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Tritium Labeling: A Unique Tool for Studying the Behavior of Humic Substances in Living Systems

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Abstract—A range of labeled humic substances was obtained by the tritium thermal activation method. The high specific radioactivity and radiochemical purity allowed direct determination of the hydrophobicity and surface activity of humic substances, and investigation of the behavior of humic substances in bacteria and plants.

Key words: tritium, labeled compounds, humic substances.

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Tritium-labeled compounds are widely used in scientific research because the physical properties of tritium nuclei are unique (the maximum energy of beta radiation is 18.6 keV, and the half-life is 12.3 years). Tritium can be introduced into practically any organic molecule, working with tritium-labeled compounds does not require any extraordinary measures for protection against radiation, and the specific activity of a compound containing 1 tritium atom per molecule is 1.07 TBq/mol.

Contemporary research on complex natural entities sets specific requirements for labeled compounds. Such compounds contain complex mixtures of macromolecules of varied composition and irregular structure and are described in terms of integral characteristics; investigation of such objects using radioactive labels therefore demands the introduction of nonselective tritium and the uniform distribution of tritium among the components of the object. In addition, the labeling procedure must be universal and the labeled preparation must have the same properties as the initial preparation.

Humic substances (HS) are one example of complex natural entities. The stochastic nature of these compounds results from the peculiarities of their formation due to the selection of biothermodinamically stable structures. The fundamental properties of HS include nonstoichiometric composition, irregular structure, heterogeneity of structural elements, and polydispersity. Chemically, HS are irregular copolymers of aromatic oxypolycarboxylic acids with the inclusion of nitrogen-containing and carbohydrate moieties. Such a structure (consisting of a framework, i.e., a aromatic carbon skeleton with alkyl and functional substituents—mostly carboxyl, hydroxyl and methoxyl—and a peripheral part enriched by polysaccharide and polypeptide fragments) is characteristic for HS of any origin.

The generally accepted classification of HS is based on the fractionation procedure. HS are subdivided into humin (insoluble over the pH range), humic acids (HA, insoluble at pH < 2) and fulvic acids (FA, soluble over the pH range). The latter two classes share the common name of humus acids. This scheme is sometimes supplemented by himatomelanic acids, which are separated by treating moist HA precipitate with ethanol.

Interest in HS is now growing around the world: production technologies are being improved, and the raw-material base is being broadened to include new types of coals, peats, slates, and peloids. Humic preparations are most widely used in agriculture as an environmentally safe alternative to fertilizers and some pesticides. Numerous investigations have shown that humic substances stimulate the growth and development of plants and increase their resistance to adverse environmental factors. Systematic use of the preparations contributes to the improvement of soil structure, buffering and ion-exchange properties, and leads to an increase in the activity of soil microorganisms and the

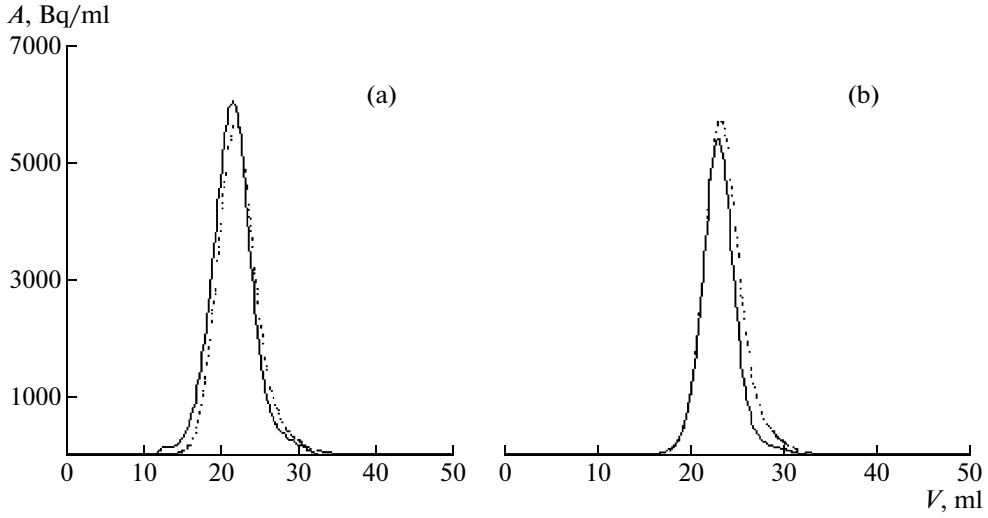

Fig. 1. HS elution profiles (for peat HA): (a) for the labeled preparation according to UV absorbance (dotted line) and radioactivity (solid line); (b) for the initial (dotted line) and labeled (solid line) preparations, according to UV absorbance.

transformation of mineral substances into forms available to plants. The adaptogenic properties of HS, a result of their ability to bind radionuclides, heavy metal ions, and xenobiotics, are worth special mention. Humic preparations increase plants' resistance to disease, drought, excess humidity, and other adverse environmental conditions.

Humic preparations are also of interest for specialists working in other areas, e.g., animal breeding and the recultivation of polluted areas. The use of HS is currently limited by the lack of research works dealing with the quantitative description of HS structure and the properties and mechanism of HS action. The preparation of radioactively labeled HS is one of the most promising ways of solving this problem.

In recent years, researchers working in the Radiochemistry Division of Moscow State University have adapted the method of tritium thermal activation for introducing tritium labels into HS. The tritium radiation was registered by liquid scintillation spectrometry, using the scintillating phase procedure [1], and by autoradiography [2]. This allowed us to investigate HS hydrophobicity and surfactant characteristics, along with HS interaction with bacteria and plants.

EXPERIMENTAL

Preparation of Tritium-Labeled Humic Substances

Tritium-labeled HS ([^{3}H] HS) were obtained using the method of tritium thermal activation. This method allows tritium to be introduced directly into complex mixtures of organic compounds and meets such labeling requirements as the introduction of nonselective tritium and its maximum uniform distribution among the components of the subject.

We have shown that optimal conditions for the introduction of tritium labels into HS by thermal activation are as follow: a target mass of 0.3–0.5 mg, an atomizer temperature of 1900–1950 K, a gas pressure in the system equal to 0.5 Pa, and a treatment time of 10 s [3]. During the reaction with tritium atoms, the target is cooled with liquid nitrogen. The obtained labeled preparation is purified by equilibrium dialysis in order to flush tritium from labile locations (OH^-, $COOH^-$ and NH_n).

Increasing the target mass leads to a decrease in specific radioactivity. This is because only molecules situated on the target surface react with tritium, since upon lyophilization HS form a dense target with a relatively small surface that is weakly permeable for tritium atoms.

Increasing the reaction time also leads to a reduction in the [^{3}H] HS yield, due to the destruction of the target's surface layer under the effect of hot atoms. The molar radioactivity of [^{3}H] HS increases upon a rise in atomizer temperature and reaches a maximum at 1900–1950 K. It should be noted that raising the atomizer temperature further promotes the formation of a larger quantity of modified tritium-labeled molecules, which leads to a reduction in the yield of the principal product. A similar temperature dependence is observed for the individual components of HS [4].

The degree to which the original and tritium-labeled preparations differed was tested by size-exclusion gel chromatography with simultaneous detection of radioactivity and UV absorbance at 254 nm. As is shown in Fig. 1, gel permeation chromatograms of [^{3}H] HS and the HS used for labeling are similar. This shows that label introduction leads neither to complete or partial destruction of HS or to significant changes in them. On the other hand, we also demon-

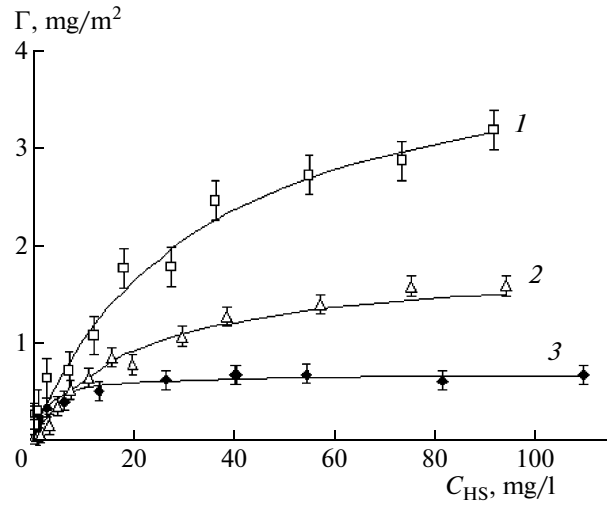

Fig. 2. Isotherms of HS adsorption on toluene–water interface: (*1*) peat HA; (*2*) peat FA; (*3*) coal HA.

strated the similarity between the gel chromatograms of tritium-labeled HS using radioactivity and UV absorbance detection (Fig. 1). We concluded from this that the label had been uniformly introduced into all of the structural fragments.

The specific radioactivity of the obtained [^{3}H] HS preparations ranged between 0.14 and 0.63 TBq/g, which was considerably higher than the specific radioactivity of [^{3}H] HS prepared by other methods (0.01–0.07 TBq/g) [5, 6]. It should be also noted that one advantage of the elaborated method is the possibility of obtaining [^{3}H] HS preparations identical to the initial HS, while modifications occur upon the radioactive labeling of HS by other methods [6–9]. For example, when FA were tritiated using [^{3}H]$NaBH_4$, the initial substance was modified, with the carbonyl groups in ketones and quinones being restored [6].

Correlation analysis of the properties of HS preparations and the specific radioactivity of the obtained [^{3}H] HS showed that there was no connection between these parameters. This is indicative of nonselective tritium introduction and the universality of the proposed approach for obtaining tritium-labeled HS.

Use of Tritium Label for Studying the Properties of Humic Substances

Hydrophobicity and surface activity are among the most important properties largely determining the biological activity of organic compounds, since these parameters influence the ability of substances to interact with biological membranes. However, direct determination of the indicated properties of HS was difficult until now, due to the problems of their analytical detection. Thanks to the possibility of determining low [^{3}H] HS concentrations in the presence of other organic compounds, we were able for the first time to perform a quantitative assessment of HS hydrophobicity and surface activity. The above properties of HSs were determined by tritium liquid scintillation spectrometry using the scintillating phase procedure [1].

Use of tritium-labeled substances and the scintillating phase method allowed us to determine the coefficients of HS distribution between water and toluene and to find the value for absorption at the interface of the two liquid phases. The dependence of HS equilibrium concentration in the organic phase on HS concentration in the water phase was linear, and this allowed us to calculate the coefficients of HS distribution between the water and the toluene (K_{tw}) [10]. The K_{tw} values ranged between 0.3×10^{-3} (soil FA) and 3.7×10^{-3} (coal HA). Since the water-octanol distribution coefficient (K_{ow}) is conventionally used to characterize the hydrophobicity of substances, the corresponding values for tritium-labeled HS were determined. A good correlation between these coefficients was found:

$$\log K_{tw} = 0.96 \log K_{ow} - 0.66 \ (r^2 = 0.96).$$

Thus, HS hydrophobicity can be evaluated quantitatively using the scintillating phase procedure and [^{3}H] HS. We have shown that HS hydrophobicity increases in the following order:

soil FA < soil HA < peat HA ≈ peat FA < coal FA.

Adsorption isotherms of HA at the toluene-water interface were obtained by the scintillating phase method. The specific radioactivity of [^{3}H] HS was sufficient for reliable determination of the HS amount at the phase interface when HS concentration in the water phase ranged between 0.1 and 100 mg/l. Adsorption isotherms obeyed Langmuir's law in most cases (Fig. 2). When concentration of the preparation in the water phase was increased to the range of 0.1–5.0 mg/l, the quantity of HS molecules adsorbed at the toluene-water interface increased rapidly. The adsorption layer became saturated in the concentration range 20–60 mg/l, while upon further increase of the substance concentration in the water phase the number of HS molecules at the interface remained practically unchanged. The maximum adsorption value Γ_{max} ranged between 0.08 mg/m^2 (soil FA) and 2.8 mg/m^2 (peat HA). The experiments performed showed that the surface activity of HS preparations increases in the following order:

soil FA < soil HA < coal HA < peat FA < peat HA.

Comparison of maximum adsorption values and average molecular mass M_w values of HS showed that maximum adsorption Γ_{max} increases upon increasing M_w [11]. Analysis of the obtained data showed that the H/C atomic ratio also influences HS adsorption at the phase interface. The maximum adsorption value Γ_{max} turned out to be linearly correlated with the product of M_w and H/C, as is shown in Fig. 3. It should be men-

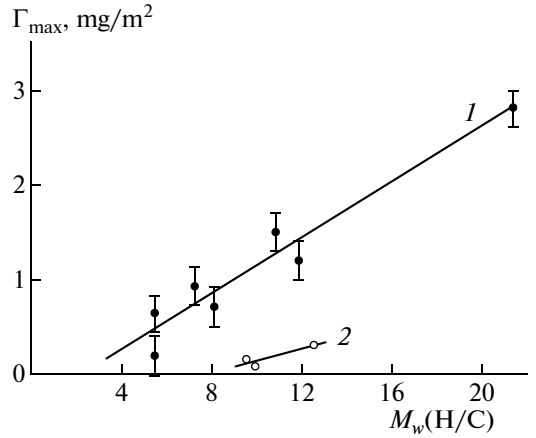

Fig. 3. Correlation between maximum adsorption values of HS on toluene-water interface (Γ_{max}) and HS properties: (*1*) peat and coal HS, (*2*) soil HS.

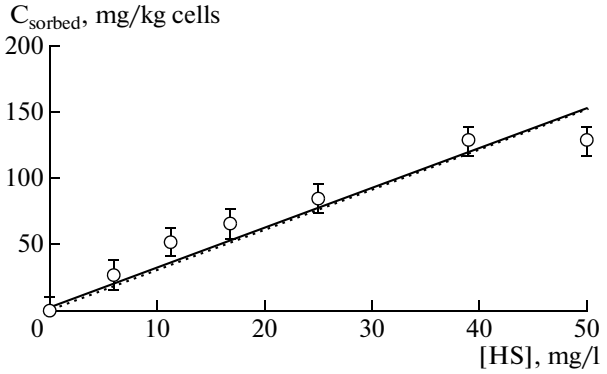

Fig. 4. Sorption of coal by cells of *E. coli* [HS]–equilibrium concentration of HS.

tioned that the HS preparations studied form two distinct groups, with peat and coal HS fitting one correlation and soil HS fitting another. This difference in adsorption properties may be explained by the fact that peat and coal HS are rich in saturated fatty acids and their derivatives are capable of forming associates with aromatic hydrocarbons [12].

Using Tritium Labeling to Study the Interactions of Humic Substances with Biological Systems

The presence of both hydrophilic and hydrophobic fragments determines the surfactant properties and membranotropic activity of humic substances [13–15]. The ability of HS to interact with biological membranes has been confirmed for phytoplankton [15], the cells of fish gills [16], bacteria [17, 18], fungi [19], and plants [20]. The varied nature of the used subjects allows us to conclude that HS sorption on biological membranes is a very common process. Little research has been done, however, on quantitatively describing this process and there are few (if any) studies dedicated to assessing HS flux into cells [21]. In the present work, tritium-labeled HS were used to study and quantitatively describe their interaction with bacteria and plants.

Gram-negative tetracycline-resistant coliform bacteria *E.coli* XL1 were used as models for the studies of HS interactions with bacteria in [22]. The obtained results show a linear correlation between HS uptake by bacterial cells and HS concentration (Fig. 4) and demonstrate that the factor of HS bioaccumulation by *Escherichia coli* ranges from 0.9 to 1.3 l/kg. The ability of HS preparations to interact with live cells changes in the following order:

$$\text{FA of soil} < \text{FA of peat} < \text{HA of soil} < \text{HA of coal} \approx \text{HA of peat.}$$

Using [^{3}H] HS, we were able to prove experimentally for the first time that HS penetrate into intracellular space. The quantities of HS penetrating the membrane range from 23 to 167 mg per kg of cells, which corresponds to 20 and 100% of the total amount of adsorbed HS in the cases of HA and FA, respectively. Comparison of HS properties and the parameters of their interaction show that a significant correlation exists between the quantities of HS entering (HS_{ent}) cell and the surface activity of the HS:

$$HS_{ent} = 49\Gamma_{max} + 28 \ (r^2 = 0.95).$$

Thus, the obtained results allowed us to demonstrate for the first time that HS surface activity plays an important role in HS interactions with living cells.

Seedlings of soft wheat *Triticum aestivum L.* were used as subjects in experiments with plants [23]. After exposing the plants to [^{3}H] HS, the plant samples were subjected to autoradiography. Quantitative analysis of autoradiograms was performed using the approach suggested in [24].

Our research allowed us to determine the quantitative characteristics of HS uptake by plants, and to show that the maximum rate of HS adsorption ranges between 6 and 54 mg/kg per hour. Autoradiography results (Fig. 5) revealed an uneven distribution of HS in plants: HS were found mostly in their roots, while considerably fewer entered their sprouts. Assuming the optical density D on autoradiograms to be directly proportional to label concentration and exposure time, we calculated the ratio of HS content in roots and sprouts as the ratio of optical densities in these parts of the plants normalized to exposure time. Under test conditions, this ratio was 40.7 ± 0.3 for coal HA and 10 ± 2 for aquatic FA, i.e. the concentration of HA and FA in sprouts was on average 40 and 10 times higher than in roots, respectively. Measuring the mass of plant roots and sprouts, we calculated that roots accumulate 22 times more coal HA than sprouts do; the analogous value for FA equaled 5. The obtained results thus demonstrate that the penetration of FA

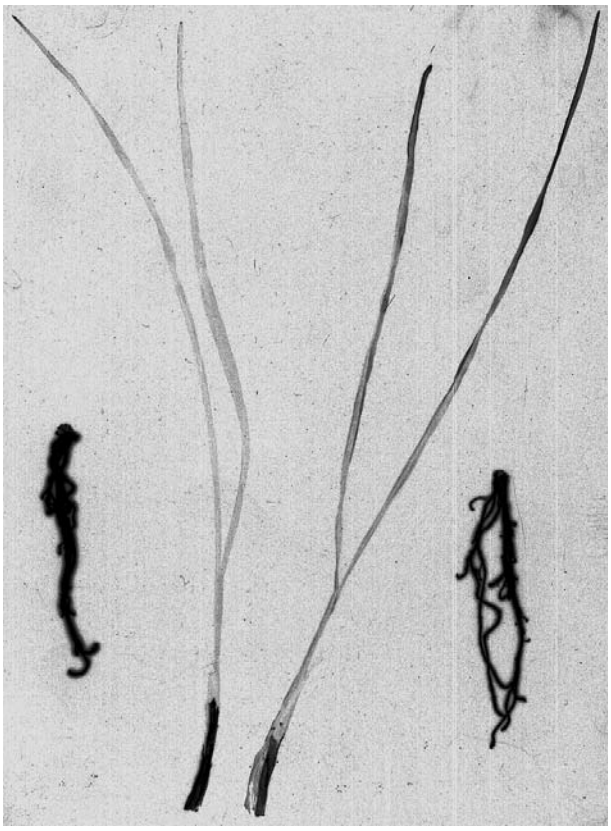

Fig. 5. Autoradiograms of wheat seedlings treated with tritium labeled coal HA (left) and aquatic FA (right).

from roots into sprouts of plants is more efficient than that of HA.

The autoradiograms obtained showed that HS distribution in roots and sprouts also was nonuniform: a local increase of HS concentration was observed in the apical regions of both roots and sprouts. Analysis of the autoradiograms showed that the optical density in root tips was on average 2 times higher than the average value for the whole root, while the analogous ratio for sprouts was 8 (see table).

We have thus elaborated a procedure for the direct introduction of tritium into HS of different origin using thermal activation. This method allows us to obtain tritium-labeled HS that meet our principal requirements: nonselective isotope introduction, uniform isotope distribution in all the components of the substance, procedure universality, and conservation of the properties of the initial preparation. The advantages of the radiochemical approach for preparing tritium-labeled HS demonstrated in the present work make it very promising for studying not only HS but also other natural polymers of complex structure and variable composition, with regard to their properties and interactions with biological systems.

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Optical densities D of various root and sprout parts of wheat plants after interacting with coal HA and aquatic FA. Exposure: 5 days, 10 minutes for roots; 1 day, 20 hours, 30 minutes for sprouts

HS type	Roots			Sprouts		
	roots	apices	D_{apices}/D_{roots}	sprouts	apices	$D_{apices}/D_{sprouts}$
Coal HA	0.38 ± 0.02	1.06 ± 0.03	2.8	0.02 ± 0.01	0.14 ± 0.01	9.7
Aquatic FA	0.43 ± 0.02	0.52 ± 0.02	1.2	0.05 ± 0.01	0.33 ± 0.01	7.0

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