ON THE METABOLISM OF TRYPTOPHAN BY *AGROBACTERIUM TUMEFACIENS*

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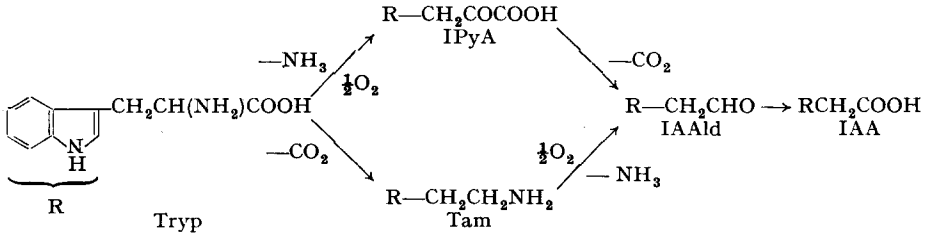
For investigations on the selective inhibition of abnormal growth in plants (*e.g.* Crown-gall) an analysis of the biosynthesis of the auxin indoleacetic acid (IAA**), being a factor of primary importance in Crown-gall development, was required.

IAA is synthesized autonomously by Crown-gall tissue *in vitro*, whereas for the culture of normal tissue IAA has to be added. This capacity might offer a possibility for selective inhibition of tumorous growth by means of analogues of intermediates in the biosynthetic chain (*cf.* WOOLLEY[1]). To make this approach, however, the components of this chain have to be known exactly.

Prior to investigations on Crown-gall tissue itself, we analyzed the formation of IAA from tryptophan (Tryp) by the Crown-gall-inducing organism *Agrobacterium tumefaciens*.

As a result of experiments with micro-organisms and higher plant[2–13,14], tryptophan is considered quite generally to be the precursor of IAA in plant tissues.

Two ways of formation of IAA have been discussed:



Of these, the oxidative deamination of Tryp (as suggested originally by THIMANN[4]), leading to the formation of indolepyruvic acid (IPyA), has been favoured in most cases.

One has to be aware of the fact, however, that other biosynthetic mechanisms may be operating *in vivo*, as has been emphasized by GREENBERG, GALSTON, SHAW AND ARMSTRONG[15], who in this respect drew attention to the possible importance of a reaction between indole and glyoxylate, resulting in the formation of indoleglycollate.

In our work with *Agrobacterium tumefaciens* it was readily shown that the occurrence of IAA in the medium depended upon the presence of Tryp.

* *cf.* J. M. KAPER, *Thesis*, Leyden, 1957.

** The following abbreviations will be used:

IAA, indole(3)acetic acid	ILA, indole(3)lactic acid
IAAld, indole(3)acetaldehyde	Tryp, tryptophan
IAld, indole(3)aldehyde	Tam, tryptamine
IPyA, indole(3)pyruvic acid	Tol, tryptophol

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The analysis of the formation of IAA and of the intermediates was performed by paper chromatography of extracts of the culture medium, and comparative chromatography of the synthetic compounds.

Special attention was paid to the occurrence of IPyA, for which, in our view, previous experimental evidence had been vague and inconclusive.

The peculiar behaviour of synthetic IPyA on chromatograms led to a rather extensive investigation of the underlying reactions, in order to distinguish between products of physiological reactions and artefacts.

MATERIALS AND METHODS

Agrobacterium tumefaciens

Strain E.III.9.6.1. (obtained from the Laboratory of Microbiology, Delft), the virulence of which had been shown in a large number of experiments on induction of Crown-gall in various plants.

Culture-medium

K_2HPO_4	0.1 %	NaCl	0.015 %
KH_2PO_4	0.1 %	$CaCl_2$	trace
$(NH_4)_2SO_4$	0.3 %	Tryptophan	0.1 %
$MgSO_4$	0.05 %	Glucose	2.0 %, aqua dest.

The salts and Tryp were dissolved in 180 ml aqua dest. in quantities calculated for 200 ml. The pH was adjusted to 7 and subsequently the solution was autoclaved for 20 min at 120°.

20 ml of a 20 % solution of glucose was autoclaved for 10 min at 110° and added aseptically to the salt solution.

Incubation

Bacteria from a test-tube culture (peptone–agar, 48 h) were suspended in 200 ml of the culture medium, the suspension was shaken in the dark at 25–27° under aerobic conditions

A culture medium without bacteria served as a blank. The blank remained colourless whereas in the presence of the bacteria a yellow colour developed during incubation.

The bacteria were removed by centrifugation at about 4000 *g*, after which both the blank and the culture-centrifugate (c.c.) were acidified and extracted with ether (freed from peroxides). From the extracts a neutral and an acid fraction were obtained in the usual way by treatment with $NaHCO_3$-solution, acidification, etc., and evaporation of the solvent.

The residues were taken up in 0.5 ml ethanol.

Chromatography

Using Whatman paper No. 1, linear and two-dimensional (ascending) chromatograms were obtained. With the linear chromatograms the compounds were applied to the paper by ticking off 3 times with a micro-pipette (0.004 ml) containing the alcoholic solution. With the two-dimensional ones this was repeated 5 to 6 times.

The solvents used were: isopropanol–ammonia–water = 10:1:1; isopropanol–glacial acetic acid–water = 4:1:1.

The spots were developed with the reagents according to Salkowski (5 % $HClO_4$–0.05 *M* $FeCl_3$ = 50:1) or Ehrlich (660 mg of *p*-dimethylaminobenzaldehyde dissolved in a mixture of 36 ml of ethanol and 8 ml conc. HCl).

Resting cells (anaerobic experiments)

Bacteria from a test-tube culture (peptone–agar, 48 h) were inoculated in 500 ml of peptone–water and aerated intensively for 48 h by shaking. Subsequently the bacteria were separated from the medium aseptically by centrifugation and washed with the salt solution to be used for the experiment (culture medium as indicated above, with omission of $(NH_4)_2SO_4$ and glucose) by repeated resuspension and centrifugation. Finally, the bacteria were taken up in 150 ml of the salt solution.

The compounds to be investigated were dissolved in a small amount of the salt solution and sterilized by means of a Seitz-filter. The solution was added to the suspension of the bacteria shortly before incubation. Indole-pyruvic acid was dissolved in an exactly equivalent amount of diluted NaOH. Tryp could be sterilized directly with the medium. The incubation mixture was

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transferred to a flask fitted with gas in- and outlet tubes. A sterile current of nitrogen (freed from oxygen by passage through an alkaline solution of pyrogallol) was passed through for 30–60 min. Then the flask was closed and shaken at the temperature required.

Radio-autography

The film used was the normal X-ray-film "Osray-Gevaert" (30 × 40 cm). In the dark room the chromatograms were attached to the film by means of paperclips.

Series of identical chromatograms were kept in the dark, in the original packing envelopes. Development took place after varying periods, thus allowing us to select the optimal conditions for the analysis.

RESULTS

Preliminary experiments

For the quantitative estimation of the IAA formed by *Agr. tumefaciens* in media containing Tryp, depending on the duration of incubation and the concentration of Tryp, the method of BENNET CLARK *et al.*[16] was followed.

After 48 h the production of IAA was maximal, but the yield did not exceed 2 %, a rather general experience with this type of experiments.

With up to 0.15 % Tryp, the yield of IAA increased, followed by a rapid levelling at higher concentrations.

With resting cells also, IAA production was observed, the main difference being that the maximum was attained within a shorter period (6–7 h).

Apart from the spot of IAA, others were present, representing most probably also indole compounds (positive Salkowski reaction) and thus possibly related to the formation of IAA from Tryp.

A comparison with chromatograms of synthetic IPyA and of tryptamine (Tam) showed the latter to be absent, whereas the presence of IPyA was considered probable. No definite conclusion could, however, be arrived at, since on the chromatogram (solvent: isopropanol–ammonia–water) of synthetic IPyA a number of spots occurred, one corresponding with IAA. Apparently the keto-acid is not stable under the conditions of chromatography and thus one has to be aware that the spots on the chromatogram of our extracts may represent products of physiological reactions and/or artefacts.

WILDMAN *et al.*[7] had already pointed out that IPyA was labile in aqueous solution, giving rise to IAA and indole-acetaldehyde (IAAld). To arrive at a correct interpretation of the chromatograms, it became necessary, therefore, to analyze the behaviour of IPyA during chromatography.

Indolepyruvic acid, synthesis and properties

While the more chemical aspects of the properties of IPyA have been reported on separately[17], the main facts of direct importance for the present problem are given here.

The synthesis of IPyA was carried out according to both ELLINGER AND MATSUOKA[18] and BENTLEY *et al.*[19], the latter method giving the best results. (For details *cf.*[17]).

($C_{11}H_9O_3N$: found C 64.7; H 4.6; N 6.6; calculated C 65.0; H 4.4; N 6.9.)

STOWE AND THIMANN[20,21] had reported the presence of IPyA in maize endosperm on the basis of a chromatographic analysis of extracts (solvent: isopropanol–ammonia–water).

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In the light of our experience it seemed doubtful whether such a conclusion could be justified, since in the solvent used IPyA does not behave as one compound. The authors[21] had already pointed out a certain instability under the chromatographic conditions.

During the course of our work STOWE[22], in a study on the production of IAA from Tryp by cell-free extracts of bacteria (including *Agr. tumefaciens*), stated explicitly that IpyA, when chromatographed, decomposes spontaneously into IAA and other unidentified compounds.

More recently, BENTLEY *et al.*[19], studying the chemical and physiological properties of IPyA, arrived at the conclusion that the compound breaks down completely under chromatographic conditions as used by STOWE AND THIMANN; this implies that the presence of IPyA in a biological extract has not yet been convincingly shown. Two of the spots were identified (R_F, colour reactions and bio-assay) as IAA and (probably) indoleglycollic acid.

In linear chromatograms of synthetic IpyA with the solvent isopropanol–ammonia–water we invariably obtained the pattern of 6 or 7 spots as indicated schematically in Fig. 1. (E not always present.)

That this result is determined by the alkaline medium becomes evident on comparison of this chromatogram with one obtained in isopropanol–glacial acetic acid–

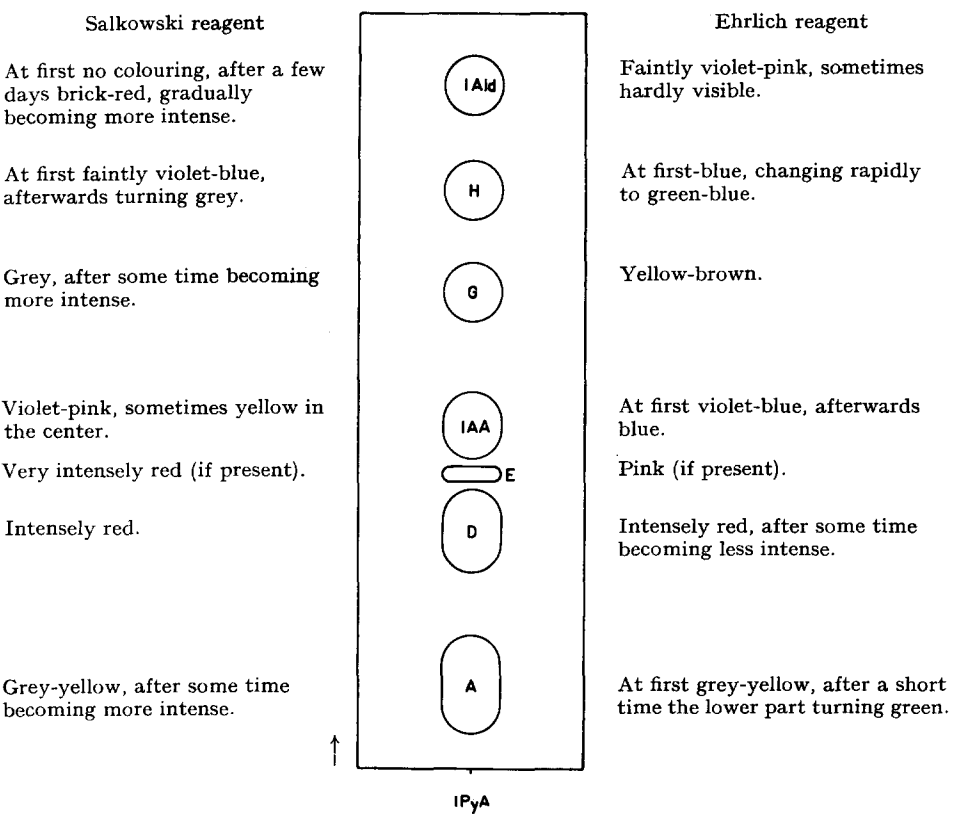

Fig. 1. Standard chromatogram of indolepyruvic acid (Solvent: isoprop.–NH_3–H_2O = 10:1:1).

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water, showing only one spot (with tailing) as reproduced in Fig. 2 (left part). Apparently IPyA is relatively stable in acid medium (BENTLEY *et al.*[19] obtained exactly the same result); this is also shown by the fact that on subsequent chromatography in alkaline medium the characteristic pattern of several spots reappears (Fig. 2).

The fact that the decomposition of IPyA takes place immediately and completely upon deposition on the paper can be deduced from the result obtained by two-dimensional chromatography, both times in identical alkaline solvent (*cf.* Fig. 3). The diagonal position of final spots, each corresponding to one of the first run, indicates the absence of IPyA on the paper. It is evident, therefore, that STOWE AND THIMANN[20,21] did not prove the occurrence of IPyA by their chromatographic procedure.

When the "standard" chromatogram (Fig. 1) is submitted to a second run in acid medium, the result is of the type as given schematically in Fig. 4. As all of the

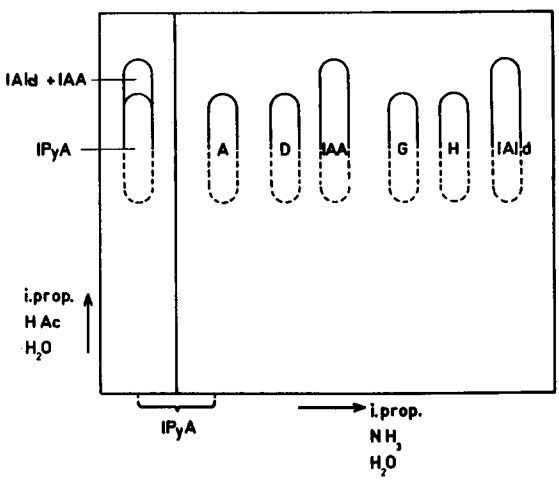

Fig. 2.

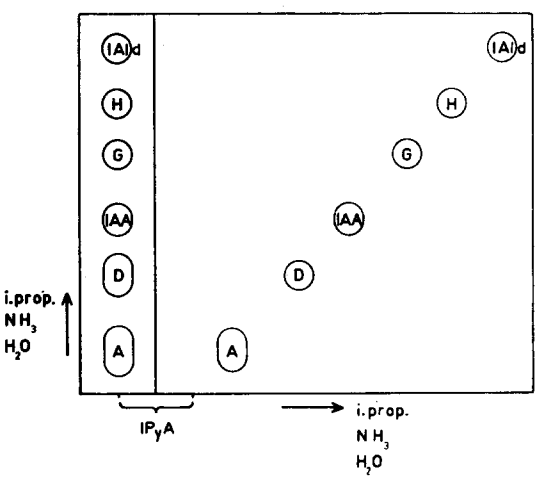

Fig. 3.

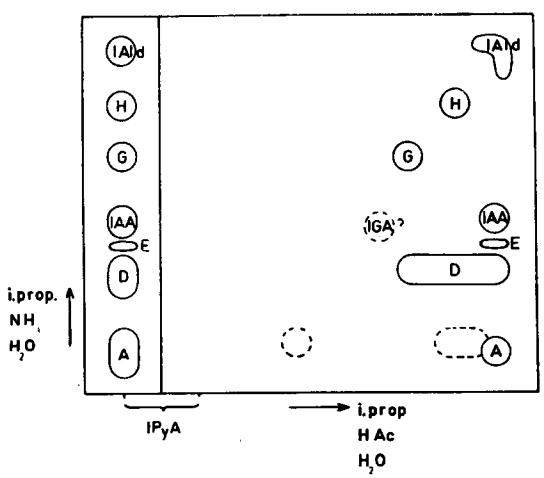

Fig. 4.

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spots can be developed by Salkowski and Ehrlich reagents, their indole character is very plausible. From the diagonal position of the spots G, H and IAld it may be deduced that most probably they represent neutral compounds, whereas the position of A, D, E and IAA would suggest that they represent acidic substances.

To identify the spots, comparative chromatography was carried out with a number of indole derivatives. As a first approximation, it soon became very probable from the R_F values and specific colours that spot IAA was identical with indoleacetic acid, and spot IAld with indolealdehyde.

In a later phase of our work we had a sample of indoleglycollic acid (sodium salt)* at our disposal, by means of which the identity of spot D as such was made probable. This is in accordance with the observation of BENTLEY *et al.*[19], who found the colour reactions of one of the spots (~ D) to correspond completely to this acid. If D were identical with indoleglycollic acid (labile toward acid) this would explain also the decomposition of D when chromatographed in acid medium (*cf.* Fig. 4).

Further information was obtained by means of ultraviolet spectra (Unicam SP 500) of both synthetic compounds and of those eluted from the respective spots on the chromatograms.

The spectra for synthetic IAA and indoleglycollic acid (Fig. 5a) are characteristic for all indole derivatives without a double bond in the side-chain in conjugation with

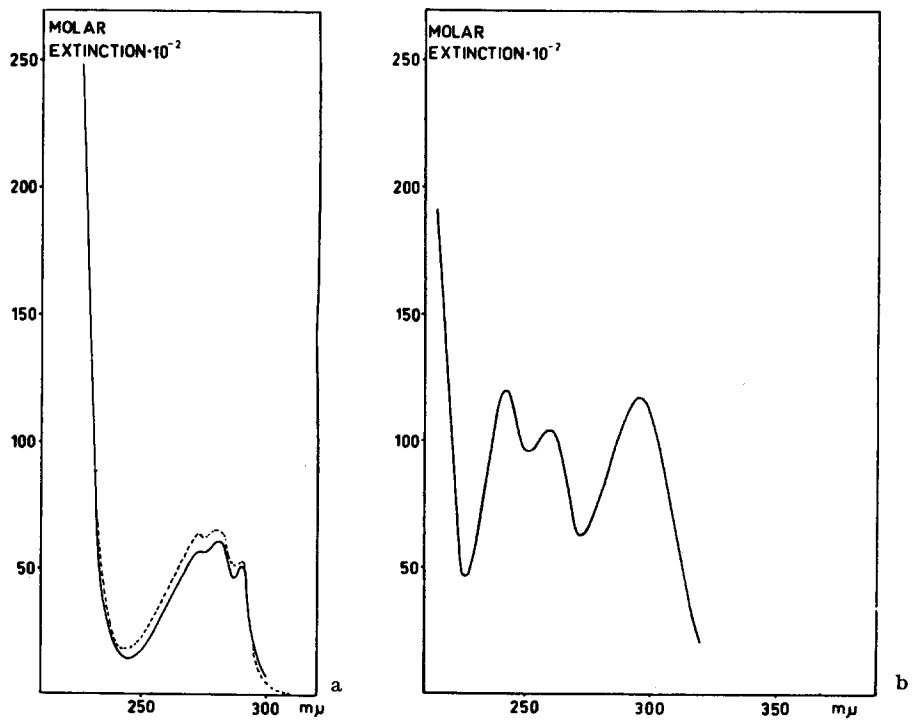

Fig. 5. Ultraviolet spectra (in ethanol). a. ——— indoleacetic acid, – – – – indoleglycollic acid; b. indole-aldehyde.

* Our sincere thanks are due to Prof. Dr. A. W. GALSTON, Department of Botany, Yale University, New Haven, for kindly providing us with the compound.

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the ring system. In that case, more pronounced maxima are present, as *e.g.* with indolealdehyde (Fig. 5b).

Since in this type of experiments the purity of the compounds cannot be guaranteed and since the quantity present in the eluate is unknown, the extinctions observed cannot be compared on a common (molar) base. This does not matter, however, since for our comparison the type of the spectra is decisive.

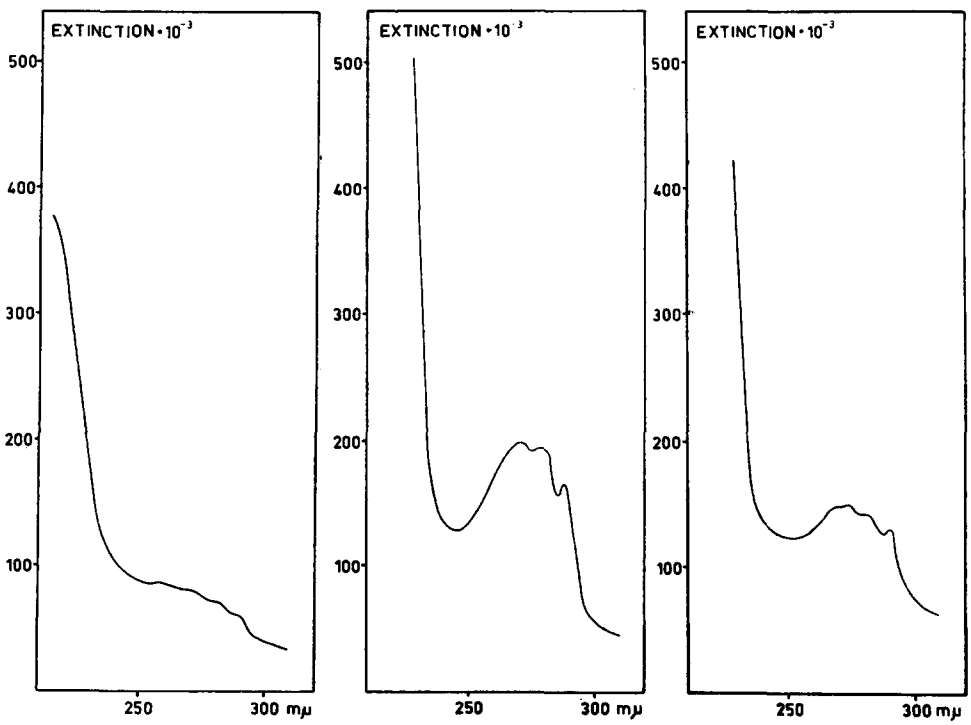
The spectra of the compounds responsible for the spots on the chromatogram of IPyA (eluted with ethanol) are represented in Fig. 6.

Whereas for A and H no distinct information can be obtained from the spectra, it is evident that D, IAZ and G represent the indole type, conformable to the structures assigned to D and IAA by chromatographic evidence. G will have to be isolated on a preparative scale for a definite identification (of the neutral compounds, indole, skatole, tryptophol and indoleacetonitrile could be excluded).

The peculiar behaviour of IPyA on chromatograms induced us to study extensively the properties of this keto-acid in solution by means of ultraviolet spectroscopy. The details are published elsewhere[17]; we should like to refer here only to the following aspects.

In neutral medium (ethanol–water = 9 : 1) the spectrum changes gradually as indicated in Fig. 7. The occurrence of isosbestic points makes it probable that an intramolecular transformation (tautomeric enol- and keto-forms) is involved.

That we are starting with the enol-form (as suggested by STOWE[22] on the grounds



Spot A Spot D IAA

Fig. 6.

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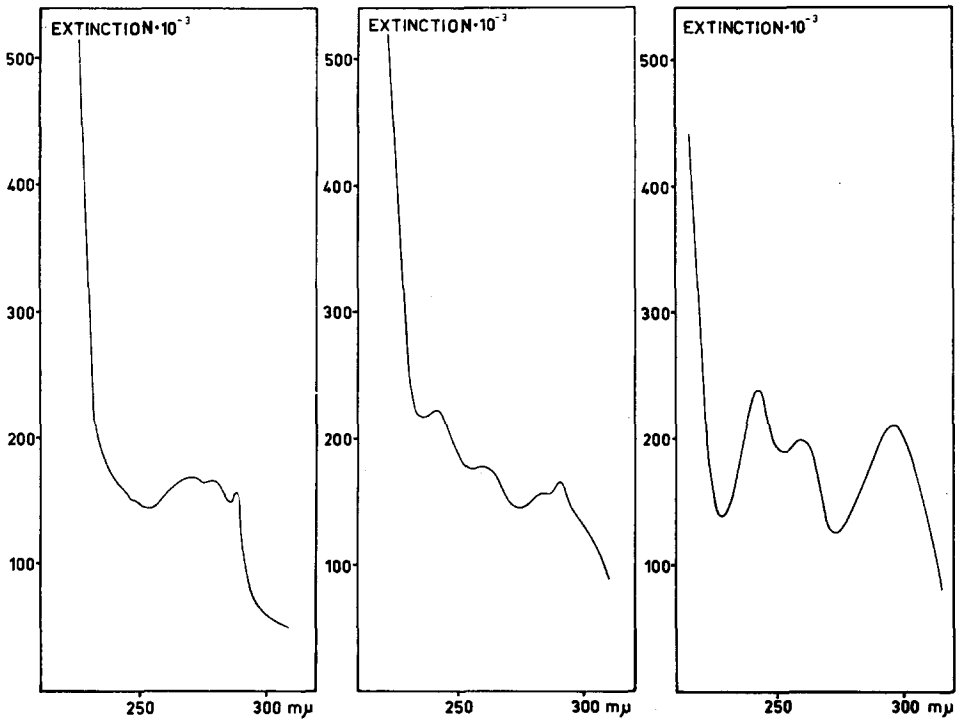
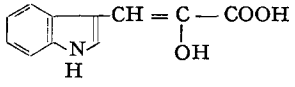


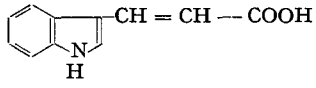
Spot G Spot H Spot IAld

Fig. 6. Ultraviolet spectra (in ethanol). Compounds eluted from the spots on the standard chromatogram of indolepyruvic acid.

of other arguments) may be deduced from the similarity of the spectrum in Fig. 7, curve 1, to that of indoleacrylic acid, as shown in Fig. 8.



enol-form of IPyA



indoleacrylic acid

By recording the spectral changes at different pH's, IPyA was found to be relatively stable in acid media, though decomposition to an indole type was observed. Such a breakdown takes place very rapidly at pH > 9, also resulting in indole derivatives. At lower alkaline pH, a highly accelerated spectral change, of the type indicated in Fig. 7, takes place.

The spectrum of IPyA in the alkaline chromatographic solvent instantly takes the indole-form, corroborating the chromatographic findings (Fig. 9).

Summarizing it must be concluded that IPyA, a potential intermediate in the metabolism of tryptophan, is liable to undergo spontaneous decomposition; consequently, the formation of artefacts under the conditions of several analytical methods has to be taken into account.

Since it appears that also in the case of other α-keto-acids that are of some importance as intermediates in cell metabolism similar transformations may occur

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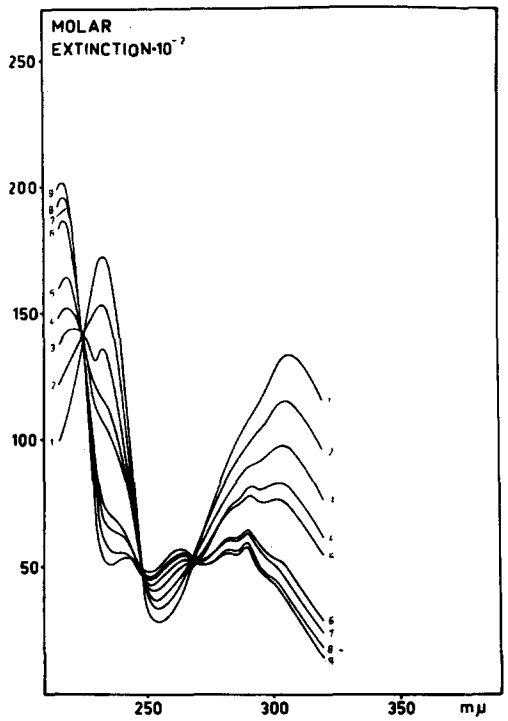

Fig. 7.

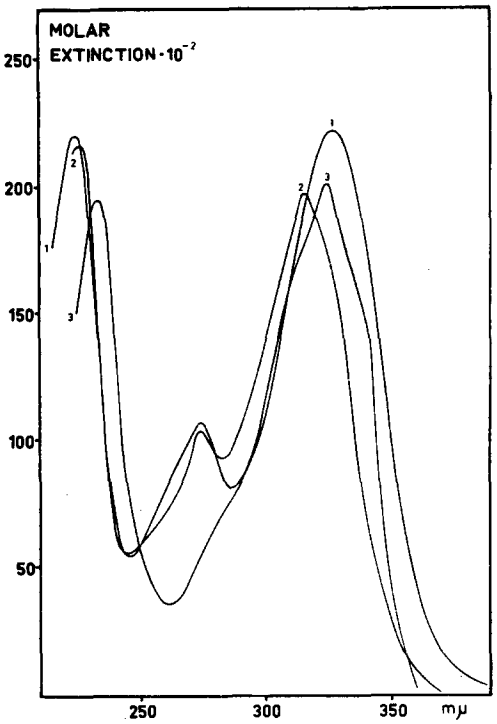

Fig. 8.

Fig. 7. Ultraviolet spectra. Indolepyruvic acid (ethanol–water). Curves 1–9, recorded after 12, 61, 110, 156, 196, 325, 366, 408 and 1500 min resp.

Fig. 8. Ultraviolet spectra. 1. Indoleacrylic acid (ethanol). 2. Indoleacrylic acid (ether). 3. Indolepyruvic acid (ether).

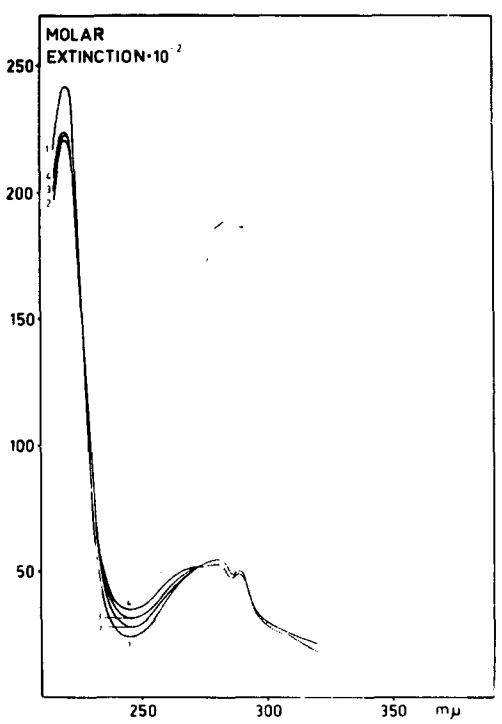

Fig. 9.

Fig. 9. Ultraviolet spectra. Indolepyruvic acid (isoprop.–NH_3–H_2O = 10:1:1). Curves 1–4, recorded after 4, 57, 233 and 1386 min resp.

(phenylpyruvic acid[23,24], *p*-hydroxyphenylpyruvic acid[25], imidazolepyruvic acid[26]), possible interference by artefacts during analysis has to be detected.

Hence, it seems advisable to undertake an extensive investigation of the relative properties of α-keto-acids.

The metabolism of tryptophan

The data from the chromatograms obtained with extracts of the incubated culture medium containing Tryp, are summarized schematically in Fig. 10.

Spots C, M, N, K. (Ehrlich: yellow; Salkowski: negative). These compounds (incompletely concentrated in the neutral fraction, *cf.* 10b and 10e), forming the complete chromatogram of the blank (culture medium without bacteria, *cf.* 10c) are apparently unrelated to bacterial activity.

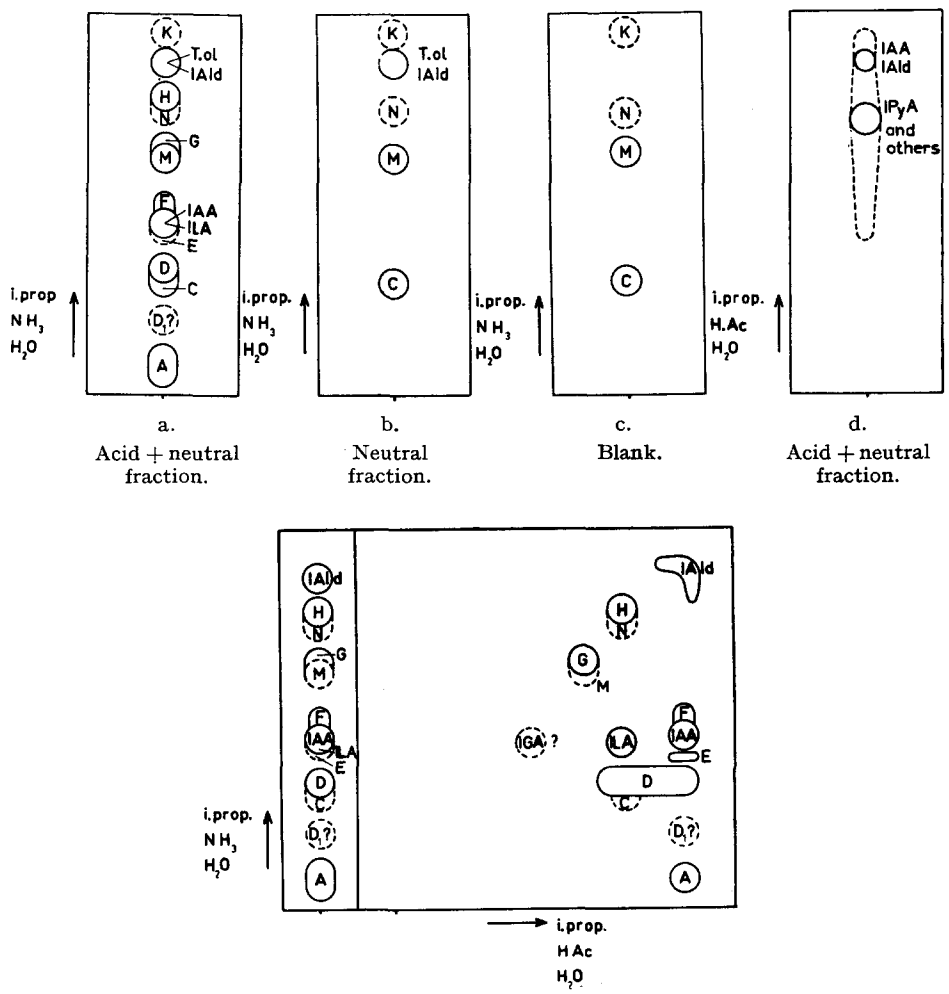

Fig. 10. Chromatograms.

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They are formed from Tryp by the extraction procedure (extraction with ether in acid medium), as could be proved by applying this procedure to an aqueous solution of Tryp, which in untreated form yielded quite normally the spot characteristic for this amino acid.

In experiments to be referred to later, the same yellow spots were obtained with other indole derivatives. The supposition that the compounds belong to the kynurenine-anthranilic acid group or to hydroxylated *o*-amino-acetophenones (*cf.* MANNING AND GALSTON[27]) could not be verified, and thus their identity remains to be established.

Spots A, D (E), IAA, G, H, IAld

By comparing the two-dimensional chromatogram of Fig. 10e with that of IPyA (Fig. 4), it becomes evident that the complete pattern of spots characteristic for IPyA is obtained from the acid fraction of the incubated culture medium. This result provides definite proof (for the first time) of the formation of IPyA in a biological medium. While in general the fact that two compounds possess the same R_F-values, is not sufficient proof that they are identical, the occurrence of a septenary pattern of spots, identical as regards R_F-values and shades of colour with that of IPyA, can be considered as a specific proof of the presence of this compound (*cf.* Plate I).

We are immediately confronted with the difficulty of establishing whether spots in this pattern (*e.g.* IAA and IAld), apart from being artefacts, are also formed as a result of physiological reactions.

That IPyA is possibly partly decomposed already during incubation, may be deduced from the fact that IAld is present on the chromatogram of both the acid and the neutral fraction.

Spot ILA

A slight difference in the shade of the colour of the spot IAA (Salkowski reagent) on chromatograms of synthetic IPyA and of the acid fraction from the culture medium, made it seem probable that in the latter chromatogram two spots were coinciding. This was verified by two-dimensional chromatography (Fig. 10e). Comparative chromatography (R_F, colour reactions) indicated that ILA is identical with indolelactic acid.

Spot Tol

The chromatograms of the neutral fraction (10b), if developed with Ehrlich reagent, showed a spot coinciding with that of IAld, but differing from it in having a blue colour. With Salkowski reagent no such distinction from IAld was observed. Comparative and co-chromatography with synthetic tryptophol in different solvents made it seem very probable that this alcohol is responsible for the blue spot.

Spot F

We have not yet succeeded in identifying this compound.

As ILA, Tol and F are not present in the IPyA pattern, they may be considered to be physiological products.

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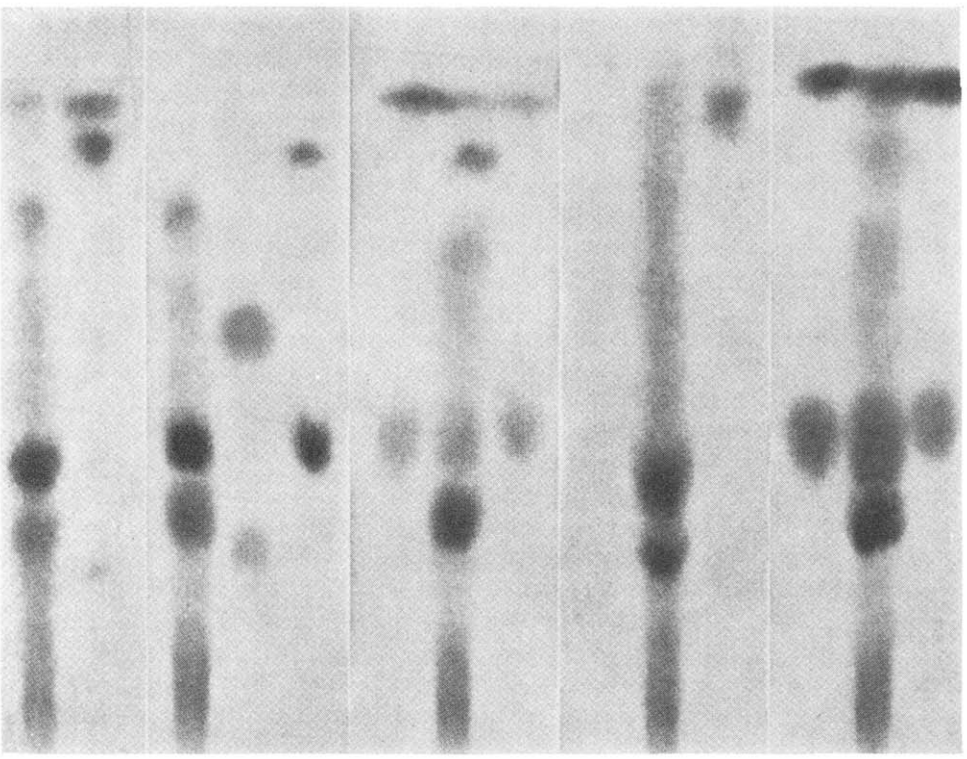
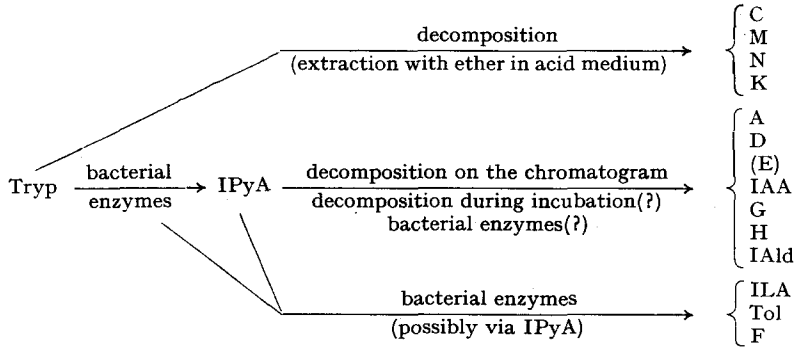


Plate I. Chromatograms. Extracts from culture media and synthetic indole derivatives (solvent: isoprop.–NH_3–H_2O = 10:1:1).

1. acid fraction;
2. neutral fraction;
3. synthetic indolepyruvic acid;
4. blank;
5. indoleacetic acid (lower), tryptophol (upper);
6. indoleacetic acid (lower), tryptophol (upper);
7. synthetic indolepyruvic acid;
8. indolelactic acid (lower), indolealdehyde (upper);
9. blank;
10. acid fraction;
11. neutral fraction;
12. indoleacetic acid (lower), tryptophol (upper);
13. synthetic indolepyruvic acid;
14. indolelactic acid (lower), indolealdehyde (upper).

No. 1–8, Ehrlich reagent, No. 9–14, Salkowski reagent. Shades of colour in 1–5 differ from those in 6–8 because of the fact that the latter chromatogram was photographed immediately after development.

At this stage of the analysis, the situation can be summarized in the following scheme:



Chemical pathways in the transformation of Tryp

In order to establish the relation between the products of physiological origin, especially IPyA, ILA and Tol, a number of experiments was carried out, incubating different compounds with cultures under aerobic or anaerobic conditions, respectively. The latter implies the use of resting cells, since the reproduction of *Agr. tumefaciens* requires aerobic conditions.

Some of the results obtained are shown in Figs. 11 and 12 (for Expt. 1, *cf.* Fig. 10e) and summarized in Table I.

It appears that under anaerobic conditions ILA and Tol are formed almost exclusively from Tryp (Expt. 2).

Expt. 3 shows that the bacteria, under the conditions normally used with Tryp, can produce ILA and Tol from IPyA. Though this does not exclude formation of ILA from Tryp along a different route, the function of IPyA as a precursor for both ILA and Tol is thereby made probable.

As in Expt. 7, the chromatograms of the blank and of the medium with bacteria were identical, no Tol being formed; ILA apparently does not serve as a precursor for Tol.

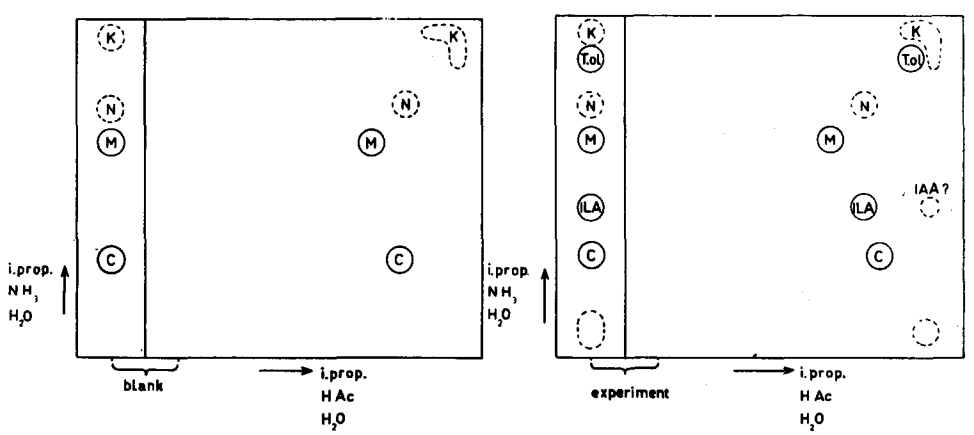


Fig. 11. Chromatograms.

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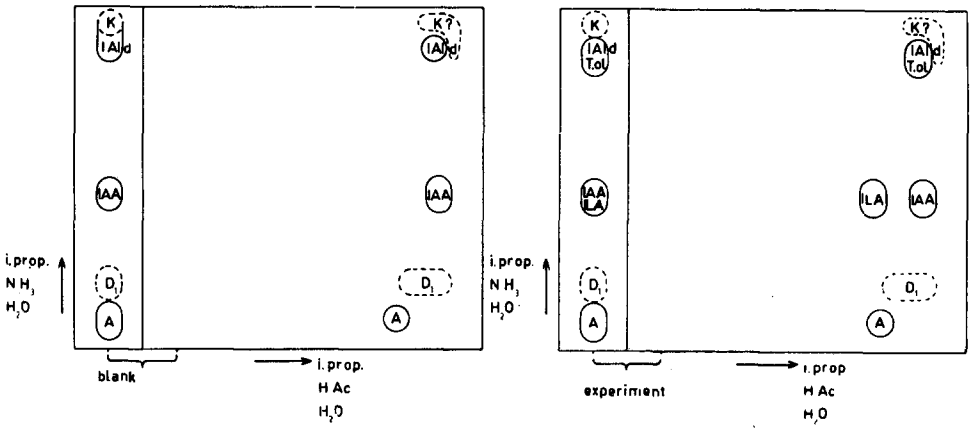
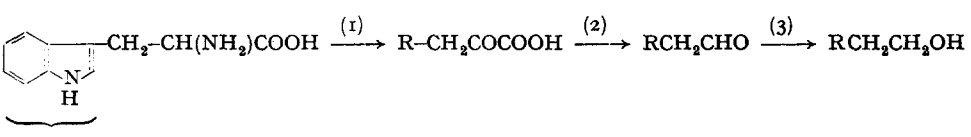


Fig. 12. Chromatograms.

TABLE I

Experiment No.	*Experimental conditions*	*Incubation with*	*Formation of*	
			ILA	*Tol*
1	Growing culture aerobic	0.1 % Tryp	++++	+++
2	Resting cells anaerobic	0.1 % Tryp	++++	+++
3	Growing culture aerobic	0.002 % IPyA	++	+
4	Resting cells aerobic	0.002 % IPyA	—	—
5	Resting cells anaerobic	0.002 % IPyA	+	—
6	Resting cells aerobic	0.1 % Tryp	—	—
7	Growing culture aerobic	0.004 % ILA	++++	—

Since it has already been shown that tryptamine is not involved in the system studied, the only plausible pathway remaining is:



This is the most probable one also for the production of ILA (by hydrogenation of IPyA). Reaction 3 may be a hydrogenation or a dismutation reaction, in the latter case resulting in additional formation of IAA.

From this point of view, the lack of ILA and Tol formation in Expt. 4 might be ascribed to too low a hydrogenating capacity in resting cells, for which, moreover, oxygen is a strong competitor. If the latter is absent (Expt. 5), some hydrogenation

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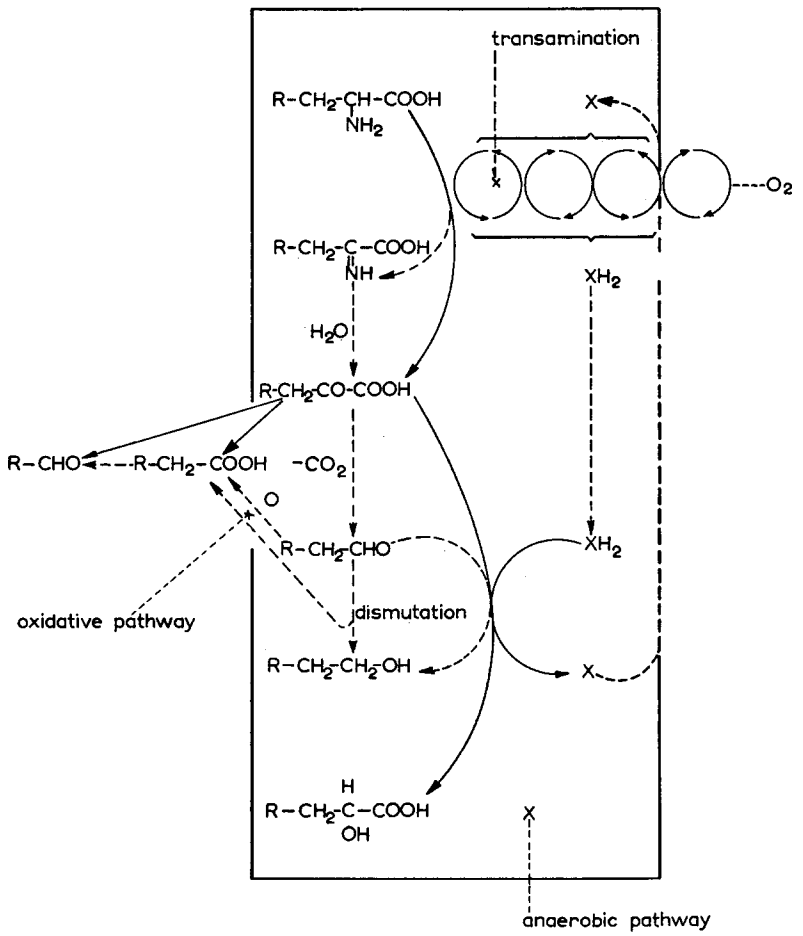
of IPyA could produce a small amount of ILA (as observed), though the absence of Tol cannot be plausibly explained*.

The fact that ILA and Tol are also not found upon aerobic incubation of resting cells with Tryp (Expt. 6) might be explained along similar lines as in Expt. 4, a complete oxidative pathway again being followed.

A scheme outlining the possible relationships revealed by these experiments is given below.



The entire scheme shows the transformations of Tryp in growing cultures under aerobic conditions, whereas the part between the lines represents the anaerobic breakdown of Tryp.

X stands for a hydrogen-carrier (dehydrogenase-system) along which the hydrogen

* Results of incubations with IPyA are sometimes erratic, owing to the instability of this acid and to the low concentrations used. In experiments with growing cultures, IPyA is added preferentially after 30 h only, when an appreciable bacterial density has been attained in the culture. Higher concentrations of IPyA often proved to be toxic.

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from the initial step (in case of oxidative deamination) or possibly after a preceding transamination (*cf.* GORDON[28]) is passed on to the oxygen under aerobic conditions.

In its reduced form (XH_2) it is assumed to be responsible for the reduction of IPyA and of IAAld, formed in a later phase.

A closed system $(X \longrightarrow XH_2) \dashrightarrow (XH_2 \longrightarrow X)$ functioning anaerobically could account for the results of the respective experiments discussed above.

In order to test such a function of $X \rightleftharpoons XH_2$, methylene blue (as a model) was added in increasing concentrations to anaerobic cultures (resting cells). If it would take over the role of $X \rightleftharpoons XH_2$ completely, the chromatographic pattern would have to be the same as in the standard experiment with Tryp. If it acts as a hydrogen acceptor only, thus replacing oxygen, it must be expected that with increasing concentrations of methylene blue the hydrogenation of IPyA will decrease, resulting in a gradual disappearance of ILA from the chromatogram. The latter proved to be the case, as is shown in Table II.

TABLE II

Experiment No.	*Concentration methylene blue M/l*	*Discolouration after*	*Formation of*		
			ILA	*IAA*	*Tol*
8	$16 \cdot 10^{-4}$	5 min	++++	+	++
9	$8 \cdot 10^{-3}$	a few h	++	++	++
10	$4 \cdot 10^{-2}$	no time lag	—	+++	++

The simultaneous increase in IAA might indicate a switching over to the "oxidative pathway" for the transformation of IPyA, most probably a dismutation reaction with IAAld, and would explain also the occurrence of a practically constant amount of Tol in all three experiments.

The nature of the dehydrogenase system in the bacteria remains to be established.

The oxidative pathway from IpyA to IAA (via IAAld) and possibly further to IAld was analysed likewise by chromatography. A complete survey could not be carried out, however, as we had no IAAld at our disposal. Moreover, the instability of this compound would have again introduced "paper chemistry" complications.

A decision whether IAA and IAld, apart from being artefacts in the decomposition of IPyA, could also be formed physiologically, could hardly be based on chromatography since in the solvents that are most suitable technically breakdown is most pronounced and even in acid media it is not completely absent.

Optimal data were obtained from experiments in which the periods of incubation with Tryp were varied (see Table III).

TABLE III

Experiment No.	*Incubation time h*	*Turbidity*	*Yellow colour medium*	*Final pH*	*Formation of*				*IPyA pattern*
					IAA	*ILA*	*Tol*	*C,M,N,K*	
11	23	+	+	6.55	++	±	±	+++	±
12	38	+++	+++	5.15	+++	++	++	+++	+++ (most pronounced)
13	48	++++	++++	4.85	+++	++++	++	+++	+++

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With increasing duration of the experiment, the yellow colour of the medium became more intense, whereas the pH value decreased considerably.

After 23 h, IAA was almost exclusively present on the chromatogram, together with a small amount of IAld (apart from C, M, N, K). The usual products of IPyA breakdown (A, D, G, H) were absent, and so were ILA and Tol. These spots did appear on prolonging the period of incubation.

If in the system:

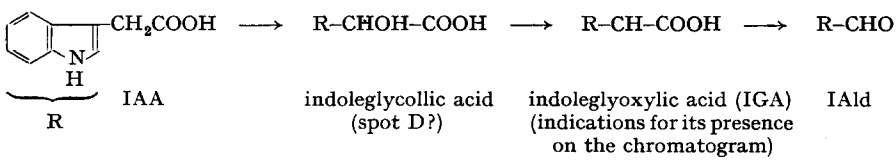
$$\text{Tryp} \xrightarrow{(1)} \text{IPyA} \xrightarrow{(2)} \text{IAAld} \xrightarrow{(3)} \text{IAA}$$

reaction (1) is supposed to be enzymic, whereas (2) and (3) may be enzymic and/or spontaneous, one is inclined to conclude from Expts. 11, 12 and 13 that at first the reactions proceed in a balanced way. With longer incubation period, reactions (2) and (3) seem to become limiting factors, resulting in an accumulation of IPyA and the subsequent formation of, *e.g.*, ILA.

That this accumulation is not caused by the decreasing pH, could be seen from an experiment (lasting 41 h) in which, at a higher buffering capacity of the medium, its pH decreased from 7 to 6.5 only, while the outcome was the same as in Expt. 12. Other possible explanations for an accumulation of IPyA, both on the basis of enzymic and of spontaneous reactions, have not yet been examined.

As regards the occurrence of IAld, it was checked whether IAA could function as a precursor under the conditions of the standard experiment. The result was negative. After incubation with IAA, no IAld was found on the chromatogram, which (being identical to that of the blank) showed the spots D, N and K only.

Though a reaction sequence:



seems plausible, from the experiment with IAA it must be concluded that under those conditions, only IPyA can be the direct source for IAld (enzymic and/or spontaneous reaction).

It is evident therefore that, though the main outlines of the process of transformation from Tryp to IAA can be discerned (*cf.* also REINERT[29] and GORDON[28]), a number of details concerning the mutual relation of the components of the chain still have to be clarified. Especially the matter of distinguishing products of physiological reactions (essential for the problem) from possibly identical products of easily occurring spontaneous side reactions will require further attention. A refinement of the time-studies, as introduced in Expts. 11–13, may contribute to a solution (*cf.* also SCHWARZ AND BITANCOURT[30]).

Experiments with tryptophan-^{14}C

In order to check more directly the relation between Tryp and the compounds indicated by the spots on the chromatograms, Tryp labelled with ^{14}C in the 2-, 9- and

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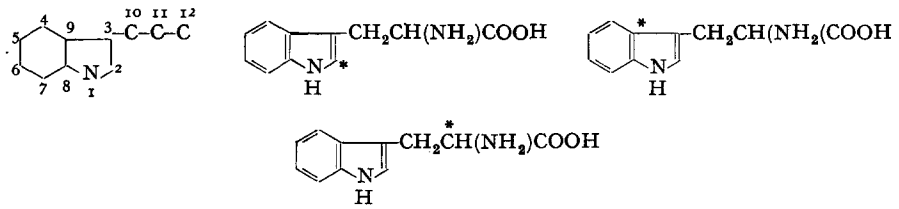
11-positions*, respectively (numbering according to skeleton below),



was used in the culture medium after an appropriate dilution with unlabelled Tryp (recrystallization of the mixture from ethanol–water).

The compounds formed were analyzed again chromatographically, the spots being traced by radioautography (control experiments with the Ehrlich and Salkowski reagents).

A diagram of the occurrence of "labelled" spots is given in Fig. 13. (The radioactivity of Tryp-9-^{14}C was rather low, so that even if no tracing was found in weak spots (colour reactions) this is not conclusive as to the presence or absence of C_9 in the compounds concerned.)

Apart from IAld (C_{11} absent), evidently all of the compounds resulting from the breakdown of IPyA on the chromatograms do possess the C_2-, C_9- and C_{11}-atoms. Their indole character is thus confirmed most definitely, considering the different pathways known for tryptophan metabolism[30]. That all of them carry C_{11} indicates that the unidentified compounds A, G and H still possess the almost (or totally) intact Tryp skeleton.

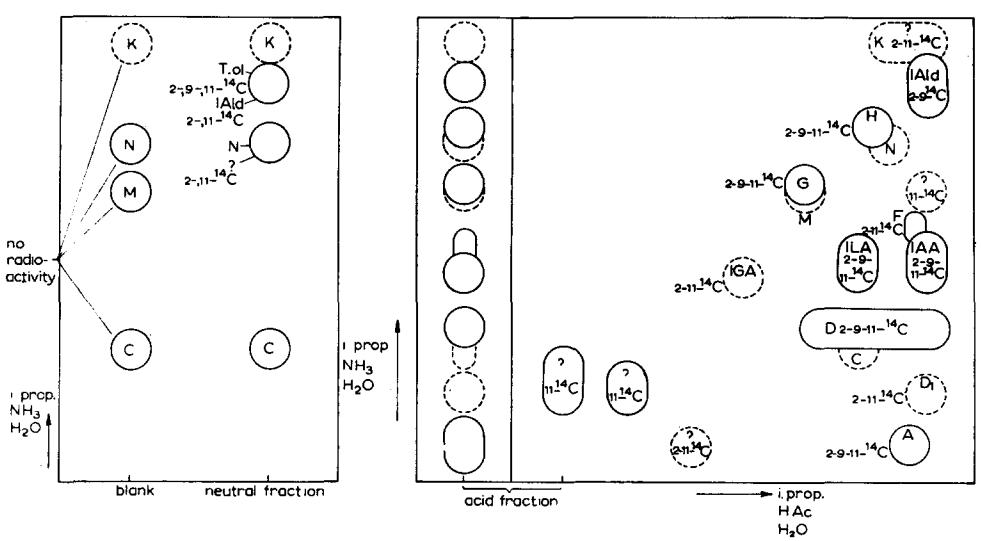


Fig. 13. Chromatograms. Indications concerning ^{14}C refer to the type of labelled tryptophan causing radioactivity in the respective spots.

* Tryp-2-^{14}C, Tryp-9-^{14}C were synthesized at the Organic Chemical Laboratory, Leyden University. Our sincere thanks are due to Prof. Dr. E. HAVINGA for having made these compounds available to us.

Tryp-11-^{14}C was a commercial preparation.

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With Tryp-11-^{14}C some additional spots, not coloured by either the Ehrlich or the Salkowski reagent, were observed. They may represent aliphatic compounds formed by fission of the side-chain ("tryptophanase" type of reaction).

The fact that C, M, N and K were devoid of radioactivity is rather puzzling and calls for further elucidation.

DANNENBURG AND LIVERMAN[32] have also applied labelled tryptophan in their experiments on its conversion to IAA by watermelon-tissue slices. The authors claim to have identified IPyA on the chromatogram (solvent isoprop.–NH_3–H_2O= 8:1:1), but in our opinion their arguments are far from convincing. The respective spot corresponds in position on the chromatogram and colour reaction (Ehrlich) to our spot A, which from its behaviour in double chromatography (Fig. 3) and from spectroscopic evidence (Fig. 6) does not represent IPyA.

The spot tentatively identified by the authors as indoleglycollic acid must represent a different compound also, since in the solvent used the more hydrophylic indoleglycollic acid must have a lower R_F than IAA, as was readily shown by comparative chromatography.

Chemical experiments with the incubated culture medium

From a larger culture (volume 20 l), by the usual extraction and separation procedures we obtained 1.5 g of an acid fraction and 0.25 g of a neutral fraction, both in the form of amorphous brown powders.

The reaction of the acid fraction with 2,4-dinitrophenylhydrazine yielded a crystalline yellow compound[17], identical as far as melting-phenomena and chromatography were concerned with a yellow compound obtained in the same way from synthetic IPyA. They could not be identified as the 2,4-dinitrophenylhydrazone of IPyA, however. Thus chemical evidence for the occurrence of IPyA is obtained in this way, though again indirectly, *via* breakdown products.

From the neutral fraction, 20 mg of crystalline IAld were isolated; this upon recrystallization from water yielded 12 mg of pure IAld (m.p. 195°, synthetic IAld 194°, mixed melting point 194°; C_9H_7ON: found C 73.6, H 4.9, N 9.6; calculated C 74.4, H 4.9, N 9.6).

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SUMMARY

In connection with investigations on the selective inhibition of tumorous growth in plants, an analysis of the formation of indoleacetic acid (IAA) from tryptophan (Tryp) by the Crown-gall-inducing organism *Agrobacterium tumefaciens* was carried out by chromatography of extracts from the culture medium.

Tryp is generally recognized as the principal precursor of IAA, oxidative deamination or transamination presumably leading to indolepyruvic acid (IPyA) as an intermediate. Its occurrence in biological media had not been proved conclusively, however. Synthetic IPyA appeared to be an extremely labile substance under the conditions of chromatography (especially with alkaline solvents), decomposing into at least seven compounds, two of which were identified as IAA and indolealdehyde (IAld) and it seems probable that indoleglycollic acid was present.

Analysis by ultraviolet spectrography pointed to an enol-form for crystalline IPyA, while in

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solution a complex, pH-dependent tautomeric equilibrium, together with decomposition reactions were found.

Most of the spots on the chromatograms of the culture media turned out to be artefacts, which are due to spontaneous breakdown of Tryp (by the extraction procedure) and IpyA (during chromatography).

The appearance of the highly specific chromatographic pattern of IPyA decomposition provided definite proof for the occurrence of IPyA in the media.

Indolelactic acid (ILA) and tryptophol (Tol) were identified as products of physiological origin. Under anaerobic conditions they are the main products, IPyA very probably being their immediate precursor.

By varying the duration of the incubation it was shown that during the first 23 h IAA is formed mainly, IPyA, ILA and Tol accumulating in the next 15 h.

By using Tryp labelled with ^{14}C in different positions and by radioautography, conclusive evidence was obtained for the indole character of the IPyA-decomposition products.

In a trial to characterize IPyA in the culture medium by reaction with 2,4-dinitrophenylhydrazine, a yellow compound was formed, identical with a similar reaction product from synthetic IPyA, which could not, however, be identified as the 2,4-dinitrophenylhydrazone of IPyA.

Pure crystalline indolealdehyde was isolated from the medium.

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