REVIEW

MULTIPLE ZONES AND SPOTS IN CHROMATOGRAPHY

ROY A. KELLER
Department of Chemistry, University of Arizona, Tucson, Ariz.
AND
J. CALVIN GIDDINGS
Department of Chemistry, University of Utah, Salt Lake City, Utah (U.S.A.)

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The tendency among workers employing chromatography to accept two or more zones on a column or spots on a paper as indicative of heterogeneity has almost led to some erroneous conclusions. The careful work of SMITH[1] AND OVENSTON[2] which detected the anomalies in their particular investigations responsible for what has become known as multiple or double zoning or spotting is deserving of praise. It is difficult to say how many "new compounds" have been reported because of this phenomenon. It is now recognized that a single pure substance may lead to the formation of more than one zone or spot[3]. Multi-zoning is particularly dangerous with mixed chromatograms where an unknown and a known compound are mixed and chromatographed. If two spots appear the compounds are assumed to be different which may not be the case at all. OVENSTON[2] advises that the unknown and suspected known be chromatographed side by side so double spotting, if it occurs, will be obvious. As OVENSTON remarks, theoretically a single substance should give a single zone but actual chromatographic systems may be far more complex than the assumed theoretical model. Explanations may be given for particular cases but no one seems to apply to all[4]. It is the purpose of this communication to review, summarize, and perhaps extend some of these explanations.

SOURCES OF MULTI-ZONING

Chemical reaction

Some solutes may, unknown to the investigator, undergo chemical conversion from one form, or species, to another prior to application to the chromatogram, after application but before development, or during the course of development. Cases of reaction on active adsorbents have been known for some time[5]. Alumina is notorious in this regard. This problem and some possible mechanisms for the action have been extensively discussed in an interesting manner by MEUNIER AND VINET[6]. If the rate of A going to B is finite but the reverse reaction is very slow, or does not occur at all, then in the course of development of A, there should be two contiguous zones, one of A and the other of B if the two compounds have different mobilities. The time

interval where the two coexist becomes shorter the faster the reaction. Rechromatography should detect this situation. In the same system, B should give one zone while A should still give two. Eventually B should be the only compound detectable.

If the immobile phase is nonparticipative in the conversion, the reaction may be oxidative or photosensitive[7,8].

Reactions that occur before development is started lead to multiple spots. BAYLY, BOURNE AND STACEY[9] found that glucose chromatographed in the presence of some ammonium salts gave three distinct, well separated spots. They later reported[10] that one of these was due to glucosylamine and another was diglucosylamine which were formed when the solution of glucose containing the ammonium salts was applied to the paper and dried in a stream of warm air prior to development. These artifacts were not formed if the origin was dried in cool air. Since the reaction attained equilibrium before development, the spots were distinct. These experiments were repeated for mannose, glucose, and galactose by RAACKE-FELS[11] who proposed for mannose a series of reversible equilibria between mannose, mannose–ammonia, mannosylamine, and dimannosylamine. The reversible reactions of mannose–ammonia proceed at rates which do not permit resolution on the chromatogram. The author proposes that the reaction is between the sugar and ammonia rather than ammonium salts, the latter being catalysts. Also, heat is apparently not necessary.

Impurities in the solution to be chromatographed

STRAIN[12] has pointed out that extracts from plants containing pigments many times contain colorless components which lead to odd results. The inexperienced researcher, intent upon the pigments, may inadvertently perform some manipulations which lead to some unusual performances of the materials and to false conclusions. These adsorbed, colorless impurities may act as displacers during development. A solution of 2 to 3 mg of lutein or zeaxanthin per 100 ml of petroleum ether–25 % acetone and containing a small amount of a strongly adsorbed material, *e.g.*, propylene glycol, when developed with the petroleum ether–acetone solvent on a magnesia column gave a dark leading zone followed by a second faint zone. The two zones appeared separated by a zone devoid of pigment. A weakly adsorbed contaminant can give a faint leading zone and a contiguous dark second zone. In both cases, prolonged development gives a single zone.

The addition of a contaminated pigment solution to a column followed by a second, uncontaminated solution of the same pigment in the same solvent may lead to two zones which later recombine on prolonged development. If the two pigment solutions are mixed before addition to the column, one zone is produced. This observation should be a warning to those who combine extracts on a column.

The appearance of a double zone is given when the initial zone contains several fold the amount of material that can be dissolved in the developer occupying this zone (overloading). The solute may be regarded as "precipitated" relative to the developer. It is dissolved at the tail of the initial dark zone, is moved through the band and appears as a faint zone, leading the initial zone and contiguous with it.

Band inversions may temporarily give the false impression of multiple zoning. If A is above B when a solution of the two in solvent 1 is placed on the column and if the order is reversed with adsorption from solution in solvent 2, then on formation of the chromatogram with solvent 1 and development with solvent 2, A will migrate through B and one zone appears to be present during the inversion. Prolonged development gives two zones.

Discontinuities in the immobile phase

Columns of kieselguhr treated with a liquid are generally accepted as the clearest example of liquid–liquid partition chromatograms[13]. The immobile liquid phase seems to consist of micro-drops dispersed through the inert support, where it is loosely held and subject to change particularly with developers which are not exactly saturated with the immobile phase. SMITH[1] chromatographed the B vitamins on kieselguhr holding one-half its weight of water or dilute buffers suspended in water-saturated *n*-butanol and developed with water-saturated *n*-butanol. If a portion of *n*-butanol containing excess water dispersed as colloidal droplets is passed through the column, the kieselguhr removes the water to give a locally continuous wet phase at the top of the column which could trap a portion of the solute. If this is developed with water-saturated *n*-butanol, the untrapped solute is washed from this region to give a band lower down on the column and leaving a band at the top. Since the immobile phase no longer exists as small droplets, equilibrium could not be established with the mobile phase and the solute is only slowly leached from the upper portion. This leads to two bands joined by a very pale region. If the column is first treated with butanol containing less than the saturation amount of water, immobile phase may be removed from the top portion of the column to give a "dry" region which can lead to double zoning on development with water-saturated *n*-butanol. Since the solubility of water in butanol is strongly temperature-dependent, these same discontinuities can be produced by a temperature variation of as little as 5° during chromatography.

OVENSTON[2] had a similar experience with silica-"Celite" columns whose adsorptive properties also depend on water content. When the column was prewashed with diethyl ether, the ether partially eluted water from the column. When the ether wash was stopped before the excess water was completely removed and the chromatogram formed and developed with a different solvent of weaker eluting power, an intermediate wet zone was formed on the column which led to double zoning at the discontinuity. Petroleum ether will remove the ethyl ether without affecting the wet zone. The two solute zones, once passed the discontinuity, will eventually reunite in this case. However, if the developer also causes the wet zone to migrate at a rate comparable to the adsorbate, they may not reunite. OVENSTON warns that double zoning is likely to occur if the column is first treated with a strong developer either in a prewashing treatment or during chromatography and is followed by a weaker developer. It would seem preferable to prewash adsorbents by decantation before packing the column to avoid such discontinuities.

A similar explanation may apply to the double zoning observed by SCHROEDER[4] on silicic acid columns.

Large amounts of inorganic salts in the solution applied to the chromatogram may produce water-logging near the origin by a salting out effect[14].

Discontinuities in the mobile phase

BOMAN[15] applied human serum albumin along an oblique line on a sheet of paper and developed the chromatogram with ethanol–phosphate buffer. The final spots should also have been on an oblique line. However, they appeared on a horizontal line. The explanation given is that the albumin travels close to the developer front and during its journey down the paper is partly adsorbed by the paper. It is soon all adsorbed to give a series of narrow elongated spots nearly parallel to the initial oblique line. The developer front appears to be followed by a "second front" which causes some change in the paper, possibly in electrical charge, which releases the albumin which then travels with this front. This front, parallel to the first, aligns the spots on a horizontal line. In certain cases, the solute appears as a double spot, one between the first and second front and one at the second front. No real discontinuity need exist in the mobile phase. If some sort of gradient of some property exists in the developer in the direction of flow and if at some particular value of this property the albumin is released, then the chromatogram gives the appearance of two fronts in the mobile phase. This sudden change of adsorbate may be pictured as similar to the sudden change in pH at the end point when a strong acid is slowly added to a strong base in the course of a titration. NUNES DA COSTA AND GUEDES DE CARVALHO[16] have observed double zoning accompanying double fronting in the paper chromatography of the borates.

Charged species and complexes

Whenever a solute can exist as one or more uncharged species and/or as one or more charged species, or is capable of forming complexes, multi-zoning can occur. This is probably the least understood cause of multiple spots.

LANDUA, FUERST AND AWAPARA[17] found that tailing and double spotting of amino acids depended strongly on the pH of the solution applied to the paper. All compounds were developed with phenol–water. The observation that the solutes could exist as one or more species and that double spots were always connected by a diffuse region is important as will be shown later. ARONOFF[18] found the same situation for lysine and that the relative intensities of the spots were in rough agreement with the distribution of ionic species as calculated from the pH of the solution applied. The lysine ions would be capable of association with the phenol to give new compounds with their own partition coefficients. WESTALL[19] has proposed a similar association to explain the separation of sodium and chloride ions on paper when developed with phenol–water. The sodium ions supposedly form a phenate which is more soluble in the phenol and shows a higher R_F.

Various dissociations may be visualized. If the solute is capable of accepting a proton

$$A + H^+ = HA^+$$

the ion formed may associate with phenate ion

$$HA^+ + C_6H_5O^- = AHOC_6H_5$$

Ionic species would be retained by the polar non-mobile aqueous phase while the unionized compound formed with the phenol would be more soluble in the mobile phase and migrate with a higher R_F. The initial distribution of charged and uncharged species would be fixed by the pH of the solution applied. If development disturbs the acid concentration, and it is difficult to see why it shouldn't since the pH of the developer is not adjusted by a buffer, the equilibria should readjust to that given by the solute in phenol–water and the chromatogram obtained should be the same in all cases, that is, independent of the initial pH. The fact that this is not true indicates a more complicated change. As will be shown, definite discrete double spots will be obtained only if the rate of conversion is slow compared to the time of development of the chromatogram. If the rate of conversion is fast compared to the time of development, one spot should be obtained. Between these two extremes, serious tailing and multiple spots joined by diffuse regions may occur. This argument, of course, makes the assumption that in the earlier stages of development, the hydrogen ion causing the initial distribution is removed from the origin and that the system is either irreversible or attempts to attain the distribution it would have in the developer. That the first part of this assumption is questionable is shown by WALDRON-EDWARD[14] mentioned below.

The rate of interconversion of charged and uncharged species is not the only factor, however. CURRY[20] observed multiple spots when he chromatographed the different acid phosphate salts in pyridine–ethyl acetate–water. The number and intensities of the spots depended upon the pH. If orthophosphoric acid is used, the faster spot predominates; if the normal salt of this acid is used, the slower spot predominates. He assumed that the spots were due to $H_2PO_4^-$, HPO_4^{-2} and PO_4^{-3} and that pyridine complexes were not involved since similar results were obtained with butanol. When orthophosphoric acid with radioactive phosphorous was used, the activity was 6000 counts/min at the spots but only 100 counts/min between spots showing the spots to be well resolved. This could be explained by a slow proton transfer

$$H_2PO_4^- = HPO_4^{-2} + H^+$$

To test this he chromatographed a mixture of labeled $H_3P^*O_4$ which ought to give $H_2P^*O_4^-$ and unlabeled Na_2HPO_4 which ought to give nonradioactive HPO_4^{-2}. Both final spots obtained were radioactive indicating an appreciable rate of proton transfer. A close examination of CURRY's paper indicates that the tagged and untagged species were mixed before application to the chromatogram. This would conceivably permit proton transfer before chromatography and one would expect activity in both spots. He did not feel that multispotting was due to the interference of inorganic

ions or impurities in the paper since the paper was acid-washed which generally is the accepted procedure for removal of such impurities. Multispotting was observed with 2',3'-isopropylidene-adenosine-5'-phosphate. ERDEM's[21] chromatograms with arsenates were very similar to CURRY's. ERDEM also obtained two to three distinct spots with divalent cadmium in the presence of ammonia. All of the spots contained cadmium and were attributed to different amino complexes. ERDEM AND ERLENMEYER[22] point out that one must assume that the kinetics of the transformation are radically different in the immobile phase than in the mobile phase. They cite examples from the literature of adsorption as justification. Their assumption requires that paper chromatography involves an adsorption mechanism rather than a partition between an immobile liquid-gel and a mobile fluid as proposed by MARTIN[23] and discussed by MOORE AND STEIN[13]. MARTIN[24] suggests that reaction rates in the liquid-gel do not differ much if at all from those observed in the pure liquid. The suggestion of radically different reaction rates in the immobile phase in paper chromatography must be regarded with suspicion unless more supporting evidence is found.

Impurities in the paper may very well cause unusual results. HANES AND ISHERWOOD[25] working with inorganic phosphate, traced "ghost spots", or retention of some of the solute at the point of application, to the presence of calcium and magnesium ions in the paper. This was eliminated by prewashing the paper with acid. These ions are also responsible for "shadows", faint regions following the principle spot but different from tailing. Multiple banded spots, which give the appearance of a well defined dark spot overlapping a fainter but equally well defined second spot, could be overcome by a strong organic acid in the developer.

WALDRON-EDWARD[14] found that D-glucosamine gave two spots when sulfate ion was present in the solution applied to the chromatogram in an amount equivalent to the amine; that the slower spot only was produced with excess sulfate and that the second spot always contained sulfate which was not present anywhere else in the developed paper. Similar effects were found with lysine, arginine, ornithine, histidine, ethanolamine, tyramine, ethylene diamine, and tetramethylene diamine. Some of these same substances were found to give unusual behavior by LANDUA, FUERST AND AWAPARA[17] and by MCFARREN[26] who investigated amino acid chromatography on buffered papers.

Earlier it was assumed that the developer separated the solutes from the inorganic ions of the buffer in the solution applied to the chromatogram thus disturbing the equilibrium. If these ions migrate with the solutes, however, they may maintain the equilibrium or the position of equilibrium may be changed only slowly by their gradual removal. Such a possibility increases the complexity of the problem. Not only are rates of conversion important but also the position of the equilibrium. The latter may be fixed by a buffered paper which may partially account for the success of this technique.

The problem of inorganic salts and their role in salting out substances, forming complexes, and migrating with developer is a formidable one. If one adds to this their possible participation in an ion exchange mechanism[27] the situation becomesworse.

Equilibrium between two species

Possibly one of the best examples of reaction between two forms of the same solute and which is free of disturbing influences such as inorganic ions is given by PARTRIDGE AND WESTALL[28] who chromatographed the sugars and related compounds on paper using phenol, *s*-collidine, and *n*-butanol–acetic acid. Neutral reducing sugars gave well defined spots indicating that the tautomeric equilibria known to occur in water did not affect the chromatogram. In butanol–acetic acid a mixture of glucuronic acid and its lactone, glucurone, gave two well separated spots; glucuronic acid gave two well defined and unjoined spots while glucurone gave a very distinct lower spot and a smaller, fainter, well separated slow spot. In *s*-collidine, glucuronic acid gave a dark slow spot and a smaller, fainter faster spot; glucurone gave two spots joined by material all the way along the chromatogram. In general, the leading spot showed tailing while the slow spot showed bearding. This observation is in accord with theory. If a substance exists in two forms which are interconverting and if the rate of conversion is slow relative to the time of development, then two spots can be obtained. There would be material joining these spots all the way along the chromatogram but its detection depends on the sensitivity of the method of revelation. The lead zone should show tailing while the slow zone should show bearding.

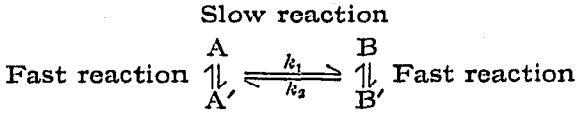
Theoretical treatments of differential migration have been given for sedimentation and electrophoresis[29–31]. If one accepts that these processes all represent the same type of phenomena, that is, differential migration of substances in a phase when subjected to some driving force, then these results should be qualitatively adaptable to chromatography. We will outline, in detail, a method for calculating concentration profiles for general differential migration methods and a method for calculating spot shapes in paper chromatograms.

INTERCONVERTING SPECIES

It is a little known fact that a large class of chromatographic anomalies can appear as a result of an involved kinetics. It is a general requirement that one of the kinetic steps be slow so that the system is not near equilibrium. The kinetic scheme discussed below, which is just one example of many possible schemes, leads to the formation of tails, comets, beards, double spots, and even triple spots, with the appropriate choice of parameters.

Suppose that a substance can exist in two different, interconvertible forms, A and B, each with a degree of permanence shown by a slow rate of conversion between them. Suppose, also, that each form undergoes the usual chromatographic exchange (adsorption or partition) between the mobile and immobile phases and back again. This exchange is denoted by $A \rightleftharpoons A'$ where A designates a molecule of the species A in the mobile phase and A′ designates a molecule of this species in the immobile

phase. A similar expression can be written for species B, *i.e.*, B $\rightleftharpoons$ B'. Both of these exchanges are assumed to proceed rapidly. The net scheme is



The slow reaction between A and B proceeds with first order rate constants, k_1 and k_2.

Zero rate constants

The investigation of the above scheme can be best approached by first assuming $k_1 = k_2 = 0$, *i.e.*, with no interconversion between A and B. Each species will migrate independently of the other and the zone of each will broaden during migration. This broadening is due to the combined result of the partitioning kinetics, molecular diffusion, and "eddy" diffusion[32,33]. Each of these phenomena can be assigned an apparent diffusion coefficient to describe its contribution and the sum of these gives an effective diffusion coefficient D which describes the spreading of the zone. When D itself is variable (due to non-constant flow rates, etc.) then a time average of D must be used. If the initial zones are very narrow bands and a diffusion model describes the spreading, then the concentration profiles, at any later time during development are Gaussian in shape.

The picture then, with zero rate constants, is of two zones migrating with their own R_F's and each spreading according to their individual values of the net diffusion coefficient D. If the mobilities of A and B are sufficiently different and zone broadening is not too great, well defined and separated spots should result. This case corresponds to the observations of BAYLY, BOURNE AND STACEY[9], and RAACKE-FELS[11].

This picture is modified for the reversible conversion of A to B with finite transition rates. The following simple argument shows the nature of the new concentration profiles. Any molecule that was originally A and has changed to B or *vice versa*, has spent part of its time migrating with an R_F characteristic of A, and the remainder of its time with the R_F of B. Hence at a later time, t, such a molecule will be found somewhere between the A and B zones at the two extremes. Thus there will appear two zones or spots with a diffuse streak between them.

The question next arises as to the amount of material in the two extreme zones relative to the material between them. Since the reactions are all first order, the end zones disappear by exponential decay. If the original material is divided with the fractions α and β in the A and B forms respectively, then at the later time t, $\alpha \exp(-k_1 t)$ will be the fraction in the A form and $\beta \exp(-k_2 t)$ will be the fraction in the B form. The total fraction of material in the intermediate streak will be $1 - \alpha \exp(-k_1 t) - \beta \exp(-k_2 t)$. At sufficiently long times, compared to both $1/k_1$ and $1/k_2$, this will approach unity and essentially all the molecules will be somewhere between the expected positions for A and B with no interconversion. Under the condition of large t, nearly every molecule will have made several A $\rightleftharpoons$ B transitions, and the concentration profile will approach a Gaussian distribution with the peak somewhere in the

center, its position depending on the relative average times spent in the A and B forms.

A quantitative evaluation of the concentration profile can be made using chromatographic theory[33a]. In its simplest form, this theory gives the probability distribution for the relative times spent in the A and B forms when the reaction scheme is, as above, $A \rightleftharpoons B$. If we let the fraction of time that a molecule spends as A be x, then the probability that this fraction is in the range x to $x + dx$ is

$$P_1(x)\,dx = a \exp[-a(1-x) - bx]\, I_0 \sqrt{4abx(1-x)}\,dx \qquad (1)$$

providing that the molecule was in the A form at the beginning of the run and in the B form at the end. In this expression, $a = k_1 t$ and $b = k_2 t$. I_0 is a Bessel function of imaginary argument.

If the molecule starts out as A and after at least one reaction cycle, ends as A, then the probability becomes

$$P_2(x)\,dx = \left[\frac{ab(1-x)}{x}\right]^{1/2} \exp[-a(1-x) - bx]\, I_1 \sqrt{4abx(1-x)}\,dx \qquad (2)$$

The only other possibility, starting with A, is that no reaction at all occurs. The probability of this case, for which $x = 0$, is $\exp(-a)$.

The remainder of the concentration profile comes from the molecules that are originally in the B form. The $P(x)$ expressions in this case are obtained from the previous equations by substituting a for b and $(1-x)$ for x. The corresponding equations, first for a molecule that begins as B and ends as A (eqn. (3)), and second for a molecule that begins as B and returns to B after one or more reaction cycles (eqn. (4)) are

$$P_3(x)\,dx = b \exp[-a(1-x) - bx]\, I_0 \sqrt{4abx(1-x)}\,dx \qquad (3)$$

$$P_4(x)\,dx = \left(\frac{abx}{1-x}\right)^{1/2} \exp[-a(1-x) - bx]\, I_1 \sqrt{4abx(1-x)}\,dx \qquad (4)$$

The probability that no reaction occurs, for which $x = 1$, is $\exp(-b)$.

The overall concentration profile is proportional to the final probability density function, $P(x)$, which is obtained by properly weighing the above expressions. Since the original fractions of molecules in the A form is α and in the B forms is β, then

$$P(x) = \alpha[P_1(x) + P_2(x)] + \beta[P_3(x) + P_4(x)] \qquad (5a)$$

$$P(x = 0) = \alpha \exp(-a) \qquad (5b)$$

$$P(x = 1) = \beta \exp(-b) \qquad (5c)$$

We have computed $P(x)$ for several different sets of the parameters a, b, α, and β. In the typical case, $P(x)$ is continuous and finite in the interval $0 < P(x) < 1$. At $x = 0$ and $x = 1$, an infinitely thin slice has an area on the normalized $P(x)$ curve of $\alpha \exp(-a)$ and $\beta \exp(-b)$. Since this cannot be shown graphically, we have included this area in a Gaussian distribution centered about $x = 0$ and $x = 1$ and with a finite standard deviation of $\sigma = 0.1$. This procedure, for a typical case, is illustrated in Fig. 1.

The distribution of material shown by the $P(x)$ curves in these figures results only from the A $\rightleftharpoons$ B reaction and has not yet included the actual chromatographic process A $\rightleftharpoons$ A′ and B $\rightleftharpoons$ B′. This situation is easily remedied, however, since a single diffusion coefficient may be used to describe the combined effects of the chromatographic process, ordinary molecular diffusion, and eddy diffusion[32–34]. In order to calculate this, we set up a numerical program which allows for the diffusion of the material in the zone described by $P(x)$. The Schmidt method has been used for this purpose[35]. The calculations of this method can be made in less than an hour with the use of a desk calculator. We have restricted our considerations to the case where the diffusion coefficients of species A and B are the same. If it seems desirable to pursue the less restricted case where they are different, this can be easily done.

The choice of the diffusion parameters is independent of those chosen to represent the kinetics of the reversible conversion of A to B. We have kept ordinary diffusion

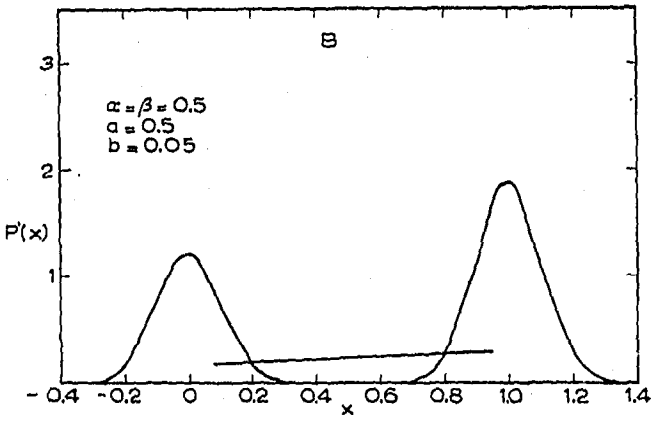


Fig. 1. The probability (concentration) density, $P(x)$.

at a minimum to emphasize the kinetic effects. In practical cases diffusion may often be a more important factor which would manifest itself simply as an additional spreading of the profile over and above that presented here. Our calculations are made with an effective diffusion coefficient

$$D = \frac{[0.1\ (R_{FB} - R_{FA})\ v]^2}{2t} \tag{6}$$

where R_{FA} and R_{FB} are the R_F values of the two species and v is the velocity of the mobile or developer phase. This value was chosen so that the root-mean-square displacement of a molecule by diffusion is one-tenth the distance between the centers of the A and B zones. The new probability density function, $P'(x)$, is obtained by adding the "diffused" $P(x)$ profiles to the Gaussian curves representing the end zones. Fig. 6 is the final result.

CALCULATION OF SPOT SHAPES

The $P'(x)$ profiles calculated above yield some representative concentration profiles which are useful if concentrations are measured directly as is often the case in the elution techniques. Quite often, however, zones or spots are observed visually,

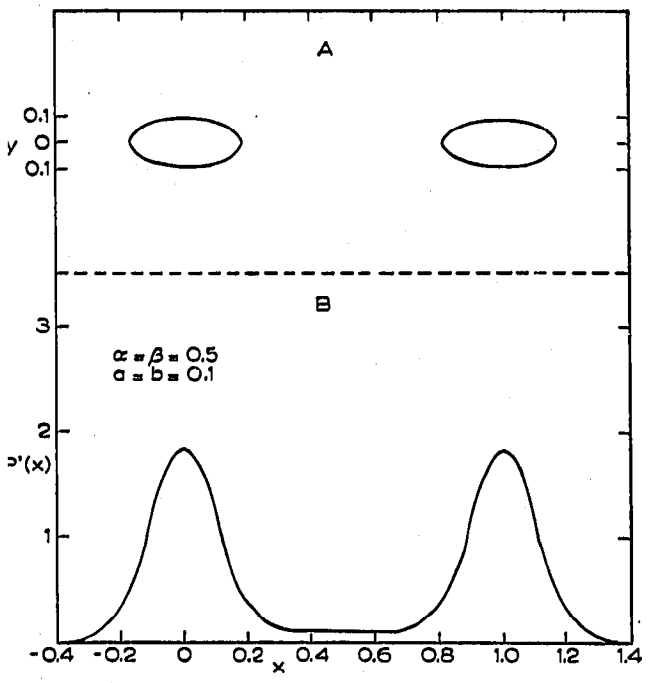


Fig. 2. Concentration profile and spot outlines for a symmetrical case. With a more sensitive method of detection, a continuous streak would exist between the two spots corresponding to the finite concentration between them.

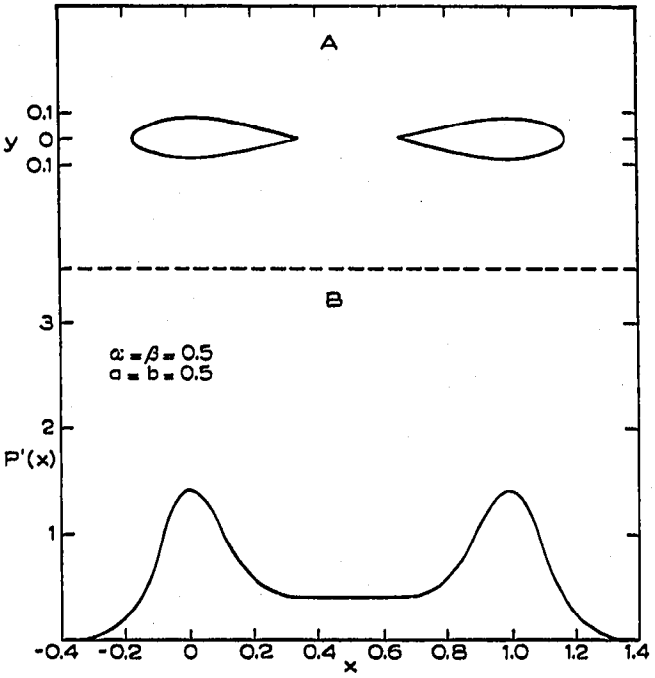


Fig. 3. A symmetrical case in which the two spots tail towards one another due to the increased concentration in the center.

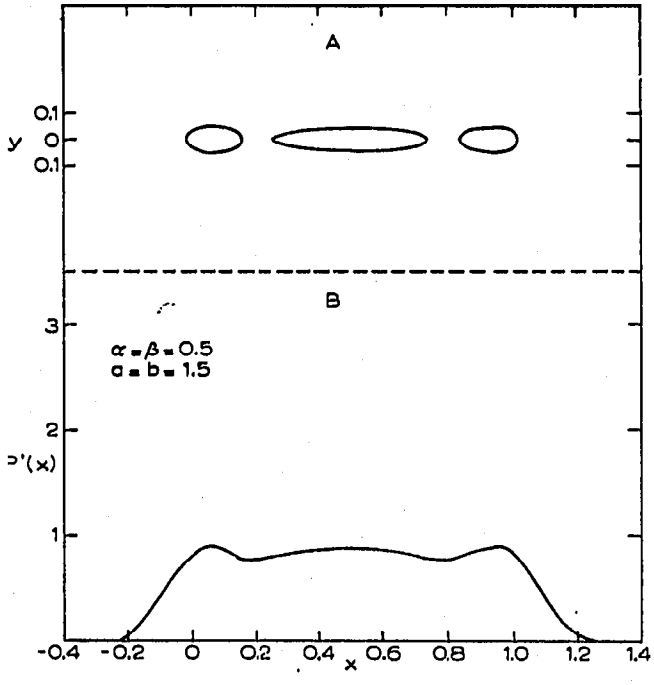


Fig. 4. Triple spots will be seen for this case if the initial spot content and the sensitivity (cutoff) fall within narrow limits. Since the minima in the concentration profile are not prominent, a streak would ordinarily appear between each spot. Otherwise the spots will be quite faint.

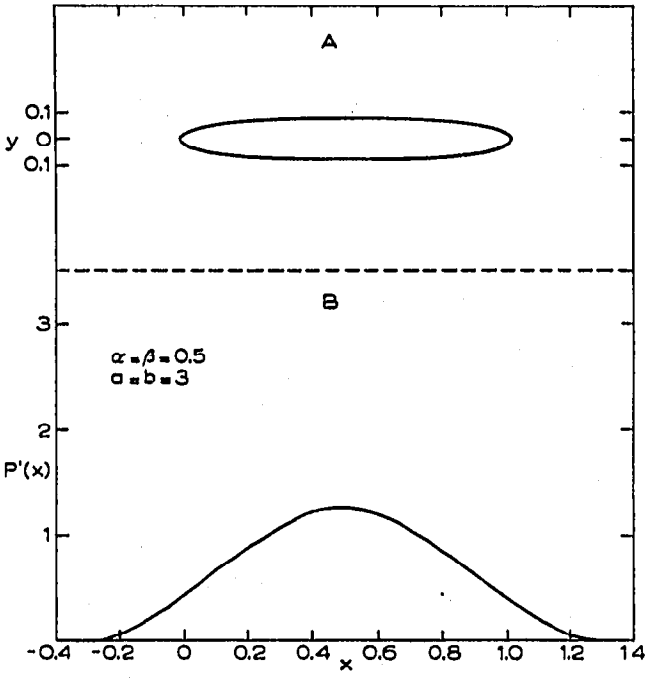


Fig. 5. Elongated spot resulting from the extended development of double spots with an origin in slow kinetic steps.

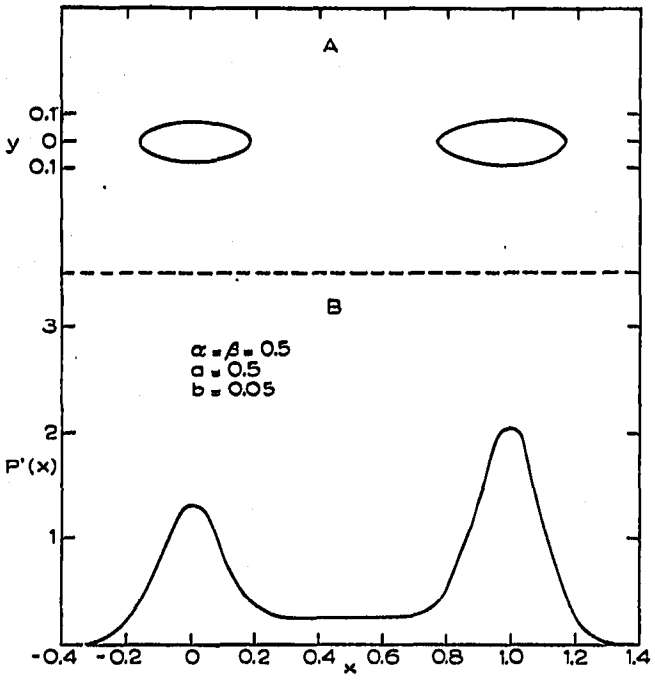


Fig. 6. Asymmetrical case. With a different spot content or cutoff this might appear as a single spot with bulging ends, double spots, or a single spot.

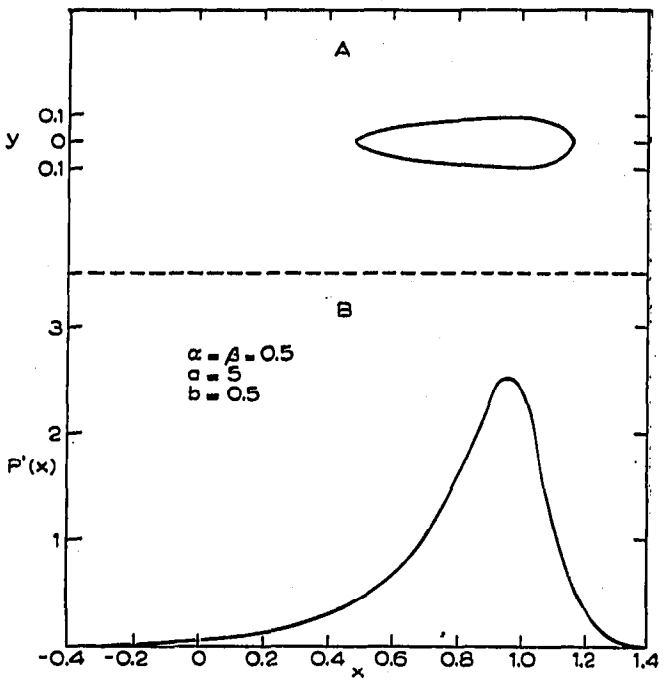


Fig. 7. Asymmetrical case in which one spot has essentially disappeared. Notice the extended tailing in the concentration profile and in the spot.

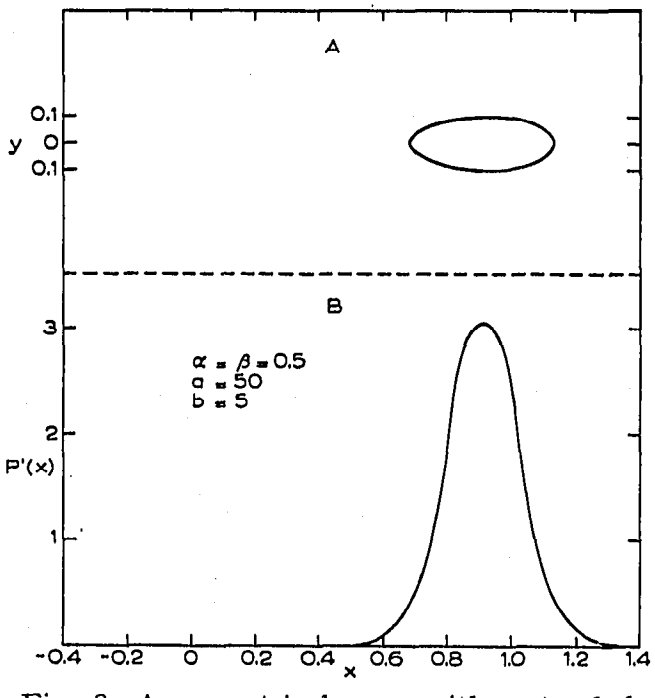


Fig. 8. Asymmetrical case with extended development. The spot appears elliptical.

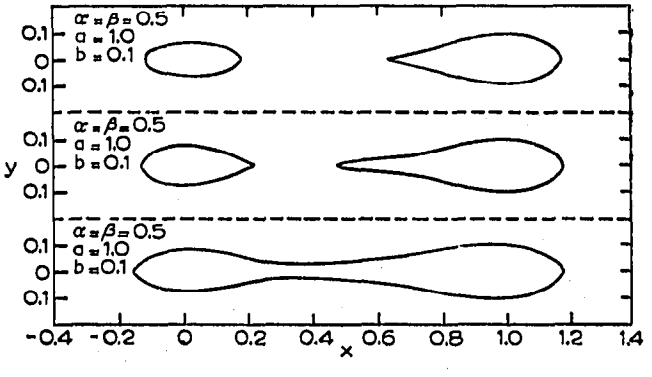


Fig. 9. Spot outlines at different cutoff values for an asymmetrical case. The range of phenomena that can be found is partially illustrated here. All the spots illustrated will, in practice, have a diffuse rather than a sharp boundary.

sometimes with the aid of a revelation reagent. In this case it is useful to express the above theory in terms of spot sizes and shapes which are encountered in paper chromatography. In this way the researcher is provided with suggestions as to the kinetic processes contributing to his chromatographic results.

In accordance with the previous communication in this series[33] we assume that the material spreads both in the direction of developer flow (longitudinal) and perpendicular (lateral) to it with different effective diffusion coefficients for the two directions. If the width of the original spot is small the lateral distribution of material will appear as a Gaussian distribution with $\sigma_y = (2D_y t)^{1/2}$ where D_y is the effective diffusion coefficient in the lateral direction. On this planar chromatogram any material is apparent as a spot (*i.e.*, within the spot boundary) when the concentration exceeds a certain critical or cutoff value (determined by the limits of visibility, etc.). This value is obtained when the two-dimensional density function equals P_0. The following equation describes the lateral extension $y - \bar{y}$, of the spot in terms of P_0

$$P_0 = \frac{P'(x)}{\sqrt{2\pi}\sigma_y} \exp\left[-\frac{(y-\bar{y})^2}{2\sigma_y{}^2}\right] \tag{7}$$

The width of the spot is $w = 2(y - \bar{y})$ and this becomes

$$w = \sigma_y \left[8 \ln \frac{P'(x)}{\sqrt{2\pi} P_0 \sigma_y}\right]^{1/2} \tag{8}$$

When w becomes an imaginary number this is to be taken as zero. Spots with widths obeying this formula have been drawn in Fig. 2 through 9 along with the $P'(x)$ profiles from which they are obtained. The cutoff point is altered by changing P_0. It should be noted that the spot might appear thicker or thinner simply by variations in σ_y.

DISCUSSION OF THE RESULTS

The theoretical analysis of concentration profiles is a general method of treating differential migration processes. In electrophoresis, for example, the same formulation applies simply by substituting the two velocities v_A and v_B for $R_{FA}v$ and $R_{BF}v$. Hence a substance composed of two slowly interconverting forms can appear as two zones and sometimes as three zones, in either electrophoresis or chromatography.

The problem of zone distribution in electrophoresis has been treated by a number of authors. CANN, KIRKWOOD AND BROWN[31] set up the one-dimensional differential equations for the distribution of two interconverting species undergoing electrophoretic migration. They were able to obtain a solution only for a limited set of parameters, but they did observe double zoning in several cases. This method seems more difficult than the present one for this type of calculation since they indicate the need of a computer to extend the range of their parameters.

The cases of zone and spot distribution that we have examined are necessarily limited. There are so many physical parameters entering the calculation that it is impossible to explore the entire range of them in a single writing. The group we have

examined is divided into two categories. In Figs. 2 through 5 we have treated the symmetrical cases where $a = b$ and $\alpha = \beta$. Asymmetrical cases are treated in Figs. 6 through 9 where $a \neq b$ and $\alpha = \beta$.

In the symmetrical cases we have shown examples where a and b successively increase. This roughly represents the calculation of a given chromatogram at successively longer time intervals. Since our profiles are plotted against the reduced variable, x, the end zones do not move farther apart with increasing time as they do in practice. It should be noticed that the principle result of increasing a and b is that of reducing the end zones and filling in the region between them. This has proceeded far enough when $a = b = 1.5$ that there appear three peaks, the center one being comparable in size to the end ones. Circumstances favorable to the appearance of three peaks are not often found since the peaks, when they appear simultaneously, are never very prominent.Three peaks would be more commonly observed with three slowly reacting species.

For larger values of a and b the end zones rapidly become insignificant. Thus where $a = b = 3$ they are swamped by the larger amount of material in the center. The corresponding zone is not Gaussian, however, until a and b become larger.

The parameters for the asymmetrical cases have been chosen arbitrarily such that $a = 10\ b$. Thus while an equal amount of material starts in each zone, the A zone is depleted more rapidly. The results show an asymmetry that is very similar to cases where $\alpha \neq \beta$. Whether the asymmetry is caused by an inequality in initial distribution ($\alpha \neq \beta$) or by an inequality in rates ($a \neq b$), or both, can be determined by further development of the chromatogram. The further depletion of material from the end zones depends directly on a and b, and if these are equal, the fractional depletion in a given time is always the same.

When a and b are unequal, as for the cases calculated, the relative depletions are unequal. Thus with $a = 10\ b$, the A zone disappears more rapidly than the B zone. This is seen in the successive Figs. 6 through 8.

With each of the Figs. 2 through 8, we have shown at least one spot outline. The encircling line represents the limit of detection around the edge of the spot. Since this limit varies with the method of detection, we have shown some examples where the concentration profile is the same but the spots differ because this limit, or cutoff, has been changed, or because the spot content is different.

With symmetrical parameters the spots are distributed equally on each side of the point $x = 0.5$. While in practice such a symmetrical distribution is not to be often expected, the calculations provide some useful prototypes. In the succession of examples we see two separate elliptical spots, two spots that are elongated towards their common center (with a different cutoff, this would become a thin streak between them), a case where triple spots are to be expected, and finally a single spot, nearly elliptical, indicating the accumulation of most of the material in the center region. This series illustrates the general tendency of two zones to merge into one on prolonged development.

The series of spots obtained with asymmetrical parameters show a similar trend.

It is noticeable that the smaller spot is more nearly elliptical while the larger spot tends to trail off in the direction of the smaller one. In the last two figures the material is nearly depleted from the left hand side and no spot appears there. Notice in Fig. 7 the definite appearance of tailing in the spot. Tailing can appear under a variety of circumstances, even when the isotherm and kinetics are linear.

The ideal spots shown here are not always closely reproduced experimentally. A number of spot imperfections, such as missing corners and bulges, can be attributed to the heterogeneity of the paper. The heterogeneous nature of flow in paper often displays itself in the uneven shape of the solvent front.

While we have computed a representative sample of spot shapes in chromatography, the meaning of the results should not be misconstrued. We have used sets of physical parameters that are certainly not unique in providing the resultant spots. An observed set of spots may have its origin in:

1. The theory outlined here with the physical constants used here to obtain a similar set.

2. The theory used here but with a different set of constants.

3. An entirely different theoretical basis as outlined in the earlier parts of this paper. In order to decide between the three possibilities a careful study of the system must, in all cases, be made. The attempt here, has been simply to show that an entire group of phenomena will often appear as a result of the intrinsic kinetics of the system.

TABLE OF SYMBOLS

k_1	rate constant for A → B interconversion
k_2	rate constant for B → A interconversion
D	effective coefficient of longitudinal diffusion
α	fraction of material originally in the A form
β	fraction of material originally in the B form
t	time
x	fraction of time that a molecule spends in A form
a	$k_1 t$
b	$k_2 t$
$P_1(x)$, $P_2(x)$, $P_3(x)$, $P_4(x)$	probability densities given in equations (1–4)
I_0, I_1	Bessel functions
$P(x)$	overall probability density before diffusion
$P'(x)$	overall probability density after diffusion
P_0	cutoff probability density in two dimensions
σ, σ_y	standard deviation due to diffusion
D_y	effective coefficient of lateral diffusions
P_y	lateral coordinate
w	width of spot
v_A, v_B	velocities of A and B
R_{FA}, R_{FB}	R_F values for A and B

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SUMMARY

Cases in which multiple spots appear in the chromatography of single substances are given. The various theoretical interpretations of multiple spotting are discussed and related to one another. Theoretical equations are derived for the evaluation of concentration profiles and spot shapes when the origin of multiple spotting is the slow interconversion of chemical species. A number of examples which give typical spot profiles are shown. Methods for determining the actual origin of multiple spotting are discussed.

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