Reports

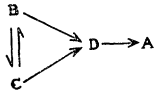
Paper Chromatography of Unstable Substances

Many organic substances are extremely unstable in solutions (sometimes also in the solid or liquid state) and are usually available only in mixture with a variable proportion of their decomposition products. Moreover, the more unstable ones go on decomposing during chemical operations and particularly during the procedure of paper chromatography. The result is that, where one spot only is expected, several may appear—an effect which is particularly troublesome in two-dimensional chromatography.

In studies of the decomposition products of indole derivatives, some of which are particularly unstable, in collaboration with Alexandra P. Nogueira, we have employed to advantage two-dimensional chromatography in which the same solvent is used in both directions (*1*). For this method, we suggest the name *double chromatography* to distinguish it from ordinary two-dimensional chromatography, in which two different solvents are used in succession.

The method can be extended to stable products which can be induced to decompose by physical means, such as exposure of the chromatogram to ultraviolet radiation before or after the first chromatography, or both.

The double chromatogram to be expected of a mixture of four substances, *A*, *B*, *C*, and *D*, decomposing according to the scheme



is shown in Fig. 1. Substances *B* and *C* are tautomers, or a monomer and its dimer, and, when separated from mixtures in which they are in equilibrium, they produce a quantity of the other substance until equilibrium is again attained. Substance *D* is a decomposition product of both *B* and *C*, and *A* is a decomposition product of *D*. Figure 1 shows that if decomposition takes place in between the first and the second chromatography, two spots of *B* and of *C*, three of *D*, and four of *A* are to be expected.

During the first chromatography, the four substances already in the mixture applied at the origin distribute themselves according to their R_f values, as is shown in Fig. 1 by interrupted circles. Since the same solvent is used in the second direction, these spots (primary spots) will appear accurately centered on the diagonal of the chromatogram after its completion. All spots appearing above or below the diagonal correspond to substances which have been produced between the first and the second chromatography and are therefore secondary spots. The tautomers *B* and *C* have produced secondary spots of each other and secondary spots of *D* and *A*. *D* has produced a secondary spot of *A*. The different spots of one substance are all equally displaced in the second dimension and are therefore aligned at the corresponding R_f (vertically in Fig. 1). The secondary spots of the decomposition products of each substance and the primary spot of the substance itself are horizontally aligned.

When comparatively large quantities of material are applied to the chromatogram, "trails" appear, which are troublesome in one- and two-dimensional chromatograms, but which in the case of double chromatograms actually furnish further evidence of the decomposition pathway of the products applied at the origin. They have been shown as interrupted lines in the diagram of Fig. 1.

Each vertical trail corresponds to a single decomposition product produced during the first chromatography. It crosses the diagonal exactly at the primary spot of that product and extends above and below the diagonal to the R_f values of its precursors. When there is one precursor only, the trail is entirely above or below the diagonal.

Horizontal trails are made up of the decomposition products of the substance at the corresponding R_f and cross the diagonal at the primary spot of that substance.

In some of our chromatograms, we have found a trail along part of the diagonal. This trail is difficult to interpret but may be evidence of polymerization of one of the substances, the spots of the monomer, dimer, and so forth, centering on the diagonal line and more or less merging into one another.

In practice, the diagonal is usually not a straight line, because the rate of flow of the solvent is not strictly the same in the first and the second chromatography; the R_f values are accordingly a little different in each direction.

As a rule, not all the spots or trails are visible, because of the small amounts of the decomposition products. Total decomposition may entirely suppress some of the primary spots, but their location is accurately determined at the crossing of the horizontal trail of their decomposition products and the diagonal. Faint or doubtful spots may be identified by their position on the diagonal and in the trails. Composite spots containing more than one substance or made up of the primary and secondary spots of the same substance can be interpreted on the same basis. The easy identification of spots and trails allows the application on double chromatograms of considerably greater amounts of the sample than in one- or two-dimensional chromatograms.

Figure 2 shows the outline of spots of a double chromatogram of indolepyruvic acid (IPyA) (*2*) after the first chromatography (*A*) and after the second chromatography (*B*). The small chromato-

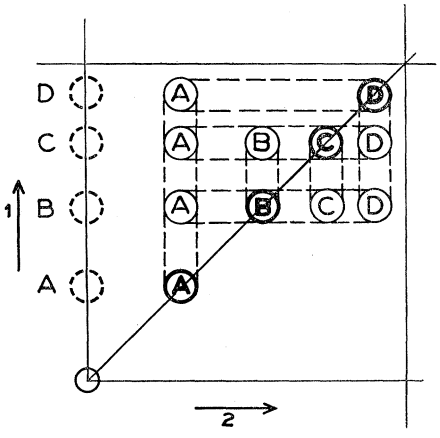


Fig. 1. Theoretical distribution of spots and "trails" of unstable substances in a two-dimensional chromatogram using the same solvent in both directions. Substances *B* and *C* are spots of tautomers which decompose into both *D* and *A* in succession. The distribution of spots after the first chromatography is shown in interrupted circles.

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gram (10- by 10-cm usable surface) was prepared, according to the ascending method used by us (*3*) with water as the solvent, in an atmosphere saturated with vapors of acetic acid. The numbers correspond to substances which have been identified by their spots in chromatograms and characterized by physical and chemical properties of the spots or their eluates (*3*). The diagonal has been traced as a curved line, the curving resulting from the accumulation of a substance in the filter paper at the front line of the first chromatography. This substance, which is not entirely removed by repeated washing of the filter paper, slows down the flow of the solvent during the second chromatography. Two horizontal rows of decomposition products at R_f values 0.14 and 0.71 are seen on the chromatogram. They cross the diagonal line at primary spots 2 and 4.

On the basis of this and other chromatograms, as well as on the basis of physical and chemical properties of the eluates, these spots are interpreted as corresponding to the keto and the enol forms of indolepyruvic acid. A secondary spot of substance 4 appears in the trail of substance 2, and vice versa. Secondary spots of substances 1, 8, 6, 7, and 5 appear on the horizontal trails of both 2 and 4 and correspond to decomposition products of these substances. Secondary spot 1*a* appears in the trail of substance 4 only, and secondary spot 3 in that of substance 2 only. Only one vertical trail, that of substance 1, has been traced, because the others were faint and because the chromatogram was made at a time when their significance was not yet well understood. Composite spots such as the cluster near the primary spot of substance 4 and the large spot of substance 8 have been interpreted according to their position in relation to the diagonal and the horizontal rows of spots as well as to their fluorescent colors.

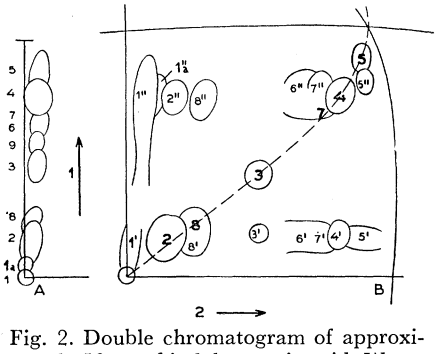

Fig. 2. Double chromatogram of approximately 50 μg of indolepyruvic acid. Water was used as solvent in both directions. The container was saturated with vapors of acetic acid. Ascending chromatography. Primary spots are distributed along the curved diagonal line. Secondary spots marked with a prime sign are from the decomposition products of substance 2, and those marked with a double prime sign are from the decomposition products of substance 4. The keto and the enol forms of indolepyruvic acid have produced, respectively, the lower and the upper row of spots of decomposition products.

KÄTHE SCHWARZ*
A. A. BITANCOURT
Plant Cancer Research Center,
Instituto Biológico, São Paulo, Brazil

References and Notes

1. This work has been supported in part by grants from the Brazilian Research Council (CNPq) and the Rockefeller Foundation.
2. The sample of indolepyruvic acid was kindly supplied by K. V. Thimann.
3. K. Schwarz, A. P. Nogueira, A. A. Bitancourt, "Spontaneous decomposition of some indole derivatives," in preparation.
* Research fellow, CNPq.

6 May 1957

Effect of Cyanide on Biosynthesis of Ascorbic Acid in vitro

It was observed in this laboratory that when rat liver tissue, homogenized in a Potter-Elvehjem homogenizer with 0.25*M* sucrose, was incubated with certain compounds under suitable conditions, an increase in the ascorbic acid value occurred over that obtained without the added substrate, the ascorbic acid being measured titrimetrically with 2,6-dichlorophenol indophenol. It was, however, noticed that in no case could a net positive synthesis of ascorbic acid be attained; that is to say, the ascorbic acid content after incubation never exceeded that present in the tissue before incubation.

In the search for a means of developing a system in which a net positive synthesis could be realized, it has been found that when rat liver homogenate is incubated with D-glucuronolactone in the presence of potassium cyanide ($5 \times 10^{-2}M$), the ascorbic acid value obtained after incubation is markedly greater than that obtained without the added substrate; the value can, in fact, be 2 to 3 times the original tissue content of ascorbic acid. Without cyanide, no increase in ascorbic acid value has been observed (Table 1). Also, when the concentration of cyanide is below $5 \times 10^{-3}M$, the influence of cyanide on the synthesis is practically nil. On the other hand, an increase in the cyanide concentration above $5 \times 10^{-3}M$ steadily increases the synthesis until a concentration of $5 \times 10^{-2}M$ is reached, when no further enhancement of the synthesis of ascorbic acid is observed. This increase in the ascorbic acid value under the influence of cyanide is not revealed when sodium D-glucuronate is substituted for D-glucuronolactone. Boiled tissue extracts give no positive results under these conditions.

Hassan and Lehninger (*1*) have shown that the enzyme system concerned in this synthesis resides in the supernatant fluid after sedimentation of the nuclear fraction and mitochondria. The aforesaid capacity of liver homogenate to convert D-glucuronolactone into ascorbic acid in the presence of cyanide has also been found to be present almost entirely in this fluid, obtained according to the method of Hogeboom *et al.* (*2*). This synthesis under the influence of cyanide is not further enhanced by the addition of cofactors like diphosphopyridine nucleotide (0.0016*M*), adenosine triphosphate (0.0016*M*), and magnesium chloride (0.004*M*), either separately or conjointly.

The results obtained with cyanide appear, however, to be at variance with those reported by Hassan and Lehninger (*1*), who observed an *inhibition* of the synthesis of ascorbic acid in the presence of 0.01*M* cyanide, although they observed none with $5 \times 10^{-4}M$ cyanide. In a repetition of their experiments it has been observed that, in their system, in which adenosine triphosphate and other cofactors are used, a small increase in ascorbic acid of the order of 0.17 to 0.23 μm is obtained with both the lactone and the sodium salt of glucuronic acid. Even in this system, however, the addition of cyanide ($5 \times 10^{-2}M$) has been found to increase the synthesis to 0.30 to 0.35 μm of ascorbic acid, and this again with the lactone as substrate and not with sodium glucuronate. It is possible that, for the formation of ascor-

Table 1. The effect of potassium cyanide on the *in vitro* synthesis of ascorbic acid. The test system contains: 0.06*M* phosphate buffer (*p*H 7.0); 0.025*M* substrate; tissue concentration 50 mg (or its equivalent of supernatant fluid) per milliliter of incubation mixture; KCN 0.05*M*. Total volume is 5.0 ml, incubated at 37.5°C for 2 hours. Negative figures indicate loss of ascorbic acid on incubation.

Expt.	Substrate	Ascorbic acid (μm) synthesized	
		Without KCN	With KCN
Rat liver homogenate	None	−0.22	0
	D-Glucuronate	−0.24	0
	D-Glucuronolactone	−0.24	+0.77
Rat liver extract (supernatant fluid)	None	−0.20	0
	D-Glucuronate	−0.20	0
	D-Glucuronolactone	−0.20	+0.61