

Radiation Induced Tritium Labeling of *p*-Aminosalicylic Acid (PAS)

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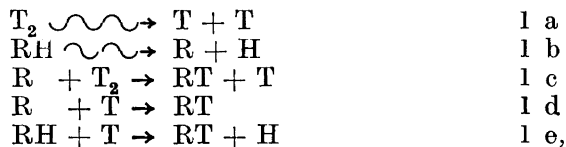
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Wilzbach's¹ method of mixing tritium gas with organic substances for labeling them with tritium was used to introduce tritium into *p*-aminosalicylic acid (H—PAS) and its sodium salt (Na—PAS) and the specific activity was studied as a function of time and agitation. Higher activities were obtained with Na—PAS and with agitation than with H—PAS or without agitation. The results are summarized in Fig. 3. Specific activities of the order of 30 mC/g could be obtained in a day.

In the last few years a number of methods have been described for the direct labeling of organic substances by exposing them to radioactive isotopes of high kinetic energy *e.g.* ²⁻¹¹. This makes it possible to label substances which previously have been found difficult or impossible to label by other methods as, *e.g.*, chemical synthesis, biosynthesis or isotopic exchange. To this group of substances belongs a great number of complicated organic molecules of biochemical importance. Since the labeling by this direct method is non-specific (a great number of other labeled organic compounds are also formed), the methods for isolating the desired product in pure form are of fundamental importance, and generally constitute the most difficult part of the process. However, very elaborate and selective purification methods are now available as *e.g.* various chromatographic methods.

An important addition to the methods of direct labeling was recently reported by Wilzbach¹ who found that if organic compounds are exposed to tritium gas, some of the hydrogen in the compounds is exchanged for tritium.

This exchange is induced by the β -radiation from the tritium. Some of these reactions may be written



where RH is the organic compound being irradiated.

The first two reactions indicate how radicals are produced by the β -radiation field. The following reactions show how these radicals may react to form the labeled compound RT; of these reactions, (1d) is least probable. The last reaction can also be regarded as a formation of RT through isotopic exchange. Ions (e.g. T^+ , T^- , R^-) may be formed in a similar manner, and these ions may also react with each other or with the radicals to form RT. The radiation field is fairly intense and amounts, for 1 Curie of T_2 -gas, to about 10^6 rad/min in the volume of the pure gas; the radiation absorbed by solid or liquid samples depends somewhat on the experimental conditions, but may well amount to 10^5 rad/min.

This kind of labeling may not necessarily be induced by the radiation from tritium: It seems quite possible to use radiation from other sources, such as from the decay of other isotopes * or from high-voltage accelerators.

For autoradiographic studies of the pharmacological properties of the tuberculostatic agent *p*-aminosalicylic acid (PAS)¹², tritium labeled PAS was needed. In a previous paper¹⁰, the labeling of PAS with recoil tritons has been reported; however, only low specific activities (~ 1.5 mC/g) were obtained. In order to obtain higher specific activities, the method of using tritium gas for the labeling of PAS has been investigated. The results are reported in this paper.

EXPERIMENTAL

Apparatus. The apparatus used is shown in Fig. 1. It consists in principle of two vessels, one for the tritium stock (F) and one for the sample to be exposed (A), and a gas-pumping mechanism. The apparatus, which is very compact, is made entirely of glass. It is operated in the following manner:

After the whole system has been evacuated, stop-cock J is connected only to vessel E, and K is closed. The tritium ampoule, F, is then broken by the magnet, and the T_2 gas is allowed to distribute itself through stop-cock D into vessel E. Stop-cock D is then switched over so that A and E are connected through stop-cock B. Air is then let into vessel L via stop-cock K, and the mercury level in E is allowed to reach just below stop-cock D, which is then closed. In this manner about 55 % of the T_2 originally present in F is introduced into the reaction vessel A.

If it is desired to remove the reaction vessel from the apparatus, stop-cock B is closed, and the T_2 gas in the volume between B and E is pumped out and transported back to F by repeatedly lowering the mercury level in E when C and E are connected, and raising the level when E and F are connected. This pumping action is effected by turning stop-cock K so that air is either let in or removed from vessel L. Part A—B can then be removed after D has been closed.

When parts A and B are removed from the rest of the apparatus, part C becomes filled with air. This air has to be removed before the tritium gas in volume A—B can be recovered so that the tritium gas will not be diluted with air. This air is therefore removed by the vacuum pump through D—E—J—K; since some T_2 is always left in the hole in stop-cock D, it will be lost during the evacuation process. The amount of this loss is 2—3 % of the total amount T_2 in vessel F. Thus, in about 20 experiments, 50 % of the T_2 has been lost in this way. Corrections (factor f_p) for this successive decrease of the T_2 -amount are made in the experiments recorded later.

Labeling experiments. The amount of PAS used in the labeling experiments was varied between 30 and 100 mg. The tritium gas was purchased from AERE, Harwell, and contained 98.8 wt % of T_2 , the rest being H_2 . The amount of tritium gas in the reaction vessel was at maximum about 2.6 curies at a pressure of about 240 mm Hg, and at minimum about 1.6 curies. All experiments were carried out at room temperature ($20 \pm 2^\circ\text{C}$).

* This has recently been shown by Turton, C. N. *Intern. J. of Appl. Radiation and Isotopes* 2 (1957) 182, who used the radiation from ^{85}Kr to improve labeling with ^{14}C in organic compounds.

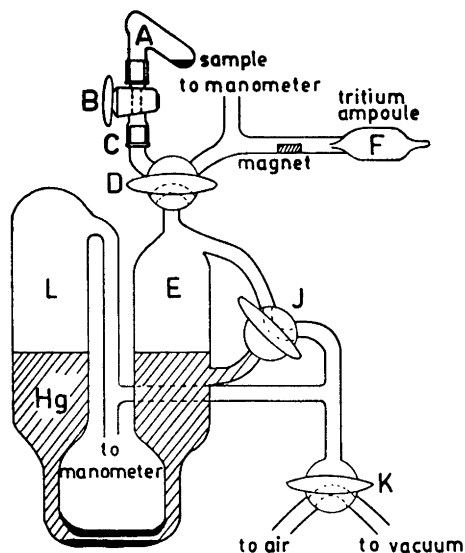


Fig. 1. Apparatus used for the exposure of organic samples to tritium gas.

Chemical procedures. *p*-Aminosalicylic acid (H-PAS, which looks somewhat similar to fineground corn meal) and sodium *p*-aminosalicylate (Na-PAS, which consists of mm-sized crystal needles) of highest commercial purity were used; the products were kindly supplied by AB Ferrosan, Malmö. In order to purify the H-PAS, which has a somewhat greyish color, the same purification procedure was used as described below for the tritiated product. All other chemicals were of analytical grade.

As the primary purification method liquid-liquid extraction was chosen. This method is very suitable for large amounts of samples and the fundamental laws governing the extraction processes are well known, *cf. e.g. Ref.*¹³

The tritiated PAS-sample was dissolved in a mixture of equal volumes of amyl alcohol and 0.1 M NaHCO₃ in water (about 10 ml for each 100 mg PAS). Under these circumstances about 95 % of the PAS goes into the aqueous phase. This aqueous phase was then washed 5 times with equal volumes of amyl alcohol. The aqueous phase was then cooled with ice and acidified with HCl to pH ~3; after about one hour PAS-HCl has precipitated and can be filtered off.

This PAS-HCl is put into 1 mM HCl, and the resulting aqueous solution is extracted with an equal volume of amyl alcohol. About 97 % of the PAS now moves over to the organic phase, from which it can be recovered by careful evaporation.

The chemical yields of a great number of such runs have been 50–70 %. The tritium yield, *i. e.* the ratio between non-labile tritium found in the purified PAS product and the total tritium content found in evaporated samples of the crude product dissolved in amyl alcohol, was found to be around 20 %.

The purity of the product was checked by paper chromatography, as described in a previous paper¹⁰, in which the principle of the purification method is more extensively treated.

Tritium measurements. All radioactivity measurements were made on solid samples in a 2 π internal gas-flow proportional counter (Nuclear Measurements Corp., Indianapolis). Corrections for resolving time could be neglected at counting rates $\lesssim 5000$ counts per sec.

The samples were prepared by dissolving a weighed amount of purified, tritiated PAS in a known volume of ethyl alcohol, and carefully evaporating 0.1 ml on flat plates heated to 55°C, *cf. Ref.*¹⁴

Table 1. Results of tritiating experiments and calculations of specific activity ($f_b = 1.14$ except otherwise stated; f_s is taken from Fig. 2).

Conditions	Sample weight, mg	Reaction time, h	Purified product		f_p	S , mC/g
			μg	cpm		
H-PAS, static runs, symbol \circ in Fig. 3	100	1	12.3	1 740	0.94	0.15
	100	1	10.0	1 080	0.69	0.15
	99	1	11.2	1 905	1.00	0.15
	104	3	13.2	4 350	0.91	0.38
	100	6	12.2	4 270	0.75	0.49
	104	24	12.6	17 600	0.97	1.43 ¹⁾
				13 700	0.97	1.35 ¹⁾
	100	24	8.7	9 800	0.73	1.39
	100	48	13.5	58 060	1.00	4.1 ²⁾
	101	165	11.6	121 860	0.77	14
H-PAS, static runs, symbol Δ in Fig. 3	31	1	10.4	4 100	0.88	0.45 ³⁾
	36	3	11.5	12 430	0.79	1.05 ³⁾
	32	24	10.0	34 390	0.82	4.1
	31	45	11.8	71 800	0.85	7.2
H-PAS, agitation runs, symbol \square in Fig. 3	102	1	10.2	9 110	0.65	0.14 ⁴⁾
	160	4	11.0	14 200	0.71	1.8 ⁵⁾
	100	24	10.0	8 600	0.63	1.34 ⁴⁾
	100	26	12.0	9 910	0.61	1.41 ⁴⁾
Na-PAS, static runs, symbol \bullet in Fig. 3	100	1	11.1	2 425	0.79	0.28
	100	6	10.0	5 420	0.77	0.70
	100	19	9.9	21 160	0.85	2.5
	107	95	11.8	102 980	0.88	10.1
Na-PAS, agitation runs, symbol \blacksquare in Fig. 3	95	1	11.6	6 950	0.59	1.09
	96	1	11.2	15 510	1.03	1.36
	98	2.5	11.0	35 710	0.94	3.5
	100	19	11.2	212 610	0.91	21

1) Double samples from same PAS-product.

2) $f_b = 1.24$.

3) $f_b = 1.50$.

4) Baking from the beginning of the experiment.

5) Baking starts after about one hour; symbol \blacksquare used in Fig. 3.

RESULTS AND CALCULATIONS

The labeling runs. Most of the labeling experiments have been »static», i.e. the solid sample has been kept with the tritium gas in the reaction vessel for a certain time without any agitation. However, it was believed that agitation would increase the specific activity of the product, so a number of runs were made in which the reaction vessel (parts A and B in Fig. 1) was removed from the apparatus and vigorously shaken in a rocking machine. It was found that with H-PAS the sample stuck to the walls of the vessel, so that in effect the sample did not move at all (called »baking» in Table 1). The introduction of broken glass pieces into the reaction vessel and careful drying of the H-PAS

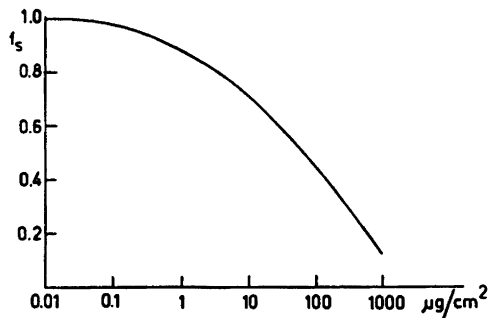


Fig. 2. Correction factor, f_s , for the self-absorption of the tritium beta-radiation in solid PAS samples¹⁴.

did not much increase the agitation of the sample. The results of these runs (see Table 1) also indicated that there was not any great difference between these "agitation" runs and the "static" runs.

This "baking" of the H-PAS samples is believed to be due to electrostatic phenomena. Since H-PAS is amphoteric, while Na-PAS is not, it was hoped that better results would be obtained with the latter. The subsequent experiments showed that no baking at all was obtained with Na-PAS. The only experiments with "true" agitation have therefore been carried out on Na-PAS.

It was thought that the static experiments with H-PAS could be extrapolated to "agitation" by means of the results on agitation of Na-PAS, *i.e.* that labeling would be equally efficient in H-PAS and Na-PAS under identical conditions. In order to check this, static runs with Na-PAS were also made; these are recorded in the fourth part of Table 1.

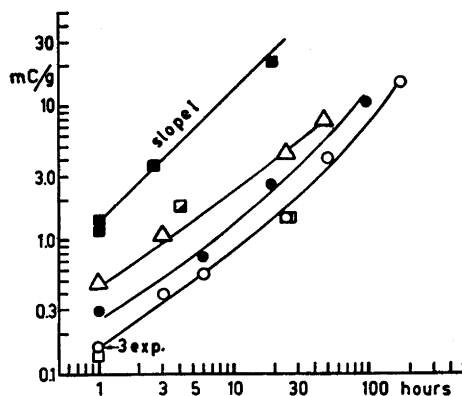
The results of the various runs are given in Table 1, where also the specific activity has been calculated.

Calculation of specific activity. In order to calculate the true specific activity, S , of non-labile tritium in the PAS product, the following equation¹⁴ was used

$$S = \frac{0.90 \times 10^{-12} \cdot I}{m \cdot f_s \cdot f_p \cdot f_b} \text{ curies/g PAS}$$

where I is the measured activity in counts/min (cpm) in a sample of weight m grams on a plate with a back-scattering factor, f_b , (for the β -radiation from tritium, f_b is 1.14 for aluminium, 1.24 for stainless steel and 1.50 for platinum¹⁴). The correction factor, f_s , for the self-absorption and self-scattering in the samples is taken from Fig. 2. In order to make all experiments comparable, corrections for the decrease in the amount of tritium gas used in the successive experiments are made through the gas correction factor, f_p , which arbitrarily has been put equal to 1.00 when the reaction vessel contains 2.6 curies of T_2 . The factor 0.9×10^{-12} is obtained from $1/2.2 \times 10^{12} \cdot f_g \cdot f_c$, where 1 curie = $2.2 \cdot 10^{12}$ dpm, $f_g = 0.50$ is the geometry factor, and $f_c = 1.0$ is the counter efficiency for tritium betas.

Fig. 3. Specific activity of tritium in non-labile positions in PAS (S mC/g) as a function of time of exposure to tritium gas (t hours). The symbols refer to: \circ 100 mg H-PAS and no agitation, \triangle 30 mg H-PAS and no agitation, \bullet 100 mg Na-PAS and no agitation, \square and \blacksquare 100 mg H-PAS and agitation, \blacksquare 100 mg Na-PAS and agitation.



Reproducibility. The reproducibility of S seems to be surprisingly good. Thus, for static runs with 100 mg samples of H-PAS, $S = 0.15 \pm 0.01$ mC/g has been obtained in three different runs with several months intermission and using two different sources of tritium gas. The poorest reproducibility so far obtained is recorded for 104 mg H-PAS during 24 h, where the deviation between two measurements amounts to about 6%. However, it is seen from Fig. 3 that some points deviate much more from the average curve drawn through the points of a set of experiments, indicating that the reproducibility sometimes is much worse.

DISCUSSION

The results of the various experiments are shown in Fig. 3, where the specific activity, S mC/g, is plotted as a function of time of exposure to tritium gas (t hours). The general tendency of S to increase with t is obvious, and also expected as long as radiation damage is negligible. When radiation damage becomes important one would expect the curves to level off and then to decrease with a further increase in exposure time t . However, the curves of the "static" experiments marked \circ , \triangle and \bullet in Fig. 3 tend rather to bend upwards at high irradiation times, which seems quite surprising.

It is thought that mainly the surface layer of the H-PAS sample is labeled. This is supported by the fact that the specific activity of the 30 mg H-PAS samples (marked \triangle), which expose about the same surface area to the tritium gas as the 100 mg samples, are almost exactly 3 times as high as that of the 100 mg H-PAS samples (marked \circ).

H-PAS is a fine powder, while Na-PAS is macro-crystalline, thus making samples of the latter more porous. It is therefore believed that the tritium gas diffuses more rapidly into the Na-PAS sample than into the H-PAS sample, causing labeling not only in the surface layer. This assumption may explain why higher specific activities are obtained with Na-PAS (marked \bullet) than with H-PAS (marked \circ) in the "static" runs.

Table 2. Comparison of specific tritium activities obtained in non-labile positions of substances similar to PAS using different labeling methods.

Substance	Method	Specific activity	Reference
Benzoic acid	recoil tritons	1 mC/g	Markowitz, Rowland, Wolfgang ¹⁵
» »	tritium exposure	14 »	Wilzbach ¹
» »	accelerated tritons	< 10 »	Wolfgang, Pratt, Rowland ⁸
Salicylic acid	recoil tritons	0.1 »	Kaplan ¹⁵
<i>p</i> -Amino salicylic acid	recoil tritons	1.5 »	Hanngren, Rydberg ¹⁰
Na- <i>p</i> -aminosalicylate	tritium exposure	~100 *	this work

* Extrapolated from the upper straight line of Fig. 3 on the assumption that no macro-decomposition of PAS occurs.

The agitation of Na-PAS causes increased labeling owing to the continuous exposure of new surfaces towards the radiation and the tritium gas; the repeated breaking of the Na-PAS crystal needles because of the agitation further increases this effect. As would be expected when the diffusion effect is eliminated through agitation, the specific activity (experiments marked ■) rises almost linearly with exposure time (slope 1 in the log-log scale of Fig. 3).

These experiments show the importance of a very intimate contact between the tritium gas and the sample. Since radiation damage does not seem to interfere for radiation times \lesssim 1 week, very high specific activities should be obtainable; the extrapolated straight line for Na-PAS (marked ■ in Fig. 3) gives, e.g., ~100 mC/g PAS after ~3 days agitation.*

In Table 2, a comparison is made of different methods of labeling organic compounds similar to PAS. The experiments refer to a reaction time of about 3 days, except in the case of accelerated tritons (0.5 h). These experiments are somewhat difficult to compare, even if the recoil tritons in all cases have been produced in a neutron flux of about 10^{12} n/cm²/sec. If the simplicity of the technique of the tritium exposure method is considered, its superiority over the other methods is obvious, especially when the sample is vigorously agitated in the tritium gas.

As pointed out in the introduction, radiation other than the β -particles from tritium should be useful in producing reactive radicals. If more radicals can be produced, with negligible radiation damage in a shorter time, still higher specific activities may be obtainable.

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* Added in proof: Later experiments have confirmed this.

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