-6} M 2,4-dinitrophenylhydrazine containing 1.0×10^{-5} M hexanal and sulfuric acid at the stated concentration. The reagent blank was 5.0×10^{-6} M 2,4-dinitrophenylhydrazine in aqueous 22.0% propan-2-ol containing sulfuric acid at the stated concentration. The blank was aqueous 22.0% propan-2-ol containing sulfuric acid at the stated concentration. Samples and blanks were incubated at 30 °C for 2 h and 10 μl injections made into the flow injection manifold for the 2,4-dinitrophenylhydrazine assay.

Varying carrier pH *by buffering with sodium formate*

Samples were aqueous 22.0% propan-2-ol solutions of 5.0×10^{-6} M 2,4-dinitrophenylhydrazine containing 1.0×10^{-5} M hexanal and 0.05 M sulfuric acid. 2,4-Dinitrophenylhydrazine (5.0×10^{-6} M) in aqueous 22.0% propan-2-ol containing 0.05 M sulfuric acid was the reagent blank. The blank was aqueous 22.0% propan-2-ol containing 0.05 M sulfuric acid. Samples and blanks were incubated at 30 °C for 2 h and 10 μl injections made into the flow injection manifold for the 2,4-dinitrophenylhydrazine assay using the optimum conditions previously determined,[11] except the carrier composition was varied as shown by adding sodium hydroxide to 0.75 M formic acid to give a range of sodium formate concentrations up to 0.10 M.

Calibration for determination of hexanal

Samples were aqueous 22.0% propan-2-ol solutions of 5.0×10^{-6} M 2,4-dinitrophenylhydrazine containing 0.05 M sulfuric acid and hexanal at 1.0×10^{-6} M, 3.0×10^{-6} M, 8.0×10^{-6} M, 1.0×10^{-5} M and 2.0×10^{-5} M.

2,4-Dinitrophenylhydrazine (5.0×10^{-6} M) in aqueous 22.0% propan-2-ol containing 0.05 M sulfuric acid was the reagent blank. The blank was aqueous 22.0% propan-2-ol containing 0.05 M sulfuric acid. Samples and blanks were incubated at 30 °C for 2 h and 10 μl injections made into the flow injection manifold for the 2,4-dinitrophenylhydrazine assay.

Determination of carbonyl compounds in autoxidised linoleic acid

Fresh linoleic acid was dissolved in potassium borate buffer (pH 9.0) and made up to a 7×10^{-5} M solution, 0.10 M in buffer, that also contained 5.0×10^{-6} M 2,4-dinitrophenylhydrazine, 0.05 M sulfuric acid and 22.0% aqueous propan-2-ol. Linoleic acid autoxidised by heating in air at 40 °C for 2 h was likewise dissolved to give a 1.5×10^{-4} M solution that was otherwise the same as the fresh linoleic acid solution. The blank was 0.05 M sulfuric acid in 22.0% aqueous propan-2-ol. Reagent blanks were prepared that contained 5.0×10^{-6} M 2,4-dinitrophenylhydrazine, 0.05 M sulfuric acid and 22.0% aqueous propan-2-ol. A standard solution of 5.0×10^{-6} M hexanal was prepared that was otherwise the same as the reagent blank. The above mixtures were all incubated at 30 °C for 2 h. Successive 10 μl samples of each solution were injected into the flow injection manifold for the 2,4-dinitrophenylhydrazine assay. Final hexanal concentrations were calculated for each solution using the calibration equation determined in the last experiment.

Determination of carbonyl compounds produced from linoleic acid by lipoxygenase

Fresh lipoxygenase (0.04 mg ml^{-1}), 2.8×10^{-4} M linoleic acid and 0.20 M potassium borate (pH 9.0) were incubated together at room temperature for 105 min and made up to final concentrations of 0.02 mg ml^{-1} lipoxygenase, 1.4×10^{-4} M linoleic acid and 0.10 M potassium borate (pH 9.0), 5.0×10^{-6} M 2,4-dinitrophenylhydrazine, 0.05 M sulfuric acid and 22.0% aqueous propan-2-ol. To obtain a matrix for the blanks and standards comparable with that of the sample, lipoxygenase that had been completely deactivated by boiling was used. Thus a reagent blank was prepared that contained 0.02 mg ml^{-1} boiled lipoxygenase (instead of fresh) and otherwise the same constituents in the same final concentrations as the sample described above. An enzyme blank was prepared that comprised the reagent blank without 2,4-dinitrophenylhydrazine. Standards containing final hexanal concentrations of 1.0×10^{-4} M, 3.0×10^{-5} M and 1.0×10^{-5} M (the expected range of carbonyl compounds to be produced by lipoxygenase action) were made that otherwise were identical to the reagent blank. All six mixtures prepared as above were derivatised at 30 °C for 2 h before being assayed for chemiluminescence on successive 10 μl injections.

Results and discussion

Optimising derivatisation temperature

The results are shown in Fig. 1. Optimum temperature for incubation was 30 °C; above that temperature presumably the position of the equilibrium reduced the proportion of 2,4-dinitrophenylhydrazine reacting with hexanal, while below 30 °C,

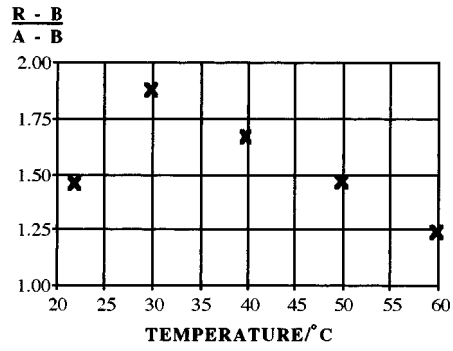

Fig. 1 The effect of temperature on the formation of hexanal 2,4-dinitrophenylhydrazone. (*A* is the mean signal for the analyte sample, *B* for the blank and *R* for the reagent blank. Samples were solutions of 5.0×10^{-6} M 2,4-dinitrophenylhydrazine in aqueous 22.0% propan-2-ol containing 1.0×10^{-5} M hexanal and 0.05 M sulfuric acid, incubated at the stated temperature for 2 h.)

the rate of reaction curtailed the extent of derivatisation within 2 h.

Optimising acid concentration for derivatisation

The results are shown in Fig. 2. Optimum derivatisation occurred in the presence of 0.05 M sulfuric acid (final concentration). We report elsewhere[12] that, in similar conditions, optimum spectrophotometric detection of hexanal 2,4-dinitrophenylhydrazone occurred at the same concentration of acidic hydrogen (0.10 M). The chemistry underlying this optimum is discussed below.

Varying carrier **pH** *by buffering with sodium formate*

The derivatised samples will be more acidic than the optimum for the assay for 2,4-dinitrophenylhydrazine. So the pH of the carrier was raised to neutralise the acid. If there was no sample dispersion, this would occur with a carrier containing 0.10 M sodium formate and 0.65 M formic acid. Therefore it was postulated that the optimum carrier would have a concentration between this and unbuffered 0.75 M formic acid, depending on the dispersion.

Both analyte and reagent blank signals showed a weak and inconsistent tendency to rise with carrier pH:

$$A = 4.794 + 22.5 \times [\text{HCOONa}] \quad r = 0.7344,\ n = 6$$

$$R = 6.437 + 21.5 \times [\text{HCOONa}] \quad r = 0.6464,\ n = 6$$

This was accompanied by a small, steady reduction in R/A as shown in Fig. 3, *i.e.*, analyte signals tended to rise by a slightly bigger factor than reagent blank signals did, as indicated in the regression equations. The effect of this is to reduce the sensitivity of the signals to changes in analyte concentration. Thus the hypothesis is found to be false; the optimum carrier is unbuffered 0.75 M formic acid.

The larger signals observed as carrier pH is increased can be explained by the greater rate of permanganate oxidation of formate ions, compared with that of undissociated formic acid; this enhances both background luminescence due to formate (the baseline is elevated by about 0.5 mV by 0.08 M formate, *i.e.*, at a pH of about 2.8) and energy transfer to the products of 2,4-dinitrophenylhydrazine oxidation. In the pH range studied, the latter tendency appears to be more pronounced. It also appears that this effect on 2,4-dinitrophenylhydrazine chemiluminescence is greater at low concentrations of this reagent, so the margin between analyte and reagent blank signals is reduced.

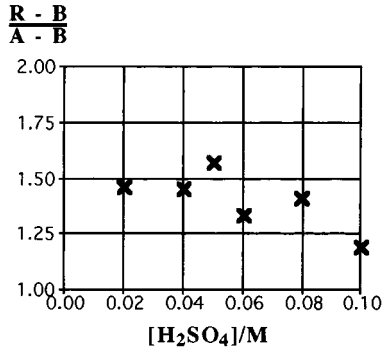

Fig. 2 The effect of acid concentration on the extent of formation of hexanal 2,4-dinitrophenylhydrazone. (*A* is the mean signal for the analyte sample, *B* for the blank and *R* for the reagent blank. Samples were aqueous 22.0% propan-2-ol solutions of 5.0×10^{-6} M 2,4-dinitrophenylhydrazine containing 1.0×10^{-5} M hexanal and sulfuric acid at the stated concentration, incubated at 30 °C for 2 h.)

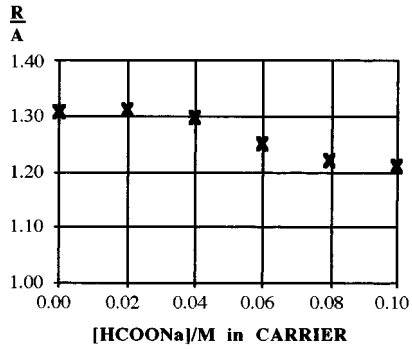

Fig. 3 The effect of buffering the carrier to vary its pH on the ratio of reagent blank (*R*) and analyte (*A*) signals obtained by oxidising residual 2,4-dinitrophenylhydrazine. (Samples were aqueous 22.0% propan-2-ol solutions of 5.0×10^{-6} M 2,4-dinitrophenylhydrazine containing 1.0×10^{-5} M hexanal and 0.05 M sulfuric acid, incubated at 30 °C for 2 h.)

Calibration for determination of hexanal

The results are set out in Table 1. log([hexanal]/M) *versus* log $\frac{R-B}{A-B}$ is linear from 1.0×10^{-6} to 2.0×10^{-5} M; ($r = 0.9931$, $n = 5$). The line of best fit has the equation:

$$\log\frac{R-B}{A-B} = 0.371 + 0.050\ \log([\text{hexanal}]/\text{M})$$

The standard deviations of the slope and intercept are respectively 0.003 and 0.0018.

Limit of detection (3*s* above blank) = 1.7×10^{-7} M (*i.e.*, 1.7 pmol hexanal detected).

Determination of carbonyl compounds in autoxidised linoleic acid

The results are set out in Table 2. Final carbonyl concentrations, expressed as hexanal, were calculated for each solution using the calibration equation already derived. The result for the standard indicated that no modification to this equation was required. The autoxidised linoleic acid was 58% oxidised, assuming one aldehyde molecule results from each linoleic acid molecule oxidised; the fresh linoleic acid was 0.33% oxidised, which is slight but significantly different from the reagent blank ($\nu = 8$, $t = 2.7121$, $p < 0.025$). The respective carbonyl concentrations of fresh and autoxidised linoleic acid, which differed by a factor of 180, indicate the wide range of

Table 1 Calibration data for the determination of hexanal from the attenuation of the chemiluminescent signal on formation of hexanal 2,4-dinitrophenylhydrazone. (Samples were solutions of 5.0×10^{-6} M 2,4-dinitrophenylhydrazine in aqueous 22.0% propan-2-ol containing 1.0×10^{-5} M hexanal and 0.05 M sulfuric acid, incubated at 30 °C for 2 h.)

[Hexanal]/M	Signal/ mV	*s*	RSD (%)	$\frac{R-B}{A-B}$
Reagent blank (*R*)	5.70	0.17	3.0	—
2.0×10^{-5}	4.36	0.24	5.5	1.36
1.0×10^{-5}	4.42	0.27	6.1	1.34
Reagent blank (*R*)	5.24	0.15	2.9	—
8.0×10^{-6}	4.16	0.23	5.5	1.31
3.0×10^{-6}	4.32	0.20	4.7	1.25
1.0×10^{-6}	4.54	0.21	4.6	1.18
Blank (*B*)	0.68	0.04	6.6	—

concentrations of lipid peroxidation products over which the chemiluminescence assay is applicable.

Determination of carbonyl compounds produced from linoleic acid by lipoxygenase

The results are set out in Table 3. The standards give a straight line which fits closely all three data points, as follows:

$$\log\frac{R-B}{A-B} = 3.907 + 0.730\ \log([\text{hexanal}]/\text{M})$$

The standard deviations of the intercept and slope are respectively 0.0128 and 0.0028. From this calibration it can be calculated that the lipoxygenase products had a carbonyl compound concentration, measured as hexanal, of 4.3×10^{-5} M, which represents 0.3 mol hexanal for each mole of linoleic acid originally present.

The calibration equation is radically different from the one that applies in the absence of the lipoxygenase–linoleic acid–buffer matrix. Signals are generally stronger, suggesting that one or more components is a source of oxidative chemiluminescence additional to 2,4-dinitrophenylhydrazine. There have been reports of other proteins emitting light on permanganate oxidation,[13] making the enzyme lipoxygenase a plausible candidate for this. Values of $(R - B)/(A - B)$ are not affected by this, as it applies equally to samples and blanks. Nonetheless, these values are much increased relative to hexanal concentration by the presence of the matrix; this increase is represented by the increased intercept constant in the regression equation. The increased slope of the regression line shows that the enhancement of $(R - B)/(A - B)$ gets greater as the hexanal concentration rises.

Table 2 Data for the determination of carbonyl compounds in fresh and in autoxidised linoleic acid and in a hexanal standard by the attenuation of the chemiluminescent signal on formation of 2,4-dinitrophenylhydrazones. (Samples were aqueous 22.0% propan-2-ol solutions of 5.0×10^{-6} M 2,4-dinitrophenylhydrazine containing the determinand and 0.05 M sulfuric acid.)

Sample	Signal/ mV	s	RSD (%)	$\frac{R-B}{A-B}$	[Hexanal]/M
Reagent blank (R)	5.44	0.25	4.6	—	—
5.0×10^{-6} M Hexanal	4.54	0.18	4.0	1.276	5.0×10^{-6}
7.0×10^{-5} M linoleic acid	5.08	0.16	3.2	1.095	2.3×10^{-7}
Reagent blank (R)	6.02	0.18	3.0	—	—
1.5×10^{-4} M Autoxidised linoleic acid	4.50	0.16	3.5	1.472	8.7×10^{-5}
Blank (B)	1.28	0.04	3.5		

Table 3 Data for the determination of carbonyl compounds produced from linoleic acid by lipoxygenase by the attenuation of the chemiluminescence of 2,4-dinitrophenylhydrazine. (The sample with final concentrations was the products of 0.02 mg ml^{-1} fresh lipoxygenase and 1.4×10^{-4} M linoleic acid pre-incubated in aqueous 0.10 M potassium borate, pH 9.0, and then mixed with 5×10^{-6} M 2,4-dinitrophenylhydrazine in 22.0% propan-2-ol that was 0.05 M in sulfuric acid.)

Sample	Signal/ mV	s	RSD (%)	$\frac{R-B}{A-B}$
Enzyme blank (B)	7.32	0.19	2.6	—
Reagent blank (R)	14.10	0.55	3.9	—
Lipoxygenase products (A)	8.62	0.30	3.5	5.215
Hexanal; 1.0×10^{-5} M	11.08	0.32	2.9	1.803
Hexanal; 3.0×10^{-5} M	9.00	0.37	4.2	4.036
Hexanal; 1.0×10^{-4} M	8.02	0.26	3.2	9.686

Discussion

Formation of hexanal 2,4-dinitrophenylhydrazone: optimum acid concentration

Nitrogen nucleophiles, RNH_2, react with carbonyl compounds by addition–elimination;[14] addition forms a carbinolamine, $>C(OH).NHR$, which is then dehydrated to an imine, $>C{=}NR$. Carbinolamine formation has three possible mechanisms:

(1) An addition reaction to form a a zwitterion, $>CO^-.N^+H_2R$, which is then transformed to the carbinolamine in one or more steps involving proton transfer.

(2) Association of reagents and catalyst to form an encounter complex, $>CO...RNH_2...HA$, which gives rise to associated zwitterion (zwitterion stabilised by catalyst) and then to carbinolamine.

(3) Concerted preassociation, in which the encounter complex converts directly to carbinolamine by two simultaneous bond formations.

The mechanism applying to any particular reaction depends on the basicity of the nucleophile, the reactivity of the carbonyl compound and the solvent. More basic nucleophiles react with reactive carbonyl compounds *via* zwitterion intermediates, which have reduced stability and lifetime if the nucleophile is less basic and/or the carbonyl compound less reactive. In all cases, the dehydration step is rate-limiting in less acidic conditions but the transition occurs at lower pH for more basic nucleophiles.

The formation of phenylhydrazones from aromatic aldehydes and ketones has been found[15] to fit this general scheme. In aqueous 50% ethanol, the concerted pathway, which is subject to hydronium ion catalysis, occurs only at pH <3 and uncatalysed stepwise carbinolamine formation occurs at pH 3 to 5. In aqueous 20% ethanol, the concerted pathway occurs only at pH <0 and zwitterion formation occurs at pH 0 to 5; at pH ≈ 1, there is a change of rate-determining step from uncatalysed zwitterion formation to the trapping of a proton by the zwitterion with general acid catalysis. General acid catalysis of phenylhydrazine ($pK_a = 5.3$) (including that by protonated phenylhydrazine)[16] is typical of moderately basic nucleophiles but not of hydroxylamine ($pK_a = 6.0$)[17] and other strong ones, for which, in acidic conditions, rate-determining carbinolamine formation proceeds *via* the zwitterion without catalysis. It has been suggested[15] that the higher the alcohol content of the aqueous solvent, the less the stability and lifetime of the zwitterion intermediate and therefore the more favoured the concerted mechanism. In the case of aqueous Girard T reagent,[18] $H_2N.NH.CO.CH_2.N^+Me_3\ Cl^-$, a weak nucleophile ($pK_a = 2.09$), hydronium ion catalysed carbinolamine formation occurs by concerted preassociation; there is no second break in the pH–rate profile, *i.e.*, no change in rate determining step, at pH ≈ 1. This is characteristic of the less basic nucleophiles ($pK_a < 3.5$).

In the formation of hexanal 2,4-dinitrophenylhydrazone, optimum derivatisation occurred at a final concentration of 0.05 M sulfuric acid; this would have pH a little above 1.0 due to incomplete second ionisation. It has been independently found[12] that optimum spectrophotometric detection of hexanal 2,4-dinitrophenylhydrazone occurred when the derivatisation mixture contained 1.0% concentrated hydrochloric acid, borate buffer and 37.5% propan-2-ol and was incubated for 2 h. This represents the same concentration of acidic hydrogen (0.10 M), but due to the presence of the borate buffer, it corresponded to a pH of about 1.3. Optimum derivatisation must be related in some way to the conditions which lead to maximum rate of reaction, so this is consistent with the mechanism proposed by Somera *et al.*[15] for arylaldehyde phenylhydrazone formation in

aqueous 20% ethanol, in which the rate determining step of the stepwise pathway changes at pH ≈ 1 from uncatalysed zwitterion formation to zwitterion protonation with general acid catalysis.

The model will apply to 2,4-dinitrophenylhydrazone formation as follows. The concerted mechanism is hydronium ion catalysed; provided that the prevailing pH is well below the pK_a, the free 2,4-dinitrophenylhydrazine concentration increases by the same ratio as hydrogen ion activity (a_H) decreases and the resulting rate is thus independent of hydrogen ion activity. The addition step (which forms the zwitterion) increases in rate as the concentration of free 2,4-dinitrophenylhydrazine increases; in conditions (pH > 0) in which this rate exceeds that of the concerted pathway, the stepwise mechanism is the prevailing one. The maximum rate occurs at the point at which protonation, the rate of which falls as a_H falls, becomes rate limiting; at this point (pH ~ 1) the rate of zwitterion formation is equal to its rate of protonation. The similarity between the kinetic behaviour of 2,4-dinitrophenylhydrazine (pK_a = 2.8) and the more basic phenylhydrazine can be understood in terms of the more electrophilic nature of hexanal and other alkanals (due to the absence of delocalisation of the charge on the carbonyl carbon) compared with the arylaldehydes studied by Somera *et al.*[15] Propan-2-ol content of the reaction medium was kept constant at 22.0%, the optimum concentration for the 2,4-dinitrophenylhydrazine assay; this appears to be similar in its effect to 20% ethanol as the medium for arylaldehyde phenylhydrazone formation.

However, the optimum detection of hexanal 2,4-dinitrophenylhydrazone by spectrophotometry was, in fact, a broad plateau covering a range of final concentrations of 1% to 4% concentrated hydrochloric acid. This pattern was not observed with chemiluminescent detection, which suggests that the signals at final sulfuric acid concentrations above 0.05 M (pH < 1) might be diminished by effects on the 2,4-dinitrophenylhydrazine chemiluminescence assay rather than by effects on the extent of derivatisation.

As the analyte signal was in all cases well above the blank, it may be assumed that the derivatisation reaction was incomplete in the 2 h available for it. But as equilibrium is approached, the rate falls, not only due to the drop in reactant concentration but also because of the increase in product concentration and consequent rate of back reaction. Thus the equilibrium constant curtails the extent of derivatisation even though equilibrium is not reached. So the extent of derivatisation during the 2 h incubation depends on both kinetic and equilibrium factors; it is these factors in combination that are optimally favourable at pH ≈ 1.

Conclusion

(1) The oxidative chemiluminescence of 2,4-dinitrophenylhydrazine can be applied to the determination of aldehydes and ketones by utilising the attenuation of the signal which occurs as 2,4-dinitrophenylhydrazine is converted to the corresponding phenylhydrazone.

(2) The optimum conditions for 2,4-dinitrophenylhydrazine to be converted by a carbonyl compound to the 2,4-dinitrophenylhydrazone are a temperature of 30 °C and the presence of 0.05 M sulfuric acid. Although this latter departs from the optimum conditions for the assay of 2,4-dinitrophenylhydrazine, no advantage is gained by buffering the formic acid carrier in order to neutralise the excess acid.

(3) The log–log calibration for the determination of hexanal is linear (r = 0.9931, n = 5) from 1×10^{-6} to 2×10^{-5} M, the equation for the regression line being:

$$\log\frac{R-B}{A-B} = 0.371 + 0.050\ \log([\text{hexanal}]/\text{M})$$

[A is the analyte signal, R is the reagent signal and B is the blank signal. $(R - B)/(A - B)$ is thus the reagent : analyte signal ratio, net of blank.] The standard deviations are 0.018 for the intercept on the y-axis and 0.003 for the slope. The limit of detection calculated at 3 s above blank was 1.7×10^{-7} M. This implies the detection of 1.7 pmol of hexanal.

(4) The attenuation of the oxidative chemiluminescence of phenylhydrazines was applied to the determination of aldehyde products of autoxidation both in fresh linoleic acid (0.33%) and after 2 h autoxidation (58%).

(5) The aldehyde products of lipoxygenase action on linoleic acid (30% conversion to hexanal) were also determined by this procedure. In this determination, the assay had to be redesigned to provide comparable matrices for samples, standards and blanks.

(6) The optimum conditions for 2,4-dinitrophenylhydrazone formation can be understood in terms of a stepwise mechanism of carbinolamine formation *via* a zwitterion intermediate. The extent of derivatisation during the 2 h incubation depends on both kinetic and equilibrium factors; these factors in combination are optimally favourable at pH ~ 1.0.

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