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DIAGONAL TECHNIQUES

INTRODUCTORY REMARKS

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SUMMARY

Diagonal techniques (flat-bed two-dimensional chromatography or electrophoresis with a physical or chemical treatment between the first and second dimensions) were discussed, and their applicability was illustrated by examples. These techniques can be used for the detection of chromatographic artifacts, for better resolution of the components of the sample, and for specific characterization and establishment of "genetic sequences" among the products of complicated reactions.

The title of this paper is a misnomer, as in the classical joke "lucus a non lucendo". This kind of chromatography is performed just in order to show up spots which do *not* lie on the diagonal. Time would not permit a thorough review on this subject and so I decided only to mention the principle, give some examples and summarize the possibilities. Overlapping with the introduction of Dr. DALLAS could not be avoided, but at least the examples I have chosen will differ from those quoted by him.

If a chromatogram is run in identical systems in two dimensions, substances which do not undergo chemical change during chromatography will be situated on the diagonal of the chromatogram. Sometimes this diagonal chain of spots is not quite straight since the conditions may not be exactly identical for both runs. For instance, paper may be wetter or drier; the adsorptive capacity of the silica gel may be higher or lower. A curved "diagonal" axis does not usually invalidate the interpretation. It is only if the variations between the two runs cause a specific change in the mobility of some of the substances, and thus an inversion of parts of the spot sequence, that complications could arise.

If some of the substances on the chromatogram undergo a chemical change after the first dimension has been seen and before the second one and if the respective products differ in their R_F values from their precursors, such products will deviate from the diagonal axis of the chromatogram (Fig. 1).

Of course, there is no reason why a two-dimensional electrophoretic separation could not be used instead of chromatography. Here again, if the electrolyte is identical for both runs and there is no chemical change of the solutes between the runs, a diag-

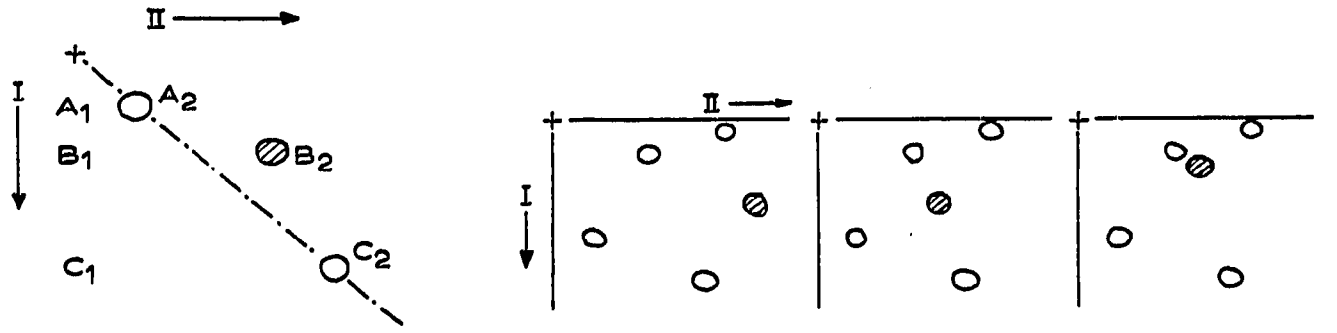

Fig. 1. Positions of spots after the first run: A_1, B_1, C_1. After the second run: A_2, B_2, C_2. Compound B was converted to its product (hatched) after treatment between the two runs.

Fig. 2. Two-dimensional chromatograms (solvent system I different from II). One of the components (hatched) was converted to its product after treatment between the two runs (center) or before the first run (right hand). Untreated substance left.

onal pattern ensues from which the spots of substances which were produced between the runs deviate.

If a one-dimensional separation does not resolve the substances sufficiently and a two-dimensional chromatogram in two different systems is necessary, at least two chromatograms must be compared. One of them without the application of the agent between the runs serves as a control; in the other one, the chromatogram is subjected to some treatment between the two runs. Spots which are found only in the latter can be attributed to the treatment (Fig. 2). Such a pair of chromatograms is obviously more difficult to interpret than a single "diagonal" chromatogram. If a site happens to be occupied by two substances giving the same detection reaction, quantitative appraisal of the spot intensity would be necessary before and after the second run, and this is notoriously difficult in two-dimensional chromatograms.

In addition to intentional physical, chemical, or biological treatments between two runs, the "diagonal" principle can be used to reveal or to elucidate some chromatographic artifacts, especially those due to a reversible or irreversible conversion of

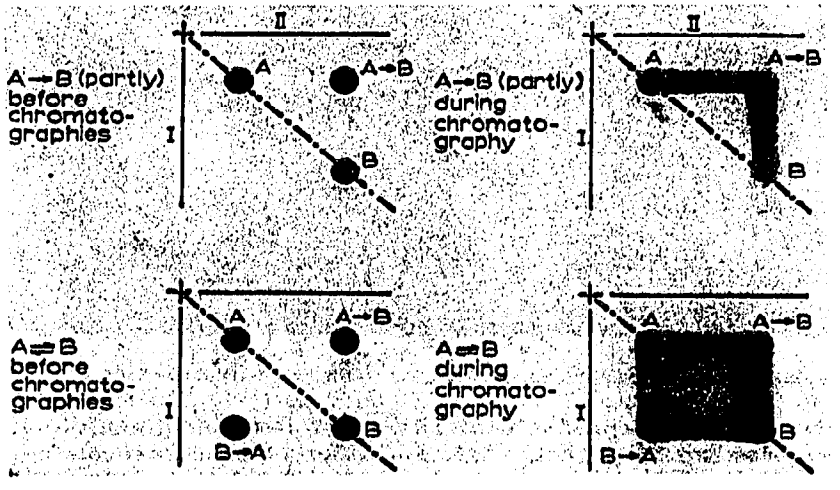

Fig. 3. Schematic drawing of the two-dimensional chromatograms (identical solvent system in both directions) when substance A is converted to B irreversibly or reversibly, before or during chromatography.

a component to another substance[1]. Let us consider several such cases (Fig. 3), substance A having a lower R_F value than B.

If such a conversion occurs during chromatography, it results in tailing. The reaction must of course take place at finite rates; if a reversible reaction is very rapid in comparison with the rate of chromatographic flow, only one spot of intermediate R_F value is formed.

The reactions expressed as A → B or A ⇌ B may be oxido–reduction, complex formation, hydrolysis or esterification, isomerization... The pattern of spots can give some information on the processes which complicate chromatography and can prevent wrong interpretation of a simple one-dimensional chromatogram.

Let us now turn to the deliberate use of "diagonal" chromatography. I shall first give a few examples and then indicate more generally further possibilities.

In order to check the stability of riboflavin and its photolytic products in dark-

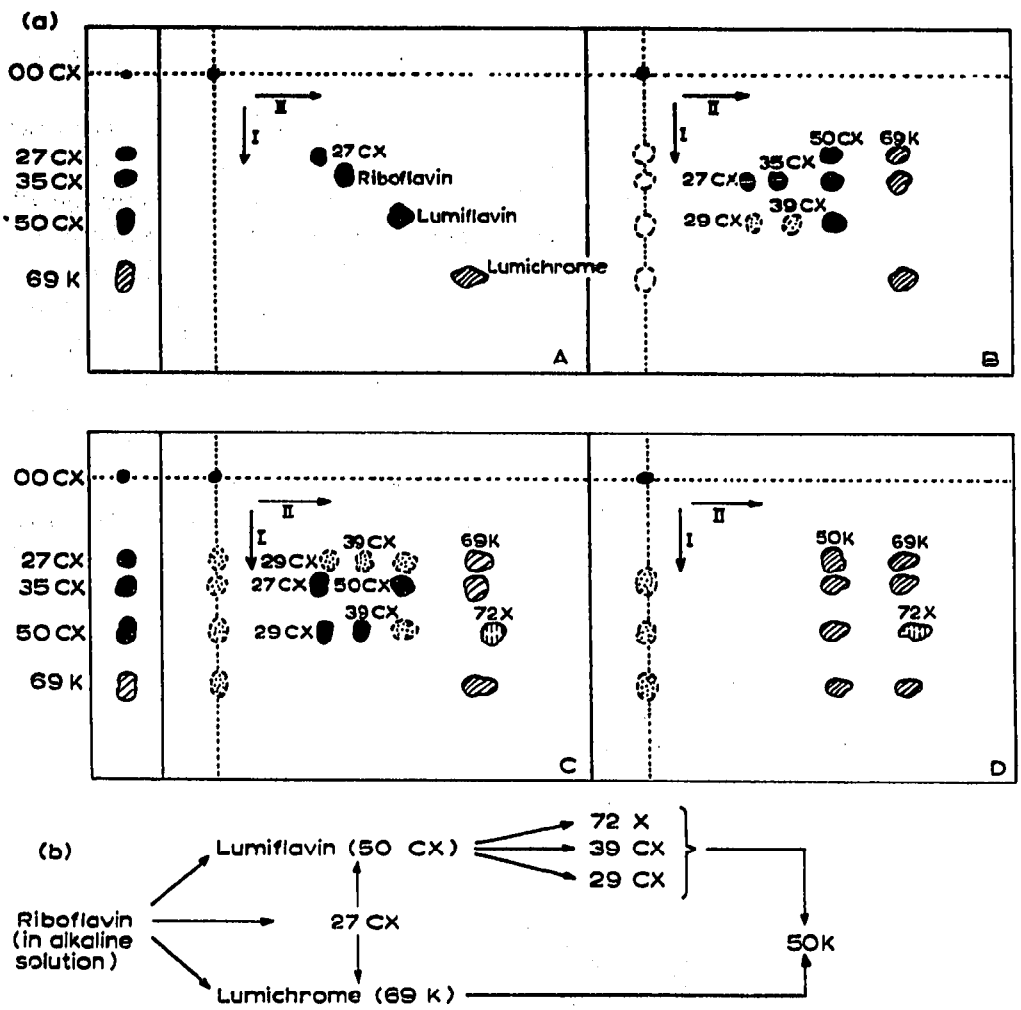

Fig. 4(a). Paper chromatogram of the photolytic products of riboflavin (see one-dimensional pattern on the extreme left) which was irradiated by visible light for different time intervals between the first and second run in 1-butanol–acetic acid–water (4:1:5). A = no irradiation; B = 1.5 h; C = 3.5 h, D = 7 h. Greenish yellow fluorescence is shown as black spots (CX), blue by hatching (K), weak fluorescence by stippling, orange yellow as short vertical lines (X)[3]. (b) The tentative "genetic" scheme of photolytic products which were derived from the chromatogram.

ness, HAIS AND PECÁKOVÁ[2] used the two-dimensional principle, developing with the same solvent system for the first as well as for the second run. The spots then occupied the diagonal position. In the full paper on this subject[3], the paper chromatograms were exposed to light between the two runs for various time intervals (Fig. 4). It was possible to check the photolytic conversion of riboflavin and its individual photolytic products into other products. The "genetic" relationships between the substances could thus be established. Let us concentrate on spot 27 CX as an example. After only 1.5 h exposure to light following the first run, it disappeared completely and spots coinciding with lumiflavin (50 CX) and lumichrome (69 K) were formed (case B). We concluded that 27 CX is an intermediate in the formation of lumiflavin and lumichrome, though not necessarily an obligatory one:

$$\text{riboflavin (35 CX)} \rightarrow 27\ \text{CX} \begin{cases} \nearrow \text{lumiflavin} \\ \searrow \text{lumichrome} \end{cases}$$

(27 CX was later identified as carboxymethylflavin, which is produced from the main intermediate, formylmethylflavin (FMF)). TREADWELL *et al.*[4] used the same approach in the study of the photolysis of riboflavin by TLC techniques.

Fig. 5 from our laboratory[5] shows a thin-layer chromatogram on which a

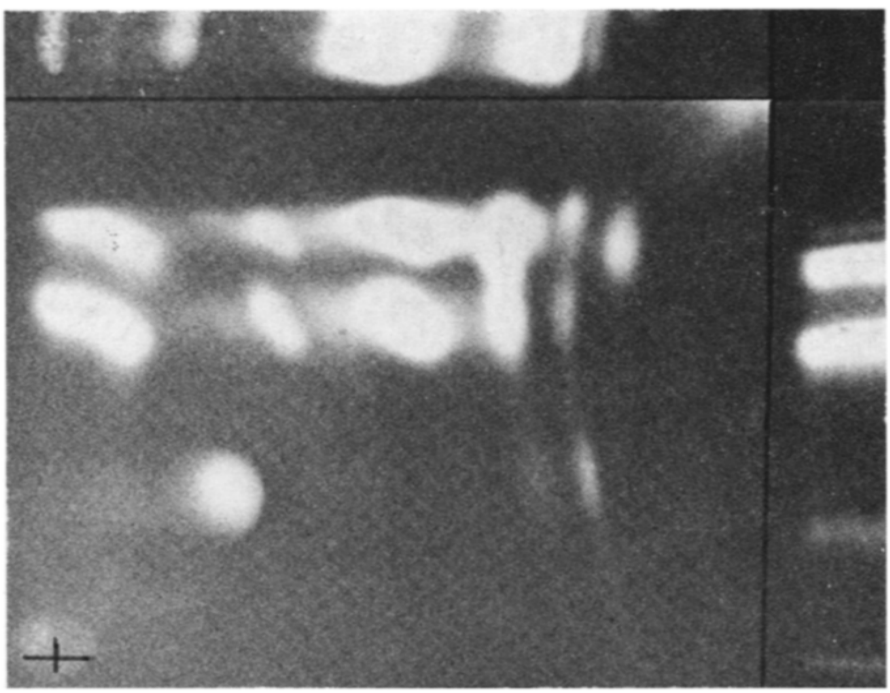

Fig. 5. A mixture, obtained by treatment of formylmethylflavin with acetic anhydride in pyridine, was chromatographed on Silica Gel TL in butanone–acetic acid–methanol–benzene (5:5:20:70) (first dimension shown vertically), dried and the chromatogram exposed to direct sunlight for 75 min. It was rechromatographed in the same system (shown from left to right). One-dimensional chromatograms of the same sample (un-irradiated) are shown along the edges: both main bands (containing three main products) are flavins (greenish yellow fluorescence), the bands with R_F 0.16 (0.24) and 0.76 (0.80) fluoresce blue. Due to the different R_F values in both dimensions the "diagonal" is curved. (CERMAN[5]).

mixture of acetylation products of FMF was chromatographed in the first dimension, the layer exposed to light and chromatographed again. The patterns of the photolytic products of the main spots of acetylated FMF seem to be similar.

In the indole field, PROCHÁZKA AND KOŘÍSTEK[6] obtained ascorbic acid in the second dimension after heating the ascorbigen separated by the first PC run, and SCHWARZ AND BITANCOURT[7] characterized some unstable indolic substances by chromatographing their degradation products in the second dimension.

BRENNER *et al.*[8] resolved isopropyl esters of methionine and of methionylmethionine after oxidation with a 15% hydrogen peroxide solution before the run in the second dimension with an unchanged solvent system. The first paper devoted specifically to diagonal chromatography and electrophoresis was that of MIKEŠ AND HOLEYŠOVSKÝ[9]. Methionine- or cysteine-containing substances (peptides) are converted to their oxidation products by exposure to performic acid vapour before the second run. (I hope that I shall not be blamed for including paper electrophoresis in this lecture, since the principle is the same.) In most cases, a strip obtained after the first electrophoretic run is attached or stitched onto a new paper sheet for the second run. In electrophoresis, the diagonal technique has proved to be a very powerful tool for the characterization of the positions of disulphide bridges in proteins (BROWN AND HARTLEY[10]). Fragments containing two amino acid sequences joined by a cystine

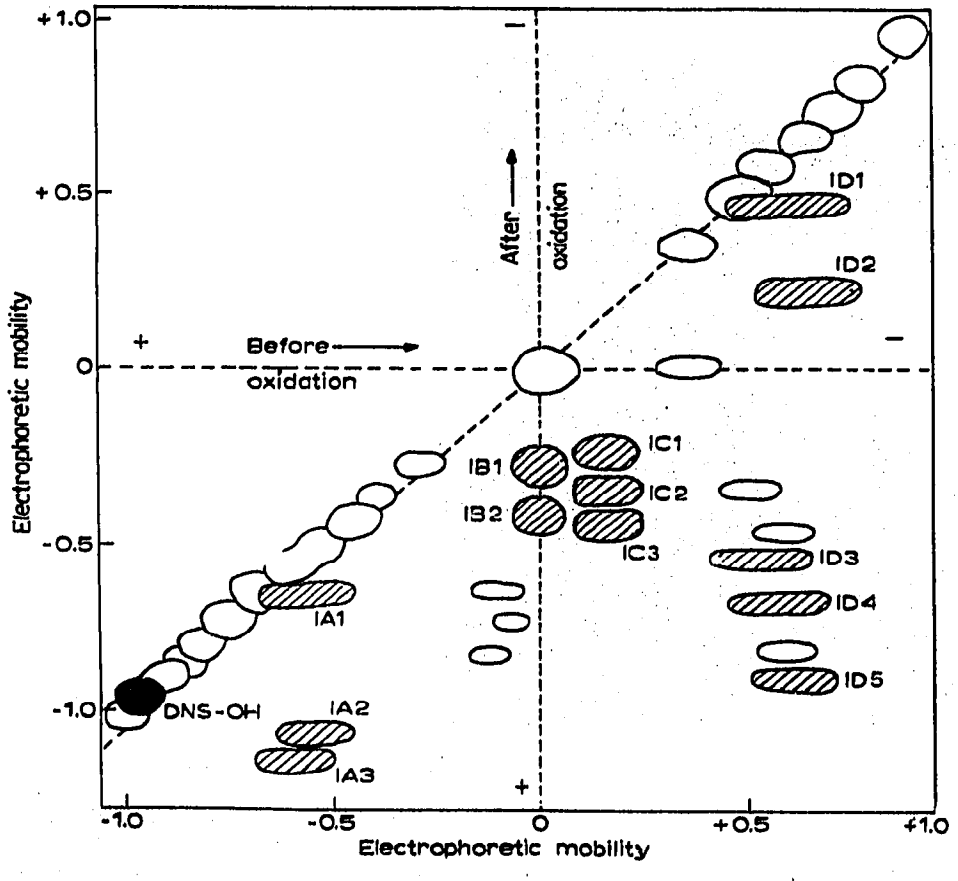

Fig. 6. pH 6.5 diagonal map of a peptidic digest of chymotrypsinogen (BROWN AND HARTLEY)[10]. Major cysteic acid peptides (formed by performic acid treatment after the first run) are hatched. DNS-OH 1-dimethylaminonaphthalene-5-sulphonic acid marker (relative mobility—1.0).

residue are converted by performic acid after the first run to cysteic acid-containing peptides (Fig. 6), which then lie off the diagonal on which the remaining peptidic fragments are placed. This method has found wide application and it has become such a routine matter that some authors using it quote neither BROWN AND HARTLEY[10] nor MIKEŠ AND HOLEYŠOVSKÝ[9]. N^{ε}-acylated lysine[11,12] or N-acylated S-2-aminoethylcysteine[13] residues, which can be deacylated by exposure to acid (or ammonia, respectively) vapour between two electrophoretic runs, can also be visualized as off-diagonal spots. Alkaline phosphatase treatment shows up similarly serine phosphate peptides[14].

EDWARDS' method[15], in which steroids are converted to their formyl derivatives during equilibration in a formic acid containing system used for the second dimension, should also be mentioned in this connection, though in this case different systems are used for both dimensions and the "diagonal" principle is not applicable.

In all these cases, reagent vapour or physical treatment, such as irradiation, are more suitable than the application of reagent solutions, since the latter may displace or deform the spots obtained after the first dimension run. Not infrequently, *e.g.* if enzymes or microbial suspensions are used to effect the specific change envisaged, spots of water-soluble compounds can spread out not only during the spraying of the reagent but during incubation as well.

The examples which I have quoted can be classified with regard to the purpose for which diagonal techniques can be used. They are as follows:

(a) The detection of chromatographic artifacts, mainly reactions taking place during chromatography.

(b) Better resolution of some compounds which have remained unresolved after the first dimension often due to overloading of the chromatogram.

(c) Specific characterization of some compounds which are modified by the treatment applied between the two runs; this is the case of peptides containing certain amino acids. Treatment with enzymes can visualize their substrates by releasing products which are not situated on the diagonal line.

(d) The establishment of "genetic sequences" among the intermediates, by-products and secondary products of complicated reactions.

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DISCUSSION

SZÉKELY: Die von Herrn HAIS behandelten Diagonal-Techniken sind sehr nützlich, leider nicht immer anwendbar. Wenn man Fliessmittel mit wenig flüchtigen Komponenten wie z.B. Phenol, Pyridin, Eisessig verwendet, verbleiben solche auf dem Sorptionsmittel und beeinflussen die Trennung in der 2. Richtung. Eine Zwischentrocknung bei höherer Temperatur kommt wegen der Zersetzungsgefahr nicht im Frage. In solchen Fällen (von nicht flüchtigen Fliessmitteln) kann der DC–DC-Trockentransfer Klarheit schaffen (vergl. *J. Chromatog.*, 42 (1969) 543–544). Dieser ist unter anderem auch zum Nachweis dünnschichtchromatographischer Artefakte geeignet, wie z.B. teilweiser Zersetzung der Komponenten beim Chromatographieren.

HAIS: Wir haben die besonders schlecht entfernbaren Fliessmittel wie Phenol nicht probiert. Was Pyridin oder Essigsäure enthaltende Lösungsmittelsysteme angeht, waren die Bedingungen bei dem zweiten Lauf zwar verschieden und die "Diagonale" war nicht linear, aber Schwierigkeiten in der Interpretation der Bilder haben wir bisjetzt nicht gehabt; ich möchte sie jedoch in Ausnahmsfällen nicht ausschliessen und so ein Trocken-Transfer wäre dann angezeigt. Hohe Temperatur zwischen den Läufen ist selbstverständlich nicht ratsam mit der Ausnahme solcher Fälle, wo man gerade den Einfluss der Erhitzung auf die Stoffe studieren will. Besonders leicht werden durch Erhitzen Stoffe gebildet, die in dem zweiten Lauf nicht wandern (feste Verbindung mit der stationären Phase oder unlösliche Produkte?).

TURINA: I think that it is possible to see from a chromatogram developed only in one direction whether investigated substance A changes to substance B reversibly or irreversibly during the chromatographic process or whether the equilibrium was established before the chromatographic process. If the substance A changes to B reversibly the distribution curve gives by numerical analysis only one function in the case when the equilibrium is fast. In the case when the function obtained by numerical analysis is composed from three elementary functions, the equilibrium is not fast, but it is possible to register it during the chromatographic process. When the substance A changes irreversibly to substance B during the chromatographic process, the function obtained by numerical analysis is composed of two elementary functions. Numerical analysis also gives the quantitative data of the kinetics of the reaction on the chromatogram.