CHROM. 5236

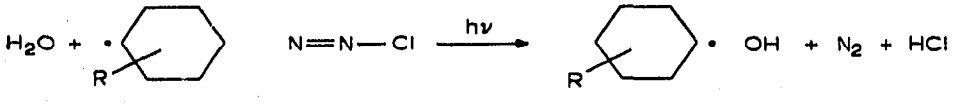
Stabilisation of the diazo reaction: Its application in cellulose acetate electrophoresis

Since the pioneer researches of PAULY[1] on diazotisation and coupling reactions, briefly designated as the "Pauly Diazo Reaction", this method has found wide applications in biological and clinical chemistry. As a great many different quantitative measurements are based upon this method it is very important to standardise the reagents and reactants to reduce quantitative errors to the minimum. Both the diazo reagent and the reaction itself are very unstable and it is difficult to avoid considerable fluctuations in results.

According to SHOSTAKOWSKI *et al.*[2] diazo compounds polymerise and form precipitates. SIMON AND BADILESCU[3] investigated the kinetics of thermal and photochemical decomposition of diazo salts. The solvent has been found to decompose the diazonium ion into nitrogen and a carbonium ion by rearranging or eliminating a proton[4] in certain cases. TOMCHIN AND PORAI-KOSHITS[5] found that during diazotisation of many aromatic compounds oxidation and not diazotisation is prevalent giving a certain amount of internal coupling products which can quantitatively influence the colorimetric evaluation of the real diazo compounds. Similar results were found by MATRKA *et al.*[6]. As regards reactants, *e.g.* aromatic amines, HAIS AND MACEK[7] found, in alkaline and even in neutral medium, that some amines are subject to interfering oxidation; brown or violet products of oxidation result. In different biological systems (*e.g.*, enzyme reactions) the diazo reagent will not only react with the given substrate (*e.g.* the aromatic amine) but also with other compounds, as was found by BURTON AND WALEY[8] for the lysine residue of aldolase and the tyrosine residue of an isomerase. MICHAELSON AND MICHAELSON[9] also described a coloured compound of a diazo salt with ascorbic acid. FEULGENHAUER AND GLENNER[10] found some interference with the colour intensity of the diazo reaction on the addition of sulphydryl compounds, such as cysteine and β-mercaptoethanol. The effect was linear with the concentration and corrected standard curves can be used with these materials. In a very early report DIEMER AND FOX[11] mentioned colour complexes between histidine and histamine and diazo salts. It was therefore necessary to find a practical modification to avoid misinterpretation of both qualitative and quantitative results of a diazo reaction. This modification would involve stabilisation of the diazo reagent, and elimination of interfering substances from the reaction mixture.

Material and methods

The necessity of always preparing and using a very fresh diazo reagent is the result of observations that the highly reactive diazonium salt will react with a second organic compound containing a carbon atom of high electron density to yield a diazo compound, with the elimination of HCl (if it is a hydrochloride). If, as a result of any delay the diazo salt becomes irradiated, it decomposes with the formation of nitrogen and in the presence of moisture forms the corresponding phenol:



This photochemical step is inherently sensitive to UV and blue light and is an oxidative process. The phenol, thus created, readily reacts with the diazo salt present by coupling. The product of such diazo coupling is highly conjugated and not water soluble and forms a precipitate. The diazo salt reagent must therefore always be freshly prepared, and even then its stability is not longer than *ten minutes*. This disturbing fact presents great difficulties especially if the reaction is to be used for quantitative spectrophotometric measurements. Stabilisation can be achieved, however, by removal of a proton from the carbon atom that holds the diazonium group. This can be done by adding proton acceptors, *e.g.* formaldehyde or formamide or dimethylformamide, to the freshly dissolved diazo salt. The solutions can be left at room temperature, in ordinary flasks for weeks and the time when any precipitate was observed is recorded. At the same time a weekly spectrophotometric measurement of the pale yellow colour was registered. We used different molar concentration of formaldehyde and dimethylformamide to find the optimal concentration of each of these reagents. The diazo salt used in this test was diazo-*p*-nitroaniline in a 0.01 *M* aqueous solution.

In our enzymological studies we have studied various enzymes which hydrolyse acyl or acylamino naphthylamide. Liberated naphthylamine was quantitatively measured by spectrophotometry after forming a diazo colour. A study of the kinetics of inhibition was done by different inhibitors. Many of them contain sulphydryl groups, and others contain hydrazide or hydrazone groups. As we have already mentioned sulphydryl groups inhibit the diazo reaction, and hydrazide forms coloured compounds with diazo salts.

In such cases removal of the free naphthylamine eventually produced and then separate coupling with diazo salt was necessary. This operation was in many cases impossible because the inhibitors were extracted together with the naphthylamine and so we again get errors. To avoid this interference, we have dissolved acyl- or aminoacyl naphthylamine in diethyl ether with a wetting agent of a very low HLB (hydrophile–lipophile-balance) in our case SPAN 80 which has an HLB of 1.8 and impregnated cellulose acetate strips with this solution which evaporated very quickly. These strips were immersed in enzyme solution containing various inhibitors, and after incubation, the strips were washed with water till all traces of the enzyme and inhibitors disappeared. The washed strips were now put in the diazo reagent, and within few minutes diazo colour developed to the maximum intensity. After further washing, the strips are either scanned or dissolved in dry ethyl acetate, a solution which is very stable, and measured in a spectrophotometer against a control cellulose acetate strip dissolved in ethyl acetate. By this method it was possible to estimate naphthylamidase activity units from the pherogram on cellulose acetate strips.

Results

Both formaldehyde and DMF are good stabilising agents for diazo salt solutions. DMF is better even than formaldehyde in that, that even when a precipitate has already formed by polymerisation it will dissolve and disappear. It seems that the formyl residue acts as the functional group in both cases, and probably enters into a conjugation reaction with the attacking Ar-N_2Cl compound. In Fig. 1, optical density readings for both stabilisers over a period exceeding three months are given. Excess of formaldehyde or DMF inhibits formation of the diazo dye.

Testing different concentrations of both α- and β-naphthylamine dissolved in

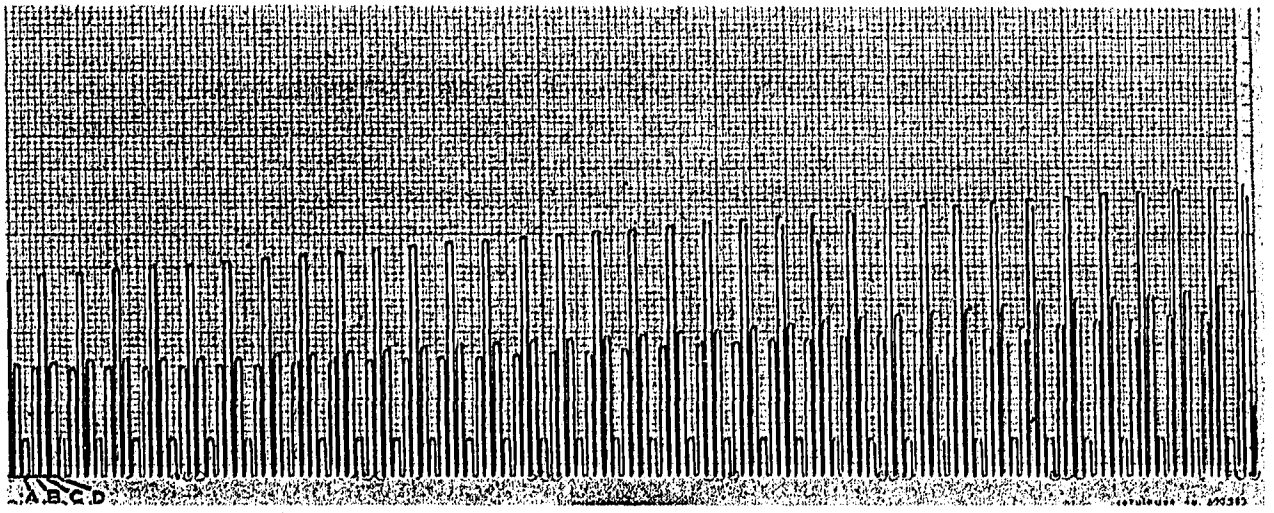


Fig. 1. Optical density readings over a period exceeding three months. *p*-Nitroaniline, diazotised 0.02 *M*. Adsorption at 450 nm; group time interval 15 min. (A) Equimolar formaldehyde; (B) 0.5 equimolarity; (C) 0.1 equimolarity; (D) 0.25 equimolarity.

1% SPAN 80 in diethyl ether and adsorbed to 1-cm² strips of cellulose acetate (Shandon — Celagram) we have found that the amounts of naphthylamine did not diminish after intensive washing with water from the unwashed control strip. The same was observed in cases when the naphthylamine strips were previously incubated with: cysteine, mercaptoethanol, *p*-CMB, INH, hydrazide hydrate, PAS, streptomycin, BAL, H_2S water and solutions of NaF, $CuSO_4$, $ZnSO_4$, $CdSO_4$, $HgCl_2$, $FeSO_4$, $KMnO_4$ and H_2O_2.

The diazo reaction is positive only after washing well, and only in this case does the colour intensity not diminish. After washing and drying the cellulose acetate strips were dissolved in ethyl acetate and the optical density was measured at the maximum absorption line. Optical density increased linearly with increasing amount of naphthylamine applied on the cellulose acetate strip according to Beer's law. It was possible to estimate the activity or inactivation of any naphthylamidase from any biological extract by this method, without the risk that any constituent of this source would interfere with the diazo reaction, because all other products except naphthylamine are washed out.

Discussion

The products of *photodecomposition* of aqueous solutions of diazonium salts, $R\text{–}N \equiv N^+ X^-$, seem to be explained by a simple cleavage of the salt to form molecular nitrogen and a carbonium ion which hydrates to form a phenol as the final product, or combines with a negative ion (*e.g.* Cl^-) at a high concentration of added ions. In less polar solvents, such as ethanol solutions, the formation of free radicals by electron transfer in the primary act of dissociation may compete favourably with the ion-forming process. Presumably this ion may be an anion, such as Cl^- or a solvent molecule which is oxidised as the diazonium ion is reduced. The quantum efficiency of nitrogen formation is a function of the molecular structure of the diazonium salt but no satisfactory comprehensive theory of these effects is available at this time. The reaction with mercaptans involves attack of the –SH ion by the diazonium ion. The alkalinity required to produce –SH ion has an untoward action on the diazonium compound,

however, eventually converting it to a form that is ineffective for coupling. The regulation of acidity of the solution is therefore achieved by the addition of reducing agents as formaldehyde, DMF, etc., which help (in this capacity) to produce azo dyes.

According to CALOUT AND PITTS[12] the only group which causes no trouble in diazotisation of *p*-nitroaniline, *m*-nitroaniline, 4-amino-3-nitrotoluene, and 2-amino-5-nitrotoluene are aldehyde groups, but they can be diazotised only in a high concentration of acid.

We were able to obtain a diazo reagent which was stable over a long period by suitable additions of formaldehyde or DMF to those salts, and this phenomenon has been more or less explained here. A special technique for the estimation of free naphthylamine by diazo coupling, despite the fact that the enzymatic reaction used for naphthylamine liberation was carried out in the presence of –SH groups, was rendered possible by immersing water insoluble naphthylamine substrates (adsorbed on cellulose acetate strips) in the enzyme-inhibitor mixture. After incubation the strips are washed to remove enzyme and inhibitors (mercaptan and hydrazide), and the remaining free naphthylamine which reacts with the diazo reagent can be dissolved in organic solvents (ethyl acetate) and its optical density measured.

This technique can be of great advantage in electrophoretic studies of enzymes which liberate naphthylamine from different substrates.

In the textile and dyestuff industry different methods have been used for the stabilisation of diazo salts, for example $ZnCl_2$ double salts, methylsulphonates, boron fluorides and 1,5-naphthalenedisulphonate. These stabilisers facilitate solubility of some difficultly soluble diazo salts, and prevent for a short time the precipitation of polymerisation products. $ZnCl_2$ and boron fluoride stabilisation have been studied spectrophotometrically by the author, but a change of colour O.D. was found with time. This is of course of not much importance in the textile industry but it is not a suitable method for quantitative analytical work. HOUBEN AND WEYL[13] mentioned the interreaction of diazonium salts with formaldehyde and DMF without any revelation about the stabilising effect of both agents on diazonium salt solutions.

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First received October 28th, 1970; revised manuscript received January 5th, 1971