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ELECTRICALLY ACTIVATED GASES AS VISUALIZING AGENTS FOR THIN-LAYER CHROMATOGRAPHY

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SUMMARY

The energetic species produced by a high-voltage discharge in low-pressure nitrogen, argon or helium can activate organic compounds spotted on thin-layer silica gel chromatoplates. These can be induced to fluoresce by subsequent exposure to volatile reagents like ammonium hydrogen carbonate vapors or ammonia. Similar results are obtained using nitrogen or nitrogen–argon mixtures fed through high-voltage sparks at atmospheric pressure. Activated compounds can be caused to fluoresce many hours after original exposure. Nanogram quantities of many compounds can be detected. Quantitation data are presented for two structurally diverse organic compounds.

INTRODUCTION

Previously we reported[1] that fluorescence could be induced in organic compounds spotted on thin-layer silica gel chromatoplates. The procedure consisted of placing the spotted chromatoplate in an evacuated chamber (0.2 torr) containing nitrogen or ammonium hydrogen carbonate, and then applying a 20,000-V discharge for times ranging from a few seconds to several minutes. When these plates were removed from the chamber and heated at 130° in air, the spots were found to fluoresce in the visible (under a 365-nm UV lamp, examined by eye).

In an effort to further our understanding of the phenomena involved, we undertook a series of experiments in which several factors were explored. These included the use of "active" nitrogen produced at atmospheric pressure, the potential for other gases as activating agents at low pressure, and exposure of activated spots to vapors which would result in, or enhance fluorescence.

In addition, we wished to measure the quantitative aspects of such a procedure using specific compounds. In previous work, we had reported a qualitative trend in fluorescent intensity with the quantity of substance used.

EXPERIMENTAL

Compounds, solvents, and reagents

The majority of the experiments were carried out with *n*-$C_{22}H_{46}$ hydrocarbon

in chloroform solution, and chlorpromazine in hexane–1-butanol solution. These were obtained from laboratory sources.

All solvents used to dissolve these substances (or dilute their solutions) were of analytical or spectroquality grade. Control experiments were done frequently with solvents alone to check for artifacts due to solvent impurities in the thin-layer plates.

Ammonium hydrogen carbonate and maleic anhydride, when employed, were obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.). Nitrogen, argon, helium, hydrogen, oxygen and ammonia gases were obtained from IWECO (Houston, Texas, U.S.A.).

Thin-layer chromatoplates

All of the experiments were conducted with "Permakote" thin-layer chromatoplates (Applied Science Labs., State College, Pa., U.S.A.). In these plates, the silica gel is bonded to the glass surface by a sintering process, and they contain no organic binder. The layer thickness was 0.23 mm. They can be reused by treatment with hot chromic acid, without noticeably changing their properties as chromatoplates. The use of these plates makes it possible to achieve an absolute minimum of fluorescent background. Other commercial plates appear to contain sufficient organic binder or impurities which result in a high fluorescent background.

It should be noted that even these plates varied in intrinsic fluorescent background after chromic acid treatment, presumably owing to inorganic fluorescent trace substances. The activity of the silica gel is rated as "moderate" by the company.

Prior to use, chromatoplates were stored in absolute ethanol. They were dried by gentle heat and then cooled to room temperature before spotting experiments were carried out.

Test procedures

For those experiments concerned with testing the effect of different activating gases in the vacuum discharge chamber, solutions containing 1 μg per 1 μl of solvent were used, with 1 μl of solution being applied. The same solutions and amounts were used in atmospheric pressure "plasma" experiments with nitrogen or nitrogen–argon mixtures. Solvents were tested from time to time, side by side with solution, and observed for possible fluorescent artifacts after activation.

The vacuum electrical discharge chamber was operated at 8.0 torr, with the test specimen of the chromatoplate placed between capacitively coupled electrodes. The time for operating the chamber ranged from 30 sec to 3 min, depending on the amount of the compound applied. After removal from the chamber, the plate was placed on a hot plate (surface temperature about 130°). For most experiments, about 2 g of ammonium hydrogen carbonate were placed adjacent to the specimen plate, and then covered with a beaker. In this way the decomposition products of ammonium hydrogen carbonate filled the beaker and enveloped the chromatoplate. The development of fluorescence was observed under an intense 365-nm UV lamp (Ultraviolet Products) for 3 min. The chromatoplate was then removed for observation or measurement in a spectrophotometer.

When ammonia gas was tested as a fluorogenic reagent, it was admitted through a glass funnel sitting on the hot plate directly over the chromatoplate.

Atmospheric pressure tests were conducted by flowing the test gas past steel

electrodes in a tee configuration, across which a 20,000-V, 0.5-MHz supply was connected. Flow-rates were varied from 5 to 30 l/min, depending upon the specific experiment. The effluent, activated gas was then directed at right angles to a spotted chromatoplate, at a distance of 2 mm from the plate surface. Almost all experiments were of 5-min duration. In several other atmospheric pressure experiments, the effluent gas was directed into a funnel arrangement under which the test chromatoplates were placed. The above described tee configuration was also supplied with a side arm, through which gases could be admitted to mix with the test gas, following activation in the spark gap.

Vacuum electrical discharge chamber

A schematic diagram of the vacuum electrical discharge chamber is shown in Fig. 1. The electrical plasma was produced by a Tesla coil-type, high-frequency generator rated at a maximum of 20,000 V, 0.5 MHz (Model BD-40, Electro-Technic Products). The high-frequency output was coupled to the chamber capacitively by simply wrapping aluminum foil externally around two sections of the central glass tube, to which the electrical connections were made. When in operation, an intense glow filled the tube within and between the aluminum foil-covered regions. Chromatoplate test specimens were placed midway between the foil electrodes. Provision was made to allow a selected test gas to be admitted to the chamber through a PTFE valve. The operating pressure was 8.0 torr.

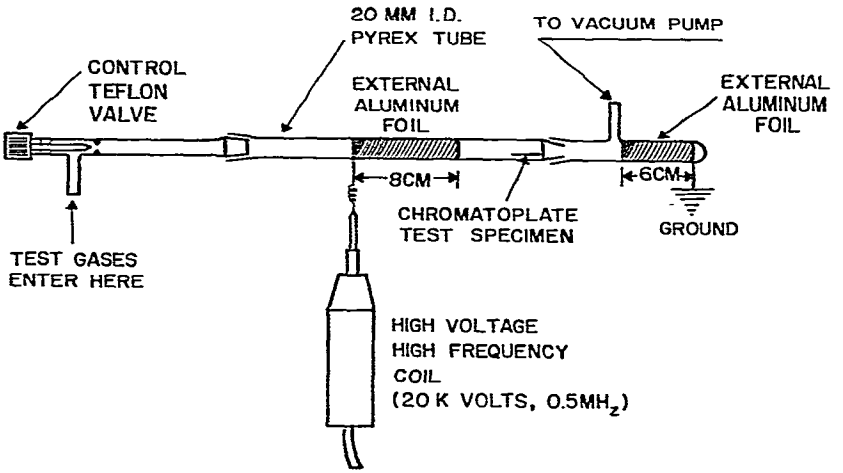

Fig. 1. Schematic diagram of vacuum electrical discharge chamber.

Atmospheric pressure "plasma" jet apparatus

Fig. 2 schematically illustrates the three apparatus configurations used for atmospheric pressure tests. The (a) configuration consists of a tee tube arrangement, with two steel electrodes introduced at right angles to the flow direction of the gas to be activated. The gas passes through the spark between the electrodes, which was derived from the same high-voltage source used in the vacuum apparatus of Fig. 1. The electrode spacing was 1 cm. A side piece was added downstream of the spark

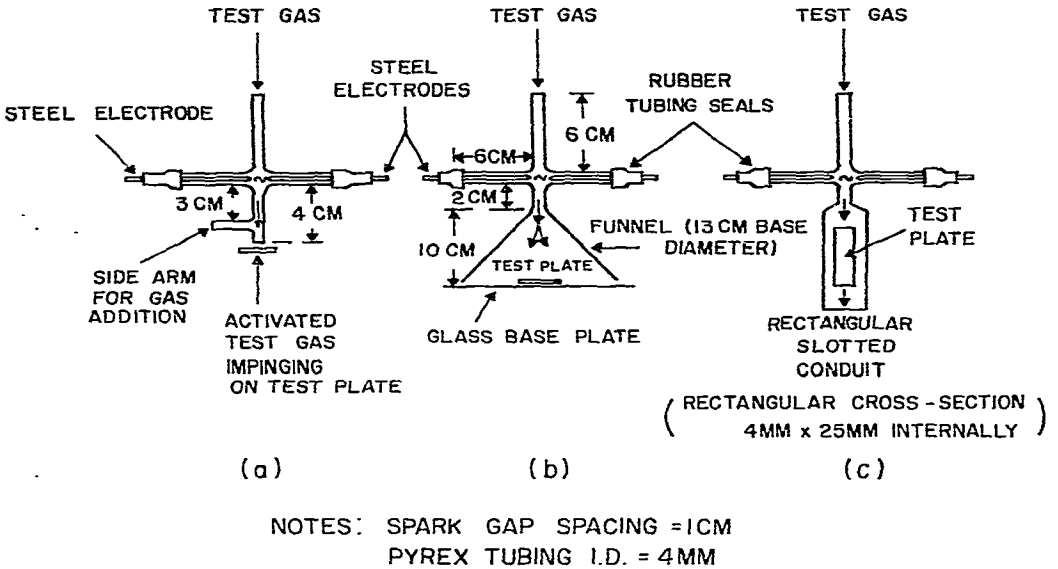

Fig. 2. Schematic diagram of three configurations used for atmospheric pressure electrical activation of gases.

gap to permit the introduction of other gases without their having to pass through the discharge.

The second configuration (b) consists of a similar tee arrangement, attached to a funnel. During operation, the funnel was placed over a sheet of glass in the inverted position with the gas flow directed downward. Test chromatoplates were placed on the glass sheet, approximately in the center of the funnel.

A third configuration (c) allows the activated gas to enter a chamber of rectangular configuration into which test plates were placed. In this way a number of spots could be tested at varying distances from the spark gap activation source.

Measurement of fluorescent intensity

Qualitative estimates of fluorescent intensity were used in experiments concerned with the effects of various activating gases, quenching gases, or the use of fluorogenic reagents. Fluorescence for these experiments was rated by eye on a 1 to 10 scale of intensity (1 = weak, 10 = strong).

Quantitative measurements were carried out for two compounds using a Carl Zeiss KM3 chromatogram spectrophotometer, at the peak emission intensity from the fluorescent spot (illuminating wavelengths 360–370 nm). Both peak signal height and integrated peak area were measured.

RESULTS AND DISCUSSION

Fluorogenic reagents following activation

In our earlier work[1], fluorescence was induced in organic compounds on the chromatoplates in a two-step procedure. The plate was first placed in the vacuum chamber and exposed to an electrical discharge at 0.2 torr in nitrogen or ammonium

hydrogen carbonate vapors. Following removal, it was placed on a hot plate in air at about 130°. Usually, moderate fluorescence was observed when removed from the chamber, and the subsequent heating enhanced the intensity.

In the work reported here, we have found that superior results are obtained by: (a) subjecting the plate to a higher-pressure (8.0 torr) electrical discharge (we term this the "activation" phase), and (b) exposing the plate to the vapors of substances capable of producing or enhancing fluorescence in the activated material. Our basis for the term "activation" will be described in the section dealing with activation by various gases.

One of the best fluorogenic agents found to date are the vapors from ammonium hydrogen carbonate. Typically, the solid ammonium hydrogen carbonate is placed adjacent to the activated chromatoplate on the hot plate, and both are covered with a beaker to confine the vapors. Slow decomposition occurs producing ammonia, water, and carbon dioxide. Within 1 min, substantial intensification of fluorescence occurs with most compounds. Three minutes was the usual exposure period used. For some compounds, removal of the chromatoplate from the hot plate and cooling to room temperature, resulted in more intense fluorescence (*e.g.* chlorpromazine, methadone, or phenobarbital). One-microgram spots of *n*-$C_{22}H_{46}$, chlorpromazine, methadone, or phenobarbital all gave a fluorescence intensity rating of 10 with ammonium hydrogen carbonate vapors. If the ammonium hydrogen carbonate solid is laid directly on the activated spots, the final intensity is even greater. This procedure, however, does not give as uniform or reproducible results.

Ammonia gas alone also was fluorogenic although the final intensity tended to be lower than that of ammonium hydrogen carbonate vapors (rating of about 8 for 1-μg spots of the previously named substances). Carbon dioxide or nitrogen containing water vapor were no more effective than heating alone in an atmosphere of helium. This indicates that it is primarily the ammonia arising from the decomposition of ammonium hydrogen carbonate that reacts in some manner with compounds activated on the silica gel chromatoplates. Other ammonium salts, such as ammonium fluoride, ammonium acetate and ammonium thiocyanate, show substantially the same effect as ammonium hydrogen carbonate.

A limited number of tests were done on *n*-$C_{22}H_{46}$ (1 μg) activated spots using maleic anhydride in place of ammonium hydrogen carbonate. The result was the most intense fluorescence thus far observed by eye, although the chromatoplate background increased substantially.

It should also be noted that the oxygen of the air, or pure oxygen, also produces fluorescence intensification of activated spots.

These tests suggest the possibility of using substances other than ammonium hydrogen carbonate to "couple" with the activated compounds for producing even more intense fluorescence and higher sensitivity.

Activation with vacuum electrical discharge

A number of experiments with 1-μg spots of *n*-$C_{22}H_{46}$ were carried out in the vacuum electrical discharge apparatus (Fig. 1) in order to determine whether there was anything unique in the use of nitrogen for ultimately inducing fluorescence. This was a tempting conclusion from earlier work in which argon, for example, gave only extremely weak fluorescence based on the old procedure[1].

Argon was first used to replace nitrogen and the anticipated faint (to nearly absent) fluorescence resulted when the plate was first removed from the chamber. However, when the chromatoplate was exposed to the vapors of ammonium hydrogen carbonate according to the present procedure, high-intensity fluorescence developed (rating 10). The same result was obtained with helium.

Clearly it is not necessary for chemical reaction to occur between the species present in the electrical discharge and the organic compounds on the chromatoplates. Argon and helium appear to act merely as energy-transfer agents, "activating" the organic substances in some as yet unknown manner. Very likely a significant part of the action of nitrogen is that of energy-transfer, although there appears to be some direct reaction as well. Perhaps bonds are broken resulting in free radicals of unusually long life, although this is highly speculative.

Some "lifetime" tests of this activated state were carried out, to determine the rate at which the unknown activated species decayed, and would no longer give fluorescence with ammonium hydrogen carbonate vapors. In one series of tests (1 μg, n-$C_{22}H_{46}$) plates which had been activated with nitrogen, argon and helium were left in the open air at room temperature (20°) for 17 h before exposure to ammonium hydrogen carbonate vapors. It was observed that some fluorescence had already developed at the end of this period owing to air alone (rating = 4). Following exposure to ammonium hydrogen carbonate vapors, the spots developed their full fluorescent intensity (rating = 10). Similar plates left in an argon atmosphere for 3 h, also came up to full fluorescent intensity when subsequently exposed to ammonium hydrogen carbonate vapors. Storage under vacuum conditions for the same time period gave the same result.

With hydrogen as the test gas, the plates came out of the chamber without noticeable fluorescence, and were extremely weak in intensity after ammonium hydrogen carbonate treatment (rating = 1). This accords with previous observations, where hydrogen in an electrical discharge appears to alter the organic compounds in some manner that precludes the production of fluorescent substances by this procedure.

Pure ammonia or pure oxygen behaves in the same manner as hydrogen in the vacuum discharge chamber.

Chemical decomposition by hydrogen or ammonia under these conditions must play a significant role, since re-exposure of plates to a nitrogen discharge environment followed by ammonium hydrogen carbonate treatment shows a negligible production of fluorescence. The action of oxygen in causing the rapid disappearance of organic compounds can be attributed to active oxidation to gaseous products.

Activation electrically at atmospheric pressure

When nitrogen is subjected to an electrical discharge under moderate vacuum conditions, a number of energetic species can be generated. This may include nitrogen atoms, N_2^+ and the relatively long-lived, electronically excited triplet state of the nitrogen molecule (the $A^3\Sigma_u^+$ state)[2]. Under vacuum conditions, this triplet state has a radiative lifetime of as much as 10–12 sec. Further, it lies more than 6 eV above the ground state (*ca.* 138 kcal). It is known that energy transfer to other atomic or molecular species is possible from this triplet state[2]. At atmospheric pressure its radiative lifetime is of the order of many milliseconds.

The potential convenience of operating at atmospheric pressure for visualizing

chromatoplates, led us to conduct a number of experiments using the three apparatus configurations shown in Fig. 2. Nitrogen (or nitrogen plus additive gases) was passed through the spark discharge powered by the same high-voltage source used in vacuum experiments. The flow-rate was approximately 5 l/min in most cases (*ca.* 20°, 1 atm pressure).

When the high voltage was turned on and nitrogen passed through the spark (1 cm long), a light purple jet was distinctly visible for a distance of more than 5 cm downstream from the spark. It could be seen issuing into the air in the case of configuration (a), and the production of ozone was evident by the odor produced. In configurations (b) and (c) the glow filled the funnel and slotted conduit chamber respectively, although the color now observed was a pale ochre, rather than light purple. All of these observations are a direct reflection of the long-lived metastable excited state of the nitrogen molecule.

The (a) configuration was the first one used in a number of experiments. A 1-μg spot of *n*-$C_{22}H_{46}$ was applied to a chromatoplate, then brought within 3 mm of the exit of the excited nitrogen gas jet. The exposure time was 5 min. At the end of that period, a faintly fluorescent spot was observed, which subsequently was exposed to ammonium hydrogen carbonate vapors. The fluorescence brightness level closely approached that attained in the vacuum discharge procedure (rating = 9). If the test chromatoplate was kept at a greater distance from the exit (*e.g.* 10 mm), the final fluorescence was much weaker. This is attributed to the mixing of oxygen from the air which had already been shown to be destructive. (It was this factor which led us to construct the (b) configuration.)

Other test gases were tried with the (a) configuration. Ammonia or argon produced no significant fluorescence. The result with ammonia is not unexpected in view of similar data in a vacuum discharge. The fact that argon alone did not produce activation under these conditions, whereas it does so in a vacuum discharge, is probably due to the relatively short lifetime of the excited argon species at atmospheric pressure, compared with excited nitrogen. An argon–nitrogen (*ca.* 20:80, v/v) mixture produced excellent fluorescent spots (rating = 10). When argon was mixed with nitrogen the character of glowing gas changed to a slightly pinkish cast and appeared to be more dense. This was especially evident in the (b) configuration, where the funnel would fill with the glowing gas.

When the nitrogen was pre-saturated with water vapor, the after-glow was absent and no noticeable fluorescence was produced. Thus, water vapor quenches the active species produced in the spark zone. Oxygen and ammonia were found to do the same. The same effect could be seen in two stages by admitting these quenching gases through the side arm shown in Fig. 1 (a). With pure nitrogen, the usual after-glow was observed downstream of the spark, and at the point where water vapor (in nitrogen), ammonia or oxygen was added, the glow would no longer be visible.

One other experiment of interest was done with the (a) configuration. Nitrogen was the test gas, with a small flow of pure methane (*ca.* 50 ml/min) admitted through the side arm. Methane did appear to produce a partial quenching of the nitrogen after-glow. When a blank chromatoplate was placed at the exit point in the usual manner, and later exposed to ammonium hydrogen carbonate vapors, a very dense fluorescence of high intensity was evident over a wide area of the plate. This fluo-

rescent material, like that produced with other substances in regular spotting tests, was readily eluted by pure water with an R_F value approaching unity.

Experiments done in the (b) configuration were generally the same as those reported for the (a) configuration. It offers the convenience of handling larger chromatoplates, without incurring contamination by the ambient air. Similar results were obtained, except that the final fluorescence was weaker than that in the (a) tests. This appears to be simply a function of the density of excited nitrogen species. Longer exposure times produce more intense spots, but no experiments were conducted beyond 10-min duration. The fluorescent rating for n-$C_{22}H_{46}$ (1 μg) under these conditions was 5.

In the (c) configuration, test chromatoplates were spotted with three 1-μg spots on n-$C_{22}H_{46}$ in a straight line, and arranged in the rectangular chamber in the direction of flow. Thus the spots varied in distance from the spark source (2, 3, and 4 cm, respectively). After a 5-min exposure to nitrogen after-glow, followed by ammonium hydrogen carbonate treatment, the fluorescent intensity of the spots diminished regularly with distance from the spark source (ratings of 8, 7, and 6, respectively). This rectangular slot configuration was selected in order to provide as high a concentration as possible of excited nitrogen species over the chromatoplate.

Miscellaneous observations

A limited set of experiments was done using ammonium azide solid to attempt to combine both the activation step and fluorogenic steps in one. The ammonium azide was prepared from sodium azide by ion exchange to hydrazoic acid followed by neutralization with ammonia solution. The latter was carefully evaporated to the solid. The application of solid ammonium azide directly on a n-$C_{22}H_{46}$ spot produced no observable fluorescence at the hot-plate temperature of 130°. Higher temperatures were avoided at this time because of the potentially explosive nature of the azide.

It has been observed consistently that clean chromatoplates left out in the open laboratory air produce a fluorescent background on chromatoplates that are treated by the procedures described in the work. The intensity of this background increases with increasing time of exposure to the air. This does not occur with dry plates stored in a vacuum or within purified gases. The effect appears to be due to adsorption of organic pollutants in the air surrounding the chromatoplates. The fluorescent substances ultimately produced are, as before, eluted by pure water with an R_F close to unity.

We also wish to report that the vacuum discharge procedure produces excellent fluorescence with the compounds sphingomyelin and beef lecithin. The rating for a 1-μg spot of each was an intense 10 value. Further, a 5 cm × 20 cm Permakote chromatoplate was used in a chromatographic analytical experiment which incorporated both a sample taken from amniotic fluid, and internal standards of sphingomyelin (3.3 μg) plus lecithin (1.6 μg). All spots were clearly visible after treatment by vacuum discharge procedure, but have not yet been quantitatively compared by spectrophotometer.

Quantitative test data

Two organic compounds have been tested to date for their quantitative response to the vacuum discharge procedure for inducing fluorescence. In all cases,

test chromatoplates were spotted with varying amounts of the compounds using a syringe. They were then exposed to 3 min of heating in ammonium hydrogen carbonate vapors. No attempt was made to optimize electrical exposure time with the amount of the substance.

The two compounds used were a straight chain hydrocarbon, n-$C_{22}H_{46}$, and the drug chlorpromazine, 2-chloro-10-(3-dimethylaminopropyl)phenothiazine. The latter has been used as a sedative and antiemetic for schizophrenic patients. Clearly, these compounds differ widely in molecular structure.

One-microgram spots of each compound were examined for their fluorescent excitation and emission spectra. For n-$C_{22}H_{46}$, the wavelength for maximum excitation occurred at 360 nm, and the wavelength for maximum emission (when illuminated at 360 nm) was 450 nm. For chlorpromazine, the corresponding excitation maximum was 370 nm, and the emission maximum 455 nm. The similarity in the spectra is quite striking. Further, these maxima correspond very closely to those obtained by Segura and Gotto[3] in another procedure used to induce fluorescence.

For quantitative measurements, two sets of chromatoplates (Permakote) were used, differing noticeably in intrinsic fluorescent background. The overall results obtained for both compounds are shown in Fig. 3. The quantities of the compounds examined ranged from 1 μg to 10 ng. The data are plotted on a linear basis of the integrated peak area *versus* the absolute amount of compound. There is approximate linearity over a portion of the range but not over the entire range. The effect of intrinsic fluorescent background of particular chromatoplate batches is clearly illustrated, there being a factor of two or more for the data shown. Obviously, the

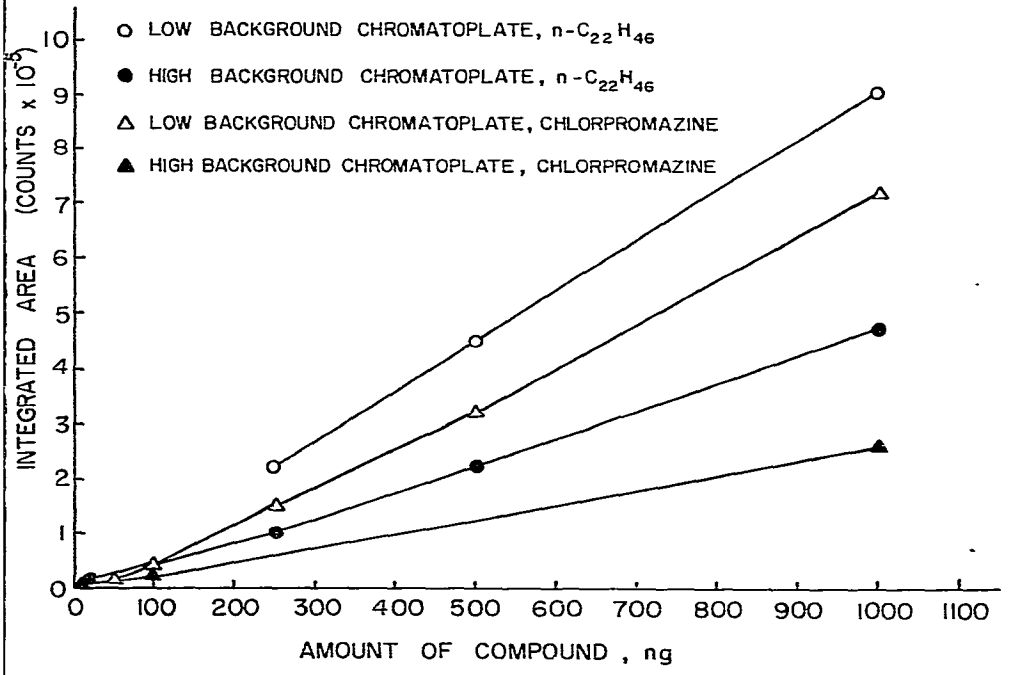

Fig. 3. Quantitative data for n-$C_{22}H_{46}$ and chlorpromazine treated to induce fluorescence by vacuum electrical discharge. Excitation output at 360 nm and 370 nm, respectively.

highest sensitivity requires the lowest possible background. The difference in background appears to be solely due to inorganic fluorescent substances.

For one particular plate, our data to date show excellent reproducibility for a given amount of a compound spotted from the same volume by syringe.

It should be noted that all the data were measured on syringe-spotted plates, which does not result in a gaussian distribution of the substance on the plate. There is a distinct difference between the center of a spot and the circular edge of that spot. The integrator associated with the spectrophotometer is programmed to deal with the gaussian situation, hence the data shown in Fig. 3 are not an accurate reflection of the correlation which would result from chromatographed substances. We intend to carry out this work in the future.

Another point of interest is shown by Fig. 3. The first three data points on n-$C_{22}H_{46}$ (low- and high-background plates) were obtained by spotting 1 μl, 0.5 μl, and 0.25 μl of a 1 mg/ml solution. Their spot diameter, after inducing fluorescence, bore the anticipated relationship between diameter and volume of solution spotted. The linearity of these three points appears to imply that the total integrated signal is directly proportional to the fluorescent area, and hence to the amount of fluorescent material produced by this procedure.

We wish to note that in the case of chlorpromazine, the substance is strongly adsorbed in the center of the point of spotting, even though the solvent spreads out to a larger diameter. We have observed the presence of small amounts of impurities in this particular source of chlorpromazine, evidenced by a faint ring surrounding the central spot (after our activation procedure).

CONCLUSIONS

Based on the work described in this paper, we have concluded the following to date:

(1) Substantially higher sensitivity is achieved by the described procedure for inducing fluorescence in spotted organic compounds on chromatoplates than was obtained by the method described in our previous paper.

(2) A major factor in ultimately producing fluorescence consists in an energy-transfer process from electrically activated gases to the organic compounds. Argon and helium, for example, work as well as nitrogen during the "activation" phase.

(3) The "activated" organic compounds on the chromatoplates have very long lifetimes. Many hours after storage under inert gas, activated spots can be induced to full fluorescent intensity by ammonium hydrogen carbonate vapors. The nature of the activated state is not known, but should be investigated for the possible presence of free radicals on the silica gel surface.

(4) While ammonium hydrogen carbonate is one of the best fluorogenic agents used to date, there is no reason to assume it is the best for any or all compounds. It should be possible to find other agents capable of coupling to the activated compounds.

(5) There is a striking commonality among the fluorescent compounds ultimately produced by the procedures described here. This is evidenced by the similarity of excitation and emission spectra for compounds of diverse chemical structure, and also by the similar chromatographic elution behavior to solvents.

(6) It appears that good quantitation will be possible with these procedures, down to the 10-ng level at least. We have readily observed (by eye) 1-ng amounts on plates which were very low in intrinsic background. Such low background is essential to operating in the low nanogram region.

(7) It should be possible to activate compounds on chromatoplates using electrical discharges at atmospheric pressure. The factor of importance appears to be the density of excited molecular states which can be achieved in the volume in which the chromatoplate is located.

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