standing for 2 hr. at 0°, the crystalline material was recovered by filtration, recrystallized from aqueous ethanol and dried *in vacuo* over P₂O₅. Yield was 3.5 g. This material was chromatographically pure and on analysis gave data identical with that previously reported (Rosenberg & Ennor, 1959).

DISCUSSION

Evidence for the presence of serine and ethanolamine in SEP isolated from earthworms was presented previously (Rosenberg & Ennor, 1959).

The present isolation of SEP in amounts sufficient to permit crystallization and chemical and physical analysis has also permitted comparison with D-SEP obtained by synthetic routes involving the use of D-serine. The results leave no doubt about the structure of the isolated material and provide proof of the presence of serine with the D-configuration in the molecule.

The yield of SEP (300 mg. from 9.6 kg. of earthworms), although small, is nevertheless believed to represent the bulk of that present in the starting material, for no loss of ninhydrin-reacting components containing SEP was detected throughout the fractionation procedures. The yield corresponds to 3.1 mg./100 g. and contrasts with the minimal content of 50 mg./100 g. reported by Roberts & Lowe (1954) in river-turtle tissue.

It has been suggested that SEP may be the precursor of lombricine (Ennor & Morrison, 1958; Rosenberg & Ennor, 1959), and the present results indicating the presence of D-serine in SEP (also present in lombricine: Beatty *et al.* 1959) are consistent with this hypothesis.

The procedure developed for the isolation of SEP has yielded lombricine as a 'by-product' in amounts (1.1 g./kg.) appreciably larger than those reported earlier and represents almost a complete recovery of this compound from earthworm tissue.

SUMMARY

1. Serine ethanolamine phosphodiester has been isolated from earthworm tissue in amounts sufficient to permit positive chemical and physical identification.

2. The serine moiety of serine ethanolamine phosphodiester has been shown to possess the D-configuration.

3. The presence of D-serine in the compound is consistent with the thesis that serine ethanolamine phosphodiester is the precursor of lombricine.

4. A simple and improved method for the isolation of lombricine is described.

We are indebted to Dr Joyce Fildes and Dr E. Spinner, Department of Medical Chemistry, for the microchemical and infrared analyses respectively; to Dr D. I. Magrath for advice and to Mr B. Thorpe for skilled technical assistance.

REFERENCES

- Beatty, I. M. & Magrath, D. I. (1959). *Nature, Lond.*, **183**, 591.
Beatty, I. M., Magrath, D. I. & Ennor, A. H. (1959). *Nature, Lond.*, **183**, 591.
Ennor, A. H. & Morrison, J. F. (1958). *Physiol. Rev.* **38**, 631.
Hurlbert, R. B., Schmitz, H., Brumm, A. F. & Potter, V. R. (1954). *J. biol. Chem.* **209**, 23.
Roberts, E. & Lowe, I. P. (1954). *J. biol. Chem.* **211**, 1.
Rosenberg, H. & Ennor, A. H. (1959). *Biochem. J.* **73**, 521.

Biochem. J. (1960) **75**, 182

Further Evidence of Tautomerism in Chromatograms of Indolyl-3-Pyruvic Acid

By KÄTHE SCHWARZ AND A. A. BITANCOURT

Plant Cancer Research Center, Instituto Biológico, São Paulo, Brazil

(Received 4 November 1958)

Indolyl-3-pyruvic acid is a very unstable substance and with the usual methods and solvents cannot be detected in chromatograms, either because it is completely destroyed (Jepson, 1958*a*; Bentley, Farrar, Housley, Smith & Taylor, 1956; Kaper & Veldstra, 1958) or because the solvents used do not separate it from its decomposition products.

With the method of 'double chromatography' (in which the same solvent is used in succession in two

directions: Schwarz & Bitancourt, 1957*a*) we have shown that in chromatograms of indolyl-3-pyruvic acid two rows of spots of decomposition products are formed, in the second development, originating from two spots that had been separated in the first development. Both rows reproduce the row of spots of the first development, including the two original spots. We have considered this as evidence of the keto-enol equilibrium of indolyl-3-pyruvic

acid existing in the solution, which is broken during chromatography when the two tautomers are separated (Schwarz & Bitancourt, 1957*b*).

In the present paper it is shown that, with due precautions against oxidation during chromatographic operations, two forms of indolyl-3-pyruvic acid can be demonstrated in chromatograms, as evidenced by the order of appearance of products in solutions of indolyl-3-pyruvic acid decomposed by several means (including changes in pH) and by the colour reactions of the two spots with seven different reagents for the indol, enol and keto groups.

EXPERIMENTAL

The sample of indolyl-3-pyruvic acid was a gift from Dr G. F. Smith (Department of Chemistry, University of Manchester). In the course of our studies it behaved exactly the same as the sample used in previous papers (Schwarz & Bitancourt, 1957*a, b*). As explained in those papers this sample was synthesized by Dr A. Ek and, according to the label on the flask received from Dr K. V. Thimann, it is the enol form, with m.p. 211–213°.

A 1% (w/v) stock solution in ethanol was used either directly for the preparation of chromatograms or for preparing more or less diluted aqueous solutions that were decomposed by ageing, heating, u.v. radiation or the pH effect given by sodium bicarbonate. Sodium bicarbonate was dissolved in water that had been boiled for 5 min. and 0.5–1.0 ml. of the 0.1M solution was added to 1 ml. of ethanolic 0.05M indolyl-3-pyruvic acid solution. The stock solution was maintained at –15°; it stayed colourless for at least 5 days, then turned reddish.

Samples of decomposing solutions were drawn at regular intervals so as to have different degrees of decomposition. To avoid decomposition during chromatography, chromatograms were short (10 cm.), which reduced the operation to a duration of about 1 hr. when the solvent was water. As a further precaution against decomposition during chromatography some chromatograms were developed at low temperature in a refrigerator (5–8°) or in a container in which the air had been replaced by nitrogen.

Whatman no. 1 paper was used in ascending chromatography. About 40 μ l./spot (4 mm. diam.) was applied at the starting line. The solvents used were: (a) water; (b) acetone–water (8:2); (c) 16% NaCl–2% acetic acid (1:1); (d) 25% acetic acid; (e) propan-2-ol–aq. 28% NH_3 soln.–water (8:1:1). With (a) and (b) the chromatogram was usually run in an atmosphere saturated with vapour of acetic acid.

Chromatograms were inspected under long-wave (366 m μ) ultraviolet light (Mineralight lamp SL 3660) and the outline of fluorescent spots was traced with a lead pencil. The outline of non-fluorescent spots which absorb short-wave ultraviolet light and quench the blue fluorescence of the filter paper was traced with a Mineralight SL 2537 lamp (254 m μ).

The following reagents were applied to the chromatogram by spraying, or by application to the chromatogram on a strip of filter paper thoroughly wetted with the reagent, or the spot was cut out from the chromatogram and dipped in the reagent. All of them give sensitive colour reactions with indolyl-3-pyruvic acid but some of them gave better

results when applied to the chromatogram while it was still wet, instead of to the dried chromatogram, as is usual.

Gordon–Weber reagent for the indole nucleus (Gordon & Weber, 1951): 0.27 g. of FeCl_3 in 100 ml. of 10% perchloric acid. Ehrlich's reagent for the indole and phenol nuclei (Jepson, 1958*b*): 2 g. of *p*-dimethylaminobenzaldehyde, 20 ml. of conc. HCl and 80 ml. of ethanol. 2:6-Dichlorophenolindophenol enol reagent (Stowe, 1955): 2 g. of 2:6-dichlorophenolindophenol in 250 ml. of ethanol and 250 ml. of water. Ferric chloride, used for the quantitative determination of the enol form of acetoacetic ester (Rothe, 1912), was approx. 5% aqueous solution. 2:4-Dinitrophenylhydrazine carbonyl reagent (Jepson, 1958*b*): saturated solution in 2N-HCl. Van Eck's carbonyl reagent (Linskens, 1955; Melchior, 1957): 5 g. of benzidine, 100 ml. of acetic acid. Ammoniacal AgNO_3 , used for detection of indolyl-3-pyruvic acid (Bentley *et al.* 1956), enol reagent (Jepson, 1958*b*), 11 g. of AgNO_3 , 33 ml. of conc. aq. NH_3 soln., 67 ml. of water.

RESULTS

All the following results were confirmed by several repeated experiments.

When the above-mentioned precautions were taken to prevent decomposition during chromatography, fresh solutions of indolyl-3-pyruvic acid chromatographed with water as the solvent in an atmosphere of acetic acid vapour, showed one spot only (spot 2, Fig. 1). This spot has a distinct purplish-blue fluorescence and a faint 'beard' of bluish fluorescence. The upper end of the beard usually exhibits a slightly increased fluorescence and occasionally forms a faint spot (spot 4, Fig. 1). In all the other solvents spot 2 was always accompanied with several spots of decomposition products.

Aerated solutions of indolyl-3-pyruvic acid in sodium bicarbonate decompose rather rapidly, as shown by the quick darkening of the solution. When sodium bicarbonate is dissolved in boiled, air-free water before mixing with the indolyl-3-pyruvic acid solution, darkening is very much delayed and starts at the free surface of the solution, gradually extending to the bottom of the tube. This is especially noticeable when the tube is long and narrow.

In chromatograms prepared in an atmosphere of nitrogen at different time intervals (Fig. 1), from samples drawn from the bottom of the tube, such solutions showed, besides spot 2, spots 3, 4 and 5. Spots 4 and 5 have a bluish and faint-yellowish fluorescence respectively, and spot 3 quenches the blue fluorescence of the filter paper and appears as a dark spot when viewed under short-wave ultraviolet light. In chromatograms of alkaline solutions of indolyl-3-pyruvic acid the maximum relative intensity of spot 2 is obtained when 1 equivalent of sodium bicarbonate is added, that of spot 4 when 2 equivalents are added to the solution.

Fig. 1. Paper chromatogram of indolyl-3-pyruvic acid (about $30 \mu\text{g./spot}$) from a freshly prepared solution treated with sodium bicarbonate. Samples were drawn at regular intervals during 30 min. of standing at room temperature (20°). Development was in water-acetic acid vapour by the ascending method at 5° . Air in the tank was replaced with nitrogen. The sample at time 0 was drawn from a fresh ethanolic solution of indolyl-3-pyruvic acid. 'Strength' (intensity of fluorescence + size of the spots) of spots 2 and 4 is indicated by the number of crosses. Spots and tails outlined with interrupted lines were very faint.

Fig. 2. 'Double chromatogram' of a solution of indolyl-3-pyruvic acid treated with sodium bicarbonate, after 15 min. About $40 \mu\text{g.}$ of indolyl-3-pyruvic acid was applied at the origin. Development was in both directions in water-acetic acid vapour at $5-8^\circ$. Fluorescence colours are indicated at the bottom. A, Outline of spots after the first development. B, Outline of spots after the second development. ■ Blue; ▨ Purple; ▤ Red; ▥ Yellow; ▧ Dark spots.

The fluorescence of spot 2 decreases markedly at first and then remains constant until the end of the experiment (30 min.). Spots 3 and 5 attain maximum intensity within 1 min. and maintain it until almost the end of the experiment. Spot 4 increases until 7 min. and begins to disappear after 30 min.

When air is not excluded from the tank, an additional spot (spot 7, Fig. 2) appears after 30 min. Spot 2 decreases continuously up to the end of the experiment and spot 4, after reaching its maximum, decreases rapidly and practically disappears in 30 min. Spot 5 and the fluorescent tail that bridges all the spots are much more conspicuous.

When the chromatogram is developed a second time with the same solvent in a perpendicular direction ('double chromatograms': Schwarz & Bitancourt, 1957a), the same sequence of spots is seen, starting from the sites occupied by spots 2 and 4 in the first development, except that spot 3 is not found in the sequence of spot 4 (Fig. 2B).

In chromatograms run with water, only spots 2 and 4 react with all the reagents, both spots responding with the same colorations. Some of these reactions, however, especially those for the enol and for the ketone groups, differ in reaction time and in intensity (Table 1). Whereas the differences are quite conspicuous with some of the reagents when they are applied to the dry chromatograms, in other cases the reagent must be applied while the chromatogram is still wet and as soon as possible after chromatography. With dinitrophenylhydrazine a marked difference in the intensity of reaction of spots 2 and 4 was observed only when the spots were cut from the wet chromatograms and dipped in 2 ml. of the reagent. After 5 min. at 30° , the solution in the tube containing spot 2 was only slightly more yellow than the reagent itself, whereas the colour was brown in spot 4. Both colorations were considerably darker after 1 hr. but kept the same relative intensity. The hydrazones in this reaction, after extraction with ethyl acetate and treatment with a solution of sodium hydroxide, imparted a slight reddish coloration to the aqueous phase with spot 2 and a dark wine colour with spot 4. Whereas spot 3 also gives a strong reaction with dinitrophenylhydrazine, it does not colour the sodium hydroxide solution as would an acid hydrazone (Friedemann & Haugen, 1943).

With dichlorophenolindophenol, which colours the whole filter paper blue or purple, spot 2 decolours it immediately and appears as a white spot. Spot 4 begins to decolour later and takes a few minutes for bleaching. Van Eck's reagent gives a yellow colour immediately with spot 4 and a delayed one with spot 2. Ehrlich's reagent for the indole and phenol nuclei, which gives specific

Table 1. R_f values and colour reactions of chromatographic spots 2 and 4 (Fig. 1)

Colour intensity and size of spots are indicated with crosses. Reaction time was instantaneous (inst.) or occurred in minutes.

Chromatography (R_f)	Spot 2	Spot 4
Water	0.07	0.85
Water-acetic acid vapours	0.15	0.80
Water-acetic acid (3:1, v/v)	0.28	?
16% NaCl-2% acetic acid (1:1, v/v)	0.09	0.57
Acetone-water (8:2, v/v)	0.71	0.82
Propan-2-ol-28% ammonia soln.-water (8:1:1, by vol.)	?	0.45*
Enol reagents		
Dichlorophenolindophenol†	++++ (inst.)	+++ (1-2 min.)
Ammoniacal AgNO_3 ‡	++++ (inst.)	+(inst.)
FeCl_3 ‡	++++ (inst.)	+(inst.)
Ketone reagents		
Dinitrophenylhydrazine§	+	+++
Dinitrophenylhydrazine + NaOH	+	+++
Van Eck's reagent†	++++ (2-3 min.)	++++ (inst.)

* Correspondence with spot 4 not certain.

† On the dry chromatogram.

‡ On the wet chromatogram.

§ Spot cut from wet chromatograms and dipped in 2 ml. of reagent.

|| NaOH reaction of hydrazone extracted in ethyl acetate from 2 ml. of reagent solution referred to in the preceding footnote (Friedemann & Haugen, 1943).

colours for different indole compounds, gives with spot 2 a yellow colour turning to greenish and with spot 4 a greenish colour which later turns yellow. Ferric chloride and ammoniacal silver nitrate, when applied to the chromatogram while it is still wet, give a strong reaction with spot 2 and a weak one with spot 4.

In chromatograms with propan-2-ol-aq. ammonia soln.-water (8:1:1) the solvent front ascends 10 cm. in 3 hr. and even fresh solutions of indolyl-3-pyruvic acid already show four spots, more or less united by a yellowish fluorescent tail. One of them, with R_f 0.45, is undoubtedly a spot of indolyl-3-pyruvic acid. It shows a bright-blue fluorescence, decolours dichlorophenolindophenol, and gives a purple colour with the Gordon-Weber reagent, pink with ferric chloride, dark brown with silver nitrate, yellow with Van Eck's reagent and brown with dinitrophenylhydrazine. The outline of the colour reactions, however, is not exactly coincidental with that of the fluorescence and extends as tails below and above the outline of the fluorescent spot. Furthermore, when such chromatograms are run in a second direction with water as the solvent, this spot, like spots 2 and 4, shows a row of spots of decomposition products.

The R_f values of spots 2 and 4 in several solvents are given in Table 1. The two spots can easily be identified in all of them, except spot 2 in propan-2-ol and spot 4 in water-acetic acid.

DISCUSSION

Since spot 2 is the only one to appear in chromatograms of fresh solutions, it is undoubtedly a spot of

indolylpyruvic acid. This is confirmed by its response to seven different reagents for this substance. After spot 2, spot 4 is the first one to appear in chromatograms of recently prepared solutions. It practically duplicates the properties of spot 2, giving the same colour reactions. In double chromatograms of decomposed indolyl-3-pyruvic acid solutions (Fig. 2), spot 2 as well as spot 4, produced in the first run, give rise, in the second direction, to rows of spots duplicating the sequence of the first run. It seems obvious that spot 2 and spot 4 represent two forms of indolyl-3-pyruvic acid.

As a keto acid, indolyl-3-pyruvic acid may be present in solutions as: (i) ionized and undissociated forms (Bitancourt & Nogueira, 1959); (ii) enol and keto forms; (iii) *cis* and *trans* stereoisomers of the enol form. The possibility that spots 2 and 4 might be the same form in two different states of dissociation seems to be eliminated by the fact that whereas both spots are present in acidic solvents in chromatograms prepared from more or less decomposed solutions, only spot 2 is present in fresh solutions in the same solvent.

Since the sample of indolyl-3-pyruvic acid used in these experiments is the result of chemical synthesis, it may contain the *cis* or the *trans* isomers of the enol form, or both. The fact that there is only one spot of indolyl-3-pyruvic acid in chromatograms from fresh solutions shows either that one of the isomers is present only in very small amounts and is undetectable with our methods, or that it cannot be separated from the other isomer. In either case, spot 4 could not possibly be the *cis* or the *trans* isomer. It should also be noted that

conversion from the *cis* into the *trans* form, or vice versa, can only be made through the opening of the double bond followed by its re-establishment. In other words, there should be at least partial conversion into the keto form and reconversion into the enol form after rotation of the distal end of the side chain. Finally the newly formed stereoisomer should be reconverted into the keto form to give the reactions of spot 4. No evidence of such a succession of conversions appears in our chromatograms.

Several workers have studied the enol-keto equilibrium of indolyl-3-pyruvic acid. Stowe (1955) states that pure indolyl-3-pyruvic acid crystallizes in the enol form and in solution tautomerizes within 20 min. at pH 8.0 and 25° largely to the keto form. Kaper & Veldstra (1958) observed the gradual change of the spectrum of indolyl-3-pyruvic acid in neutral medium and suggested that it was caused by tautomerization of the enol form. As the result of careful u.v.-spectroscopic studies, G. F. Smith (personal communication) has found that 'indolyl-3-pyruvic acid, which in aqueous solution exists almost entirely in the enol form, is converted into β -indolyl-CH₂·CO·CO·O⁻ on being treated with one equivalent of alkali in the absence of oxygen. If oxygen is not excluded, auto-oxidation occurs. The anaerobic action of the alkali presumably first produces β -indolyl-CH:C(OH)·CO·O⁻, which is energetically not favoured, and immediately tautomerizes to the keto form.'

We have found no mention in the literature of the separation of enol-keto tautomers by paper chromatography, but such a separation is to be expected if the tautomers have sufficiently different R_f values and if the reconversion of the two forms, resulting from the re-establishment of equilibrium, proceeds at a slower pace than chromatography. As may be seen in Fig. 1, in the short time that elapses between the addition of sodium bicarbonate to the solution of indolyl-3-pyruvic acid and the preparation of the chromatogram (approx. 1 min.), the substances of spots 3-5 are formed at the expense of spot 2, as indicated by the immediate decrease of its intensity. From then on, during 20 min., little change is observed in the intensity of the spots. This, and the fact that the chromatogram prepared from a fresh solution (on the left of Fig. 1), shows no decomposition products, is a proof that under our experimental conditions there is no oxidation in the chromatogram during chromatography and during the 30 min. it takes to prepare the chromatogram.

Factors that are known to affect the enol-keto equilibrium such as pH, heat, quality of solvent and ageing of solutions (Knorr, Rothe & Averbek, 1911) produce a decrease of spot 2 and an increase of spot 4. The almost immediate decrease of spot 2 as a result of alkalization would result both from

tautomerization yielding spot 4 and from decomposition yielding spots 3 and 5. The tail of spot 4 indicates the continuance of tautomerization during chromatography. Spot 4 attains its maximum between 7 and 15 min., obviously at the expense of spot 2. As mentioned above, Stowe (1955) observed the conversion of the enol form into the keto form in 20 min. The fact that spot 4 decreases later and is greatly reduced after 30 min. whereas spot 2 stays the same may be due to spot 4 being more readily oxidized than spot 2.

Propan-2-ol-aq. ammonia soln., an alkaline, slow and aggressive solvent, gives in chromatograms of freshly prepared solutions, developed during 3 hr. at room temperature, one spot only of indolyl-3-pyruvic acid (R_f 0.45) as evidenced by six colour reactions. In chromatograms developed with propan-2-ol during 15 hr. indolyl-3-pyruvic acid is completely destroyed (Jepson, 1958*a*, b).

The question of which of spots 2 and 4 is the enolic form of indolyl-3-pyruvic acid is of interest. The difference in the colour reactions of these two spots support the conclusion that spot 2 is the enol form and spot 4 the keto form of indolyl-3-pyruvic acid. Both spots give the five reactions for enol and for ketone reagents but in one way or another the enol reactions are stronger with spot 2 and the ketone reactions with spot 4 (Table 1). The reaction is slow for spot 4 with dichlorophenolindophenol, a reagent for the enol form (Stowe, 1955; Linskens, 1955), and the same is true for spot 2 with Van Eck's reagent, specific for the carbonyl group (Linskens, 1955; Melchior, 1957).

Spot 2 responds to Ehrlich's reagent with a yellow colour, changing to green, whereas spot 4 gives a greenish colour, later turning yellowish. It would seem that tautomerization proceeds in spots 2 and 4 during the drying of the filter paper. This may also explain the fact that, with some reagents, differences between spot 2 and 4 are much sharper when the reagent is applied to the chromatogram when it is still wet. The enol reagents ferric chloride and silver nitrate give a much paler reaction with spot 4 when the chromatogram is still wet whereas the reaction is strong with spot 2 whether the chromatogram is wet or dry. Ferric chloride also gives a colour reaction with other indole derivatives but in that case the reaction is much slower than with enolic compounds.

In previous papers it was concluded from spectroscopic considerations (Schwarz, 1957) and from the strong reaction of spot 2 with dinitrophenylhydrazine on the dry chromatogram, in contrast with the faint reaction of spot 4, that spot 2 corresponded to the keto form and spot 4 to the enol form of indolyl-3-pyruvic acid (Schwarz & Bitancourt, 1957*b*). The evidence shown here, however, points to the opposite conclusion. We

have since ascertained that the difference in reaction was mainly due to the very small amounts of the substance of spot 4 produced in our early chromatographic experiments. Furthermore, we have obtained, with dinitrophenylhydrazine, a faint reaction with spot 2 and a strong one with spot 4 by dipping the spots, cut from the chromatograms, in the reagent instead of applying it to the dry chromatogram.

Since spot 2 is the first to appear in chromatograms of freshly prepared solutions the evidence from the colour reactions is that the sample of crystalline indolyl-3-pyruvic acid received from Dr Smith is in the enolic form. In this connexion it is noteworthy that in a recent experiment Dr Smith (personal communication) has obtained an u.v. spectrum corresponding to pure enol from his crystals of indolyl-3-pyruvic acid dissolved in anhydrous peroxide-free dioxan. The preparation proved to be rather stable since E at 328 $m\mu$ did not change at all after the first reading, made within 10 sec. of dissolution. Dr Smith thinks this is a reasonable indication that indolyl-3-pyruvic acid must have been in the enol form in the crystalline state.

As mentioned above, Stowe (1955) states that indolyl-3-pyruvic acid crystallizes in the enol form and, from spectrographic studies, Kaper & Veldstra (1958) arrived at the same conclusion. Hydroxyphenylpyruvic acid and phenylpyruvic acid show an analogous behaviour, that is, crystalline acid represents the enol and tautomerizes in aqueous solutions (Knox & Pitt, 1957).

SUMMARY

1. A freshly prepared solution of indolyl-3-pyruvic acid treated with one equivalent of sodium bicarbonate soon begins to decompose. Paper chromatograms of samples drawn at regular intervals show the fading of the original spot (spot 2) and a corresponding appearance of three other spots (3, 4 and 5). The most conspicuous of these, spot 4, first increases and, after 20 min., decreases and fades out.

2. Good separation of spots 2 and 4 is achieved only when precautions are taken against decomposition during chromatography. With a slow solvent like propan-2-ol, in alkaline medium, a number of decomposition products crowd the chromatogram and only a single spot with the characters of spots 2 and 4 can be identified.

3. Colour tests for indolyl-3-pyruvic acid

(Gordon-Weber reagent, Ehrlich's reagent, dichlorophenolindophenol, ferric chloride, ammoniacal silver nitrate, dinitrophenylhydrazine and Van Eck's reagent) applied to chromatograms show that only spots 2 and 4 respond to all of them, suggesting that spots 2 and 4 represent two forms of indolyl-3-pyruvic acid.

4. The relative intensity of spots 2 and 4 was shown to depend on pH, temperature, ageing and quality of solvent, i.e. factors influencing tautomerization.

5. It is therefore suggested that spots 2 and 4 correspond to the enol and keto tautomers of indolyl-3-pyruvic acid respectively, and that the tail in between is due to tautomerization during chromatography.

We are most grateful to Professor G. F. Smith for the sample of indolyl-3-pyruvic acid and for his personal communication. We wish to thank Professor O. Rothe, Brazilian Research Council, and Professor H. Hauptmann, University of São Paulo, for their encouragement and advice, and M. Meneghini, of this Institute, for discussing the problem. We are also indebted to Dr H. Mahler, University of Indiana, Bloomington, for valuable advice on chromatography. One of us (K.S.) is a Research Fellow of the Brazilian Research Council.

REFERENCES

- Bentley, J. A., Farrer, K. R., Housley, S., Smith, G. F. & Taylor, W. C. (1956). *Biochem. J.* **64**, 44.
 Bitancourt, A. A. & Nogueira, A. P. (1959). *Science*, **129**, 99.
 Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
 Gordon, S. A. & Weber, H. P. (1951). *Plant Physiol.* **26**, 192.
 Jepson, J. B. (1958*a*). *Biochem. J.* **69**, 22*r*.
 Jepson, J. B. (1958*b*). In *Chromatographic Techniques*, p. 114. Ed. by Smith I. London: Heinemann.
 Kaper, I. M. & Veldstra, H. (1958). *Biochim. biophys. Acta*, **30**, 401.
 Knorr, L., Rothe, O. & Averbeck, H. (1911). *Ber. dtsch. chem. Ges.* **44**, 1138.
 Knox, W. E. & Pitt, B. M. (1957). *J. biol. Chem.* **225**, 875.
 Linskens, H. F. (1955). *Papierchromatographie in der Botanik*. Berlin: Springer.
 Melchior, H. (1957). *Plants*, **50**, 262.
 Rothe, O. (1912). Dissertation: University of Jena.
 Schwarz, K. (1957). *Arch. Inst. biol. (Def. agric. anim.)*, *S. Paulo*, **24**, 81.
 Schwarz, K. & Bitancourt, A. A. (1957*a*). *Science*, **126**, 607.
 Schwarz, K. & Bitancourt, A. A. (1957*b*). *Arch. Inst. biol. (Def. agric. anim.)*, *S. Paulo*, **24**, 183.
 Stowe, B. B. (1955). *Biochem. J.* **61**, ix.