

Humic Acids with C^{14}

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Received July 3, 1958

Souhrn

1. Listy cukrovky (*Beta saccharifera*), jež asimilovaly aktivní kysličník uhlíčitý $C^{14}O_2$, byly v zemině podrobeny pochodu humifikace. Po třech až pěti měsících humifikace byla usušená zemina extrahována ether-alkoholem, pak natrium nebo kaliumhydroxydem a kyselinou chlorovodíkovou byly huminové kyseliny odděleny od fulvokyselin. Všecky frakce byly radioaktivní.

2. Rostliny kukuřice (*Zea mays*) vyrostlé ve zředěném Knopově živném roztoku s přidanou aktivní huminovou kyselinou jevíly v kořenech i v listech radioaktivitu. Mezi kořeny a listy však byl velký rozdíl. Byla-li aktivita kořenů po dvou až dvanácti dnech asi 100 až 200 imp./min, byla aktivita listů kolem 20 imp./min počítáno na jednu rostlinu nebo asi 300 imp./min/10 mg u kořenů a na hranici měřitelnosti u listů.

3. Když na spodní část rubu prvního listu kukuřice byla nanесena kapka roztoku a suspension aktivní kyseliny huminové, šířila se velmi málo i v tomto listu; aktivita druhého a třetího listu byla i po pěti dnech na hranici měřitelnosti; obdobně v kořenech. Také z kapilár zabodnutých do mesokotylu se kyselina huminová šířila obdobně, po pěti dnech byla na hranici měřitelnosti.

4. Autoradiogramy se plně shodovaly s výsledky získanými počítačem. Kořeny byly prokresleny zřetelně a více méně intenzivně, kdežto listy se neobjevily nebo jen jako slabé stíny.

5. Poněvadž aktivita živného roztoku během kultivace stoupla, nepřijaly rostliny ani to množství huminové kyseliny, jež bylo rozpuštěno v objemu vytranspirované vody; z toho snad lze soudit, že rostliny přijímaly velké molekuly, a proto pomalu. Rozhodně nelze mluvit o hromadění v pletivu.

6. Naše pokusy nerozhodují, proniká-li nezměněná kyselina huminová do buňky. Můžeme podle nich pouze uzavírat, že, přidáme-li radioaktivní huminovou kyselinu do vody nebo do živného roztoku, objeví se radioaktivita v kořenech a později slabě i v listech. Připustíme-li že v rostlinných orgánech naměřená aktivita byla působena aplikovanou kyselinou huminovou přímo, znamená to, že proniká do rostliny pomalu, není hromaděna a z kořenů se po rostlině šíří pomalu.

Je možné, že se radioaktivní uhlík dostane do rostliny v molekule kyseliny huminové, není však vyloučena možnost, že se do kořenů a z kořenů do listů dostane teprve po rozkladu velké molekuly, eventuálně teprve po její mineralisaci. Ale při rychlé mineralisaci by se v rostlině jistě šířila rychleji.

Summary

1. Leaves of sugar beet (*Beta saccharifera*), which had assimilated active carbon dioxide $C^{14}O_2$, were subjected to the process of humification in soil. After three to five months of humification the dried soil was extracted with

ether-alcohol, then with sodium or potassium hydroxide; the humic acids were separated from fulvic acids with hydrochloric acid. All fractions were radioactive.

2. Maize plants (*Zea mays*) grown in diluted Knop's solution with the addition of active humic acid showed radioactivity in the roots and leaves. There was, however, a marked difference between the roots and the leaves. While the activity of the roots after two to twelve days was about 100 to 200 cpm., that of the leaves was about 20 cpm. as calculated for one plant, or about 300 cpm./10 g. for roots and at the limits of measurement for leaves.

3. When a drop of solution and suspension of active humic acid was placed on the lower surface of the first leaf, it spread very little even in this leaf; the activity of the second and third leaves was at the limits of measurement after five days; the same applies to the roots.

4. Autoradiograms were fully in keeping with the results obtained with the counter. Roots were clearly marked and more or less intensely, while the leaves did not appear or appeared only as faint shadows.

5. These experiments do not resolve the question of whether unchanged humic acid penetrates into the cells. It can only be concluded, on the basis of these experiments, that if radioactive humic acid is added to water or to nutrient solution, radioactivity appears in the roots and later weakly in the leaves. If it is assumed that the activity measured in the plant organs was caused by humic acid applied direct, this means that it penetrates into the plant slowly, it is not accumulated and spreads throughout the plant slowly from the roots.

Preparation of active humic acids

As the source of active material we used sugar beet (*Beta saccharifera*) leaves extracted with water. They assimilated active carbon dioxide $C^{14}O_2$ and, in the water extract and in the non-dissolved residue (LIEBSTER, CHRASTIL and BABICKÝ 1957), contained both radioactive assimilates and anabolic substances formed from them. These were subjected to the normal course of humification. Thus, labelled humic acids could be formed both by the humification of the radioactive compounds contained in the beet leaf, and also, through the exchange of active carbon with the carbon of humic acids already contained in the mixture with earth. We did not deal with this question, which had no essential effect on the result of the experiment. Still less is it possible as yet to judge in what group and in what manner active carbon is bound. In the meantime, however, it may be regarded as decisive that the humic acid fraction prepared in the usual way, i. e. by alkali extraction and precipitation with acid, and the other fractions, were radioactive (PRÁT, ČATSKÝ and MELICHAR 1957, PRÁT 1958).

As the substrate for humification and as inoculation material we chose leafmold (fine earth sieved through a two millimetre sieve), which did not effervesce in hydrochloric acid. In the second series we therefore omitted the preliminary extraction with hydrochloric acid.

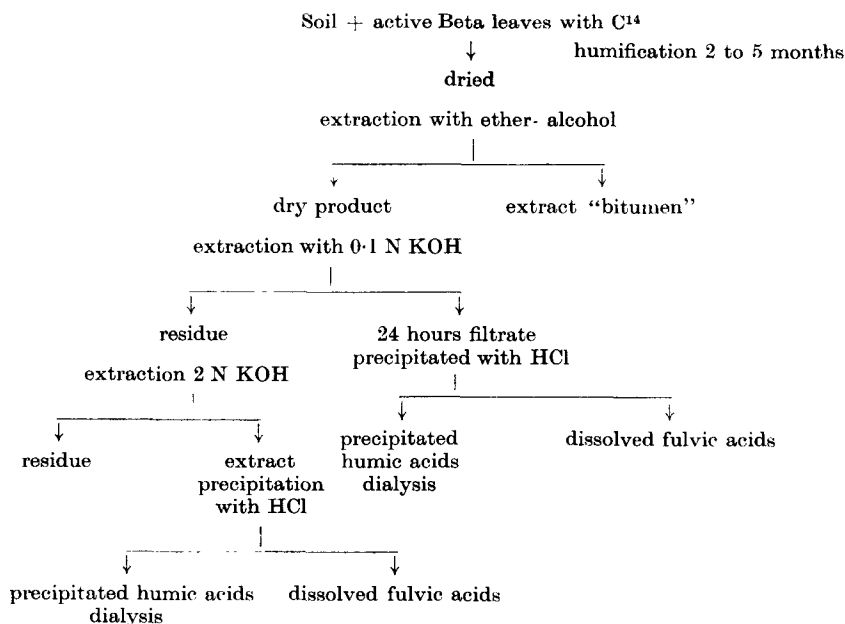
In the first experiment we used about 396 mg. of dry beet leaves with an activity of 394 μC , i. e. about 1 $\mu\text{C}/\text{mg.}$, content of C¹⁴ about 119 mg. We exposed these leaves to humifying conditions in a mixture of 25 g. of dry leafmold, 1.5 g. dry normal beet leaves and 20 ml. of water. The material was mixed and put in a covered dish in a desiccator with 20 ml. 0.1 N NaOH in the dish and test-tube. After sixteen weeks (5. VII to 24. X. 1956) the soil was dried at a temperature of 85° C, extracted with 50 ml. 0.1 N HCl; following filtration it was extracted with 0.1 N NaOH. The filtrate was precipitated with 2 N HCl and on the following day the sediment (brown flakes) was centrifuged. The clear yellow supernatant was active, but dialysis of fulvic acids has hitherto only been partially successful, because they passed through the membranes used. The sediment of humic acids obtained by centrifuging was dried (preparation a). It was again dissolved and precipitated to clean it. The procedure in the second experiment was similar. 10 g. of fresh leafmold sieved through a 2 mm. sieve was mixed with 30 g. of fresh beet leaves, 170 mg. of dry radioactive beet leaves and 6.5 ml. of distilled water were added. Humification proceeded under conditions similar to those of the first experiment. After about 4 months (5. I. to 2. V. 1957) the material was dried at a temperature of 52° C. The dried sample was first extracted in Soxlet's apparatus for 24 hours with 50 ml. ethylalcohol and 50 ml. ethyl-ether. The clear yellow extract later turned greenish; when dried in the air the residue ("bitumen") was dark green.

50 ml. of 0.1 N KOH was poured on to the soil that had been extracted with alcohol-ether, the filtrate was precipitated after 24 hours with HCl and the brown flakes obtained were centrifuged after a further 24 hours. The sediment was purified by dialysis against distilled water and dried at ordinary temperatures (preparation b).

The ratio of the activities of "bitumen", fulvic acids and humic acids to the extracted residue was about 6 : 6 : 10 : 25. This residue of soil was then extracted again with hot 2 N KOH (40 ml.) for about 30 min. It was left to cool, centrifuged on the second day; the supernatant was precipitated with HCl, the second (less soluble) fraction of fulvic acids, humic acids and humin (preparation c) being thus separated; these were considerably more active than those from the first extraction, 0.01 ml. giving 285 cpm. The process of preparation can be indicated as follows: (Scheme on the page 74).

Measurement of activity

The activity of all samples was measured by V. Oppltová of the Radiobiological Department of the Institute of Biology, Czechoslovak Academy of Sciences. She used mainly Friesecke and Hoepfner's apparatus; mica 1 mg./cm². or 0.99 mg./cm². The preparations were dried on stainless metal dishes or on aluminium foil. The solutions were evaporated and dried on them. Plants, both separated leaves and well-washed roots, were ground and the pulp was dried on aluminium foil. Insofar as any sap could be obtained by dripping from the pulp, this was dried on one foil and the remaining pulp on another.



In the case of leaves, in particular the dry weight of the older plants was greater; this caused variations in the thickness of the material; under these conditions the autoabsorption of the preparation was then strongly marked.

The background was calculated in every case from ten-minute measurements and gave 12 to 18, usually 14 cpm. Even measurements on different days varied to a relatively small extent. Otherwise the period of measurement was always 3 min.

Repetition of measurement of the same sample on different days showed close agreement (115 : 112).

On account of the relatively low activity of the samples, measurements were taken in different positions in order to be able to ascertain whether the number of impulses drops when the sample is at a greater distance.

Experiments with plants

Maize plants (*Zea mays*), "horse tooth" variety, were germinated in Petri dishes in distilled water. They were then cultivated in Knop's solution and after five to eight days transferred to Knop's solution diluted by about one quarter. Radioactive humic acid with a total concentration of humate of about 0.01% was added to this solution. The activity of the preparations varied.

The plants were cultivated individually in test-tubes with 20 ml. of solution and the stand with the tubes was put into a large desiccator. Before the desiccator was opened the air was extracted through a safety flask, although the

experiments showed that under the experimental conditions no measureable amount of active carbon dioxide escapes.

At the start of the experiment the plants had roots of about 6 to 15 cm. (they were selected at approximately the same length in each series), coleoptiles were about 2 to 4 cm. long (sometimes the first leaf had broken through). During the experiment the plants grew normally, the daily increase for the roots being 1 to 2 cm., and side roots were also formed normally. The plants developed 2 to 3 leaves of normal green intensity during the experiment. Some plants formed adventitious roots over 10 cm. long at the base of the stem. The first two series were cultivated in the laboratory in diffused daylight, the rest in digestors under Tesla lamp TZ 20/R 36.

In the first series the solutions remained clear or were slightly clouded. In series V to VI the control solutions (without plants) remained clear, while the solutions in test-tubes with maize plants clouded within 2 to 4 days and there was a sediment of brown flakes. Control solution only clouded after over a month.

Examples of Experiments

II. Maize was cultivated in diluted Knop's solution with active humic acid preparation b. The activity of the residue from 20 ml. of solution at the start of the experiment (9. X. 1957), i. e. the amount for one plant, was 1320 cpm. Results of activity measurements are compared in the following survey:

Date	Days in solution	cpm./plant	
		roots	leaves
10. X.	1	4	0
14. X.	5	16	2
21. X.	12	75	14

Even after twelve days activity is weak, i. e. about 70 cpm/10 mg. for roots while for leaves it is about 5 cpm/10 mg. Adventitious roots of one plant formed after twelve days showed activity of 6 cpm, i. e. about 12 cpm/10 mg.

V.

Date	Days in solution	cpm. residue from 20 ml.	cpm./plant			
			Roots	Leaves		
				subst.	sap	total
21. XI.	0	3162	—			
23. XI.	2		41	8.6	5	14
26. XI.	5		128	19	15	34
29. XI.	8		232	7	5	12
3. XII.	12	4034	221	5.2	11.5	17

These values after eight to twelve day correspond for roots to about 300 cpm/10 mg., for leaves there are only single impulses.

VI. Humic acids prepared 1. XI. 1956 (preparation a).

Date	Days in solution	cpm. residue from 20 ml.	cpm./plant				
			Roots		Leaves		
			in solution	advent.	subst.	sap	total
17. XI.		2202					
19. XI.	2		58	—	3.7	0.3	4
22. XI.	5		132	—	2	3	5
29. XI.	12	2450	177	3	16	6	26

VII. Following up the experiments on resorption of humic acids from solution by roots we tried to observe the activity after the addition of humic acid through the leaves. We used two methods, the application of drops of the solution on the leaves, and according to Němec's method, by piercing the maize mesocotyl with a capillary filled with a drop of the solution and containing the suspension of humic acid.

The maize plants in this experiment had roots 15 to 25 cm. long, the first three leaves were developed; they were cultivated individually in test-tubes with 20 ml. of Knop's solution diluted to a quarter. From 22. XI. to 27. XI. the plants grew normally.

VII. a. A drop (0.01 to 0.1 ml.) of solution with a suspension of active preparation c was placed on the lower surface of the first leaf. It was difficult to keep the drops on the smooth leaf surface, but those which stayed dried on well. After five days the first leaves with these drops were dried for autoradiography, while the younger leaves and the roots were ground as before and dried on folio and measured; these upper leaves and the roots in solution did not come into direct contact with the active preparation and activity could spread to them only from the first leaves.

The average of 15 measurements gave a value of 3.7 cpm. in roots and 2.7 cpm in leaves, i. e. an activity within the bounds of measurement.

VII. b. 0.05 to 0.1 ml. of solution with a suspension of the active preparation was drawn into the capillary, which was thrust into the mesocotyl of maize or above the mesocotyl. The results of measurements taken after seven days were 6.2 cpm in roots and 9 cpm in leaves.

Autoradiograms

Autoradiograms fully confirmed the results obtained by measuring with the counter.

According to the intensity of radiation the roots were printed slightly grey to nearly black on the autoradiograms, while activity hardly spread at all to

the leaves so that they were not shown at all on the radiograms. It was only after 12 days that faint autoradiograms of the leaves began to show as hardly noticeable shadows after one month's exposure on the X-ray films Foma.

It is also necessary to deal with the question of the possibility of adsorption of active compounds at the surface of the roots. From autoradiograms it is often possible to observe increased activity at the surface. Localised structures within the root tissues, particularly meristems, young lateral roots, and vascular bundles show much more intensively black. Active compounds do not spread from the roots in a regular manner. When their time of action is short the part of the root above the solution is less clearly shown, the lower parts of leaves are not shown at all and only the tips of the leaves are clearly black.

In the leaves, both from cultures with active bicarbonate and from those with humic acids, the vascular bundles are the first and most intensively marked.

Since the humic acids are easily precipitated from solution, it is necessary to count with their adherence to the root surface. Autoradiograms show that sometimes, in isolated cases, places appear on the root surface which are strongly black, thus indicating localised precipitation.

Discussion

From the literature quoted and from our experiments it is evident that radioactive carbon in carbon dioxide or from a solution of inorganic salts (bicarbonates) penetrates very rapidly into plants. According to KURSANOV (1957) radioactive carbon spreads in herbaceous plants at a rate of 3 cm./min., that is 2 m./hour. In recent literature there is a wealth of evidence on the rapid spreading of radioactive carbon in plants. In volumes of Plant Physiology for recent years, and elsewhere, very many different experiments with various isotopes have been described (cf. M. v. ARDENE).

On the other hand, with maize plants cultivated in a solution of active humic acid the roots, even after five days, showed a relatively low activity and only after 12 days did it rise. From the total amount of radioactive carbon in the solution however, only a small part was resorbed. In the leaves, even after the end of this period, the activity was at the border-line of measurement or weak. Thus, active compounds spread from the roots into the leaves very slowly. This is also confirmed by the behaviour of adventitious roots, which grew above the level of the solution into the damp air and were either not radioactive or were radioactive to a much lesser extent than roots immersed in the solution.

The activity of sap pressed from the pulped leaves (unfiltered) varied greatly; however, it is not possible to say that activity accumulates in them; either a considerable part of the radioactive compounds in the leaves was not dissolved or they remained adsorbed on the solid residue of the leaves.

KUZIN and MERENOVA have studied plants, which had grown for 7 days on soil to which radioactive organic matter had been added (1955). In 1955 they already demonstrated that carbon is taken up by the roots from dissolved organic compounds and rapidly conveyed to the leaves, while on the contrary

plants sown on soil with insoluble components of green manure took up radioactive carbon slowly ("medlennoe usvoenie") (p. 251).

Our experiments do not, however, enable us to speak of permeability of cells to humic acid, i. e. if it penetrates unchanged into the cell. The only conclusion we can draw from them is that if we add radioactive humic acid to water or to nutrient solution radioactivity appears in the roots and later weakly in the leaves. If we assume that the activity measured in the plant organs was caused directly by the applied humic acid, it means that the acid penetrates slowly into the plant, that it does not accumulate and spreads from the roots slowly. It is necessary to consider the possibility that radioactive carbon gets into the plant in the humic acid molecule, but the possibility cannot be excluded that it gets into the roots and from them to the leaves only after the disintegration of large molecules, or even after their mineralization. We must consider the possibility that humic acids added to the solution may be decomposed by exoenzymes from the roots or by microbial activity and reach the roots only following this. But in this case, too, they get to the plant slowly. In the meantime it is difficult to bring this fact into line with the repeatedly confirmed experience that the influence of humic acids on the growth of roots is exerted positively from the first hours or days.

Since the activity of the nutrient solution was increased during cultivation, the plant does not take up even that quantity of humic acids which was dissolved in the corresponding volume of transpired water; it can perhaps be judged from this that plants took up large molecules and that for this reason the process was slow. It is definitely not possible to speak of accumulation in the tissues.

This fact points to the possible conclusion that during cultivation humic acid was not mineralized as far as carbon dioxide, because this could escape into the air and would be taken up by the plant much more quickly. Alternatively, mineralization is so slow that the activity of its products is very low.

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Гуминовые кислоты с C^{14}

С. ПРАТ, Ф. ПОСПИШИЛ

Резюме

1. Листья сахарной свеклы (*Beta saccharifera*), которые ассимилировали активный углекислый газ $C^{14}O_2$, подвергались процессу гумификации в земле. После 3—5-месячной гумификации высушенная почва экстрагировалась смесью эфира со спиртом и потом гуминовые кислоты отделялись от фульвокислот с помощью едкого калия, или едкого натрия или соляной кислоты. Все фракции оказывались радиоактивными.

2. У растений кукурузы (*Zea mays*), выращенных в разведенном питательном растворе Кнопа с прибавлением активной гуминовой кислоты, в корнях и листьях наблюдалась радиоактивность. Однако между корнями и листьями была большая разница. Если активность корней через 2—12 дней составляла около 100—200 имп./мин., то активность листьев была около 20 имп./мин. в пересчете на 1 растение, или же около 300 имп./мин./10 мг у корней и только следы активности наблюдались у листьев.

3. Когда на нижнюю часть обратной стороны первого листа кукурузы наносили каплю раствора и суспензии активной гуминовой кислоты, активность весьма незначительно распространялась и в этом листе. Активность второго и третьего листа через 5 дней бывала на пороге измераемости так же и в корнях. Из капилляров, воткнутых в мезокотиль, гуминовая кислота распространялась таким же образом и через 5 дней наблюдались лишь следы активности.

4. Авторадиограммы полностью соответствовали результатам, полученным с помощью счетчика. Корни вырисовывались отчетливо и более или менее интенсивно, тогда как листья не появлялись или появлялись только в виде слабых теней.

5. Так как активность питательного раствора в течение культивации повышалась, растения не впитали даже того количества гуминовой кислоты, которое было растворено в объеме воды, выделившейся при транспирации. Можно полагать, что в растения поступали крупные молекулы и поэтому скорость их поступления была медленной. Во всяком случае нельзя говорить о накоплении в тканях.

6. Наши опыты не дают ответа, проникает ли гуминовая кислота в клетки в неизменном виде. Можно только сказать, что, если радиоактивную гуминовую кислоту

прибавить к воде или к питательному раствору, то радиоактивность появляется в корнях и позднее и слабее — и в листьях. Даже если допустить, что отмеченная нами в органах растений активность была обусловлена прямо применением гуминовой кислоты, это означает, что гуминовая кислота проникает в растения медленно, не накапливается и из корней медленно распространяется по всему растению.

Возможно, что радиоактивный углерод попадает в растение в молекуле гуминовой кислоты, но не исключена возможность, что он попадает в корни и из корней в листья только после распада крупной молекулы, или же только после ее минерализации. Однако при быстрой минерализации активность, несомненно, распространялась бы в растении быстрее.

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