

A technique for intense radiolabelling of pollen and pollen extract with ^{99m}Tc

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Methods for radiolabelling Poa praetensis whole pollen grains and pollen extract with ^{99m}Tc are described. Structurally intact and antigenically potent dry pollen grains with specific activities as high as 4.2 mc. per milligram pollen were obtained. The radiolabel is apparently confined to the nucleophilic groups of pollen outer wall. An almost identical procedure may be used for radiolabelling pollen extract.

Several methods for binding ^{99m}Tc to a variety of molecular species, such as human serum albumin, are currently in use.^{1, 2} These techniques depend upon the conversion of Tc (VII) to a reduced form. This paper describes a system for radiolabelling whole *Poa praetensis* pollen and pollen extract with ^{99m}Tc , which involves the use of Sn (II), Sn (0), and pH adjustment. Optimum parameters for effective radiolabelling are presented. The impact of this relatively simple and efficient method for preparing radioactively tagged pollen grains and pollen extract is considerable, and the method should allow an accurate description of the distribution of inhaled pollen and thereby greater study of the early pathophysiology of allergic phenomena.

METHODS AND MATERIALS

General procedure

Whole *Poa praetensis* pollen (Hollister-Stier Laboratories, Burbank, California), or the high molecular weight components of a 1:100 extract of this pollen, were used for radioactive labelling. A 3 ml. aliquot of a reducing agent solution containing either Fe (II) or Sn (II)[†] was added to a preselected weight of 30-mesh granular tin (obtained from J. T. Baker, Phillipsburg, New Jersey) in a 10 ml. pyrex beaker, and the pH (Radiometer PHM 26 c, Copenhagen, Denmark) of the mixture was adjusted to an initial value, optimally 2.2, using 0.1 N HCl or NaOH. The solution was then stirred for 3 minutes with a polyethylene-covered magnetic stir bar, decanted, and centrifuged for 2 minutes

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[†]Made from $\text{FeCl}_2 \cdot 7\text{H}_2\text{O}$ or $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ crystals obtained from J. T. Baker, Phillipsburg, N. J., dissolved in 0.01 N HCl.

TABLE I. Outline of experiments

Experiment no.	Studied parameters	Experimental conditions	Results
1	Reducing agent	10 ⁻⁴ M Fe (II) or Sn (II)	Sn (II) 29% more efficient than Fe (II)
2	Sn (0) concentration	0 to 100 mg.	50 mg. Sn (0) increases efficiency 34%
3	Sn (II) concentration	0 to 10 ⁻⁷ M	90% efficiency with 10 ⁻⁵ M Sn (II)
4	Initial pH	0.5 to 4.0	(see Fig. 1)
5	Initial incubation time	0 to 420 sec.	Efficiency not improved after 60 sec.
6	Terminal pH	1.0 to 6.0	(see Fig. 2)
7	Terminal incubation time	0 to 320 sec.	Efficiency not improved after 60 sec.
8	^{99m} TcO ₄ ⁻ concentration	0.2 to 12.2 mc./mg.	(see Table II)

TABLE II. Effects of ^{99m}TcO₄⁻ concentration on labelling efficiency and label intensity

Millicuries added per milligram pollen*	Label intensity†	Labelling efficiency (%)
0.2	0.13	85
0.5	0.38	81
0.9	0.71	79
2.3	1.30	56
12.2	4.21	35

*All other labelling parameters maximized as detailed in Table III.

†In millicuries per milligram dry pollen following lyophilization.

at 3,500 r.p.m. (Aloe Conical Centrifuge, St. Louis, Missouri) to remove any precipitate. One milliliter of the supernatant was added to the species to be radio-labelled in a 10 ml. pyrex beaker. The pH was readjusted to its original initial value, and the reaction mixture underwent a one-minute "initial incubation period." Two milliliters of ^{99m}TcO₄⁻ (obtained from Iso-Med., Inc., Hawthorne, California) of appropriate activity in physiologic saline was then introduced, and the pH was adjusted to a terminal value, optimally 3.6. The radiolabelling mixture was then stirred while undergoing a "terminal incubation period" normally one minute in length.

Following radiolabelling, the tagged whole pollen was separated from unbound ^{99m}Tc by two 10 second centrifugations at 1,000 r.p.m. the whole pollen being resuspended in 3 ml. distilled water prior to each centrifugation. To remove any small particles released by the pollen during the labelling procedure, a 3 ml. slurry of the washed radiolabelled pollen was vacuum filtered with a 20 μ mesh nickel sieve (Perforated Products, Inc., Brookline, Massachusetts) fitted in a 47 mm. Nucleopore filter holder (General Electric, Pleasanton, Illinois), the labelled pollen being retained on the filter. The pollen was rinsed once with 3 ml. distilled water while suspended on the sieve. After the radiolabelled pollen was washed off the filter with 4 ml. distilled water, it was centrifuged for 10 seconds at 1,000 r.p.m., and the supernatant was removed. Finally, the wet labelled whole pollen grains were returned to their dry natural state by lyophilization (New Brunswick Scientific Cryolizer, New Brunswick, New Jersey).

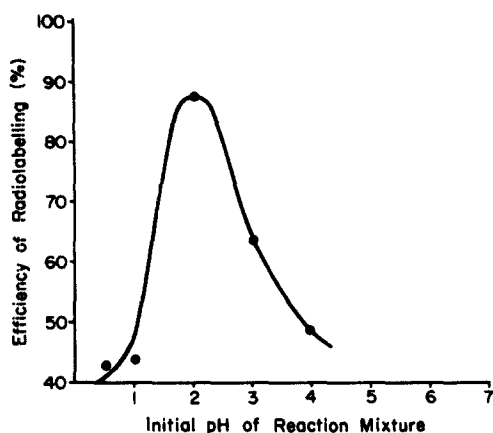


FIG. 1. Efficiency of radiolabelling as a function of initial pH of mixture.

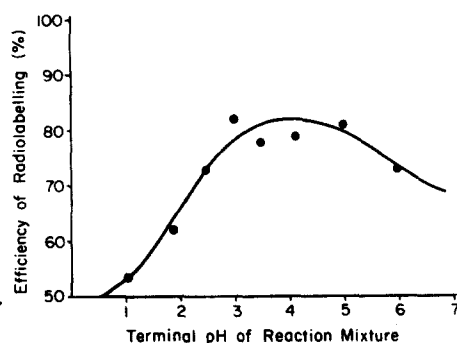


FIG. 2. Efficiency of radiolabelling as a function of terminal pH of mixture.

The pollen extract was prepared by suspending 30 mg. of *Poa praetensis* pollen in 3 ml. of distilled water for 12 hours, followed by a centrifugation at 3,500 r.p.m. for 3 minutes to remove the pollen grains from the extract. The extract was then passed through a 100 ml. G-25 Sephadex column (Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey), and only the initial fraction starting at the void volume* was collected. After the presence of high molecular weight species had been confirmed by ultraviolet spectrophotometry (Beckman DU spectrophotometer, Fullerton, California) at 280 m μ , the purified extract was lyophilized to dryness, yielding about 10 mg. of solid material. This was then dissolved in one ml. of distilled water and was subsequently radiolabelled in the manner described previously. Free ^{99m}Tc was removed from the tagged extract by repassage through the G-25 Sephadex column, taking advantage of the fact that the unbound species of technetium eluted much later than the radiolabelled antigen.³

The efficiency of radiolabelling the whole pollen or the extract was calculated by dividing the radioactivity found on the labelled pollen material by the total activity of all the unbound technetium in the washes and filtrates plus the activity on the tagged species.

In order to develop the optimum radiolabelling technique, a variety of experiments were attempted. Following the general procedure just outlined, the effect of each major reaction parameter was tested and analyzed. A complete listing of the experiments performed appears in Table I.

RESULTS

Stannous (II) was found to be a better reducing agent for $^{99m}\text{TcO}_4$ than Fe (II) for the following reasons: (1) 10^{-4}M Fe (II) yielded an efficiency of only 21 per cent while 50 per cent efficiency was obtained with Sn (II) under the same conditions (Table I). (2) The Fe (II) method required more drastic pH

*"Void volume" is the elution volume for a substance completely excluded from the gel.

TABLE III. Recommended radiolabelling methods

Whole pollen	Pollen extract
1. 10 mg. whole <i>Poa pratensis</i> pollen	1. Suspend 30 mg. pollen in 3 c.c. distilled water for 12 hours; then centrifuge the mixture at 3,500 r.p.m. for 3 minutes.
	2. Pass the supernatant through a G-25 Sephadex column collecting the high molecular weight fraction starting at the void volume.
	3. Lyophilize the extract to dryness and suspend it in 1 c.c. distilled water.
Incubate 0.05 g. 30-mesh granular tin with 3 c.c. of 10^{-5} M Sn (II) at pH 2.2 for 3 minutes. Decant solution and centrifuge for 2 minutes at 3,500 r.p.m. Add 1 c.c. of the supernatant to the species being radiolabelled, readjust the pH to 2.2, and incubate the mixture for one minute. Now introduce 2 c.c. of $^{99m}\text{TcO}_4^-$ of appropriate activity into the reaction mixture, raise the pH to 3.6, and incubate for one minute.	
2. Wash the radiolabelled pollen by suspending it in 3 c.c. of distilled water and centrifuging it at 1,000 r.p.m. for 10 seconds. Remove supernatant and repeat.	4. Separate the labelled pollen extract from any unbound technetium species by passing it through a G-25 Sephadex column and collecting the first fraction eluted containing activity.
3. Pour a 3 c.c. slurry of the radiolabelled pollen in water onto a $20\ \mu$ sieve under vacuum and wash once with a 3 c.c. aliquot of distilled water. Rinse the pollen off the filter with 4 c.c. water. Centrifuge for 10 seconds at 1,000 r.p.m. and remove supernatant.	5. Reduce the eluate containing the radiolabelled extract to the desired volume by lyophilization.
4. Lyophilize pollen, returning it to its dry natural state.	

changes; low pH values have a deleterious effect on the pollen, while high pH values cause formation of iron hydroxide, which often precipitates. (3) Since Sn (II)—Sn (IV) is a stronger redox couple than Fe (II)—Fe (III), reduction of Tc (VII) necessary for radiolabelling is more thermodynamically favorable when Sn (II) is used.⁴

One of the major drawbacks that has been associated with the use of Sn (II) as a reducing agent for radiolabelling polypeptides with ^{99m}Tc is the inherent instability of Sn (II) cationic species in solution.⁵ By incubating Sn (II) solution with solid tin, Sn (II) is kept in a reduced form.⁶ An initial incubation of Sn (II) with solid granular tin at a low pH for 3 minutes increased the efficiency of the radiolabel almost 34 per cent over trials where this step was omitted (Table I). This Sn (II)—solid tin mixture was then decanted and centrifuged to remove any undissolved tin as well as any tin hydroxide precipitates that may have formed. This step is essential since we have found that tin hydroxide flocculates also label quite effectively with ^{99m}Tc , thereby leading to erroneous efficiencies and large radiolabelled nonpollen particulate masses that are very difficult to separate from tagged pollen grains.

It was determined that an apparent Sn (II) concentration of about 10^{-5} M yields the most intense labels (Table I). It should be noted that as the concentration of Sn (II) is decreased below this level, there is very little change in radiolabelling efficiency. This is probably due to large amounts of Sn (II) produced by the dissolving solid tin in excess of the initial Sn (II) concentration.

The pH during the initial incubation period of the Sn (II)—whole pollen

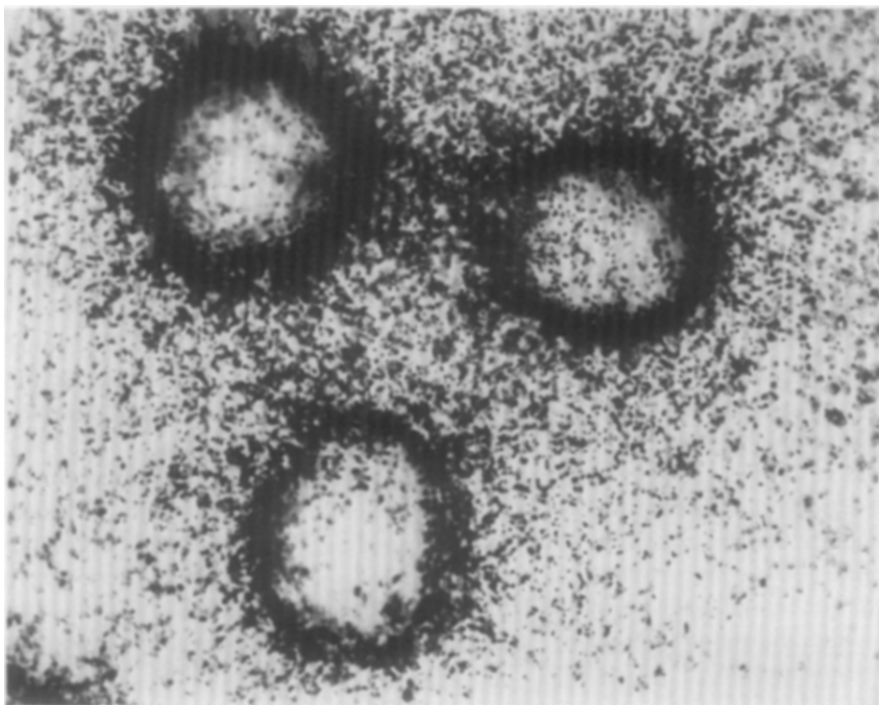


FIG. 3. Radioautogram of lyophilized whole *Poa praetensis* pollen. Diameter of individual grains is 25 μ .

mixture was found to be extremely critical. Fig. 1 demonstrates that labelling efficiencies drop precipitously if the pH of the mixture is outside the 1.5 to 2.5 range, implying that careful pH adjustments should be made during this step. It was also found that the efficiency of labelling was not significantly increased by incubating this mixture for longer than one minute (Table I). Since we have noted that *Poa praetensis* pollen undergoes continuous leaching and breakdown in aqueous media, the duration of all incubation periods involving whole pollen should be kept to a minimum.

After addition of pertechnetate, the pH of the reaction mixture was increased by titration with 0.1 N NaOH. Fig. 2 demonstrates that the terminal pH of the reaction mixture should be adjusted to a value in the 3-5 pH range. However, since the likelihood of precipitation of tin hydroxide increases as the pH is raised, it is advisable to keep the pH below 4. During terminal incubation it was found that high labelling efficiencies may again be achieved after only one minute (Table I).

The effects of ^{99m}Tc concentration in the reaction mixture were quite pronounced, as can be noted in Table II. It is evident that labelling efficiency decreases considerably when more than 1 mc. of $^{99m}\text{TcO}_4^-$ is added per milligram of pollen. However, despite decreasing efficiency, it is possible to achieve radio-labels as high as 4.2 cm. per milligram of lyophilized pollen by greatly increasing the amount of $^{99m}\text{TcO}_4^-$ added to the reaction medium. This level of activity is

enough to allow *individual* pollen grains to be resolved with a scintillation camera. It is evident from Table II that as the concentration of pollen present in the reaction mixture is increased, the labelling efficiency rises, but activity per milligram of pollen falls. This implies that for high radiolabel intensity, some labelling efficiency must be sacrificed. Conversely, if high labelling efficiencies are desired, increasing the ratio of pollen to ^{99m}Tc will allow efficiencies of greater than 90 per cent.

Since whole pollen grains had been exposed to potentially disruptive treatment, an effective procedure for removing all disrupted pollen grains and particulate matter was needed. It was determined by microscopic inspection and gamma well counting that centrifugation following the terminal incubation period effectively removed all ^{99m}Tc that was free or bound to soluble extracted pollen, as well as most of the degenerated pollen casings or ghosts produced during radiolabelling. During centrifugation these species remain in the supernatant, while the whole grains form a pellet at the bottom of the centrifuge tube. Since *Poa praetensis* pollen is larger than $25\ \mu$ in aqueous media, all smaller particulate matter was filtered off easily by passing a slurry of the tagged pollen through a $20\ \mu$ sieve under vacuum. Sieves of 5, 8, and $15\ \mu$ sizes were also tested, but the $20\ \mu$ sieve produced the most satisfactory results with a maximum of 2 pollen ghosts per 100 whole pollen grains as determined by visual inspection with a microscope.

An extensive study of radiolabelling pollen extract was not made, but efficiencies of over 90 per cent were consistently obtained with label intensities of one mc. of radioactivity per milligram of lyophilized extract. The procedure utilized is outlined in Table III, which describes the recommended radiolabelling methods for both extract and whole pollen.

Scratch tests were done on 5 sensitive subjects for comparisons of the allergenic properties of the tagged versus the unlabelled species. Reactions were identical between labelled and unlabelled whole pollen grains and between equal dilutions of labelled or unlabelled pollen extract.

DISCUSSION

Technetium ^{99m} was chosen as the labelling isotope because of its unique qualities of relatively short half-life (6 hours), moderate gamma energy (143 KeV), and high chemical reactivity. These properties permit the production of highly labelled pollen that may be easily monitored externally—yet it is relatively safe for human use.

A chemical mechanism that describes the process by which ^{99m}Tc binds to whole pollen grains can be postulated based upon an understanding of the chemical properties of technetium and a knowledge of pollen morphology. Transition metals like technetium in a cationic state will complex with proteins or with molecules possessing nucleophilic or electron donating groups.⁷⁻⁹ Technetium (VII), as it exists in $^{99m}\text{TcO}_4^-$, is reduced by Sn (II) to ^{99m}Tc cation, which presumably bonds to nucleophilic groups found on pollen grains.

Morphologically, the outer wall of the pollen grain consists of a tough, chemically resistant covering called the exine and a layer directly beneath this

known as the intine.¹⁰ Exine of the pollen wall contains a substance called sporopollenin, which is composed of a mixture of polymerized carotenoids and carotenoid esters possessing electron-donating constituents that might bond with a reduced form of technetium.^{11, 12} In addition, since pollen grain exine is permeable to molecular species in aqueous media,¹³ intine cellulose and polypeptide nucleophilic groups may also complex with reduced technetium.^{11, 13}

This postulate is partially supported by an autoradiogram of several labelled whole pollen grains demonstrating localization of radiolabel to the outer pollen wall (Fig. 3). These and other labelled pollen grains are morphologically identical to pollen grains found in the native state by phase and light microscopy.

Since *Poa praetensis* pollen extract is mostly polypeptide and protein, the high labelling efficiencies observed presumably occur by a similar mechanism.

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