Scand., 1954, v31, 317.

21. Frank, R., Sognnaes, R. F., Kern, R., *Calcification in Biological Systems*, 1960, R. F. Sognnaes, Ed., Pub. #64, AAAS, Washington, D.C.

22. Hartles, R. L., *Brit. Dent. J.*, 1961, v111, 52.

23. Patterson, D., *Nature*, 1954, v173, 75.

24. Thunberg, T., *Acta Physiol. Scand.*, 1947, v14.

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Comparison of the Physico-Chemical and Physiological Behavior of Natural Inulin with Its Radioactive Derivatives.* (28433)

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The commercial availability of C^{14} and H^3 labelled inulin derivatives† has prompted use of these substances as radioactive inulin tracers in the measurement of glomerular filtration rates and extracellular spaces. Although evidence has been presented(1,2,3) indicating the validity of the labelled derivatives as physiological inulin tracers, it will be shown that subjected to certain *in vitro* conditions in which molecular size, diffusibility, or charge were important variables, the labelled derivatives utilized in this study *separated* from the non-labelled compound.

While measurements of urine specific activity at various times after a single intravenous injection of inulin-C^{14}OOH and inulin to a dog revealed no glomerular differentiation (constituting evidence that both substances filtered at equivalent rates in this physiological process) the possibility of a more rapid rate of extrarenal removal of radioactive inulin from the circulation was suggested by data presented below. These findings emphasize that careful attention must be given to establishing the validity of the labelled derivatives as inulin tracers in any particular situation before employing them as analytical tools.

Materials and methods. The radioactivity labelled inulin derivatives were purchased from New England Nuclear Corp., Boston, Mass. Several lots of inulin-C^{14}OOH were used, No. 35-211-5 (S.A. 3.0 μc/mg), and No. 35-92-21 (S.A. 1.36 μc/mg), and lot No. 46-114-5 (S.A. 27 μc/mg) of inulin-OCH^3. Non-radioactive inulin was Pfanstiel (4045), Difco (446208) or Warner-Chilcott 10% solution (Control No. 205191).

Tritium and carbon^{14} were counted in the Technical Measurement Corp. LP-1 liquid scintillation counter. Ordinarily 0.1 ml of aqueous inulin-containing solution was mixed with 3.0 ml absolute ethanol and 10 ml toluene, containing 0.3% diphenyloxazole (DPO) and 0.01% 1,4-di-2-(5 phenyloxazolyl) benzene (POPOP). Urines containing much inulin precipitated under these conditions and 1 ml of 1 M Hydroxide of Hyamine 10-X was added to each counting vial to keep the inulin in solution. Internal standards of labelled toluene were added and counted to verify a consistent counting efficiency.

Paper electrophoresis was performed in 0.075 μ Veronal buffer at pH 8.6 with the Spinco Model R apparatus. Carbon^{14} activity of the papers was counted on the Forro paper chromatogram scanner coupled with Nuclear-Chicago Model 1615-B ratemeter and Texas Instruments Rectiriter recorder.

* This paper is based on work performed under contract with U. S. Atomic Energy Commission at University of Rochester Atomic Energy Project, Rochester, N. Y.

† Tritium label is introduced by reacting inulin with dimethylsulfate, only a small amount being used so as to methylate a very low proportion of the hydroxyl groups. In synthesizing the carbon^{14}-labelled inulin derivative a cyanohydrin is formed with $KC^{14}N$ and subsequently hydrolyzed to give inulin-C^{14}OOH. This additional carboxyl group undoubtedly was responsible for the anionic behavior of the C^{14} labelled derivative. Both labelled derivatives are re-crystallized from ethanol and stated by the supplier to possess molecular weights between 3000-4000.

Dialysis and ultrafiltration experiments were performed using cellulose casing obtained from Visking Co.‡ and A. H. Thomas Co.§ The mean pore diameters as reported by these suppliers are about 24 Å and 48 Å, respectively, based on water flow measurements. The A. H. Thomas membrane was considerably thinner and solutions were ultrafiltered through it at a faster rate. Ultrafiltrations were all carried out with the centrifuge-type apparatus(4) operated at 37°C. All solutions were 0.15 M, either in saline or Krebs-Ringer buffer.

Chemical inulin determinations were performed in a Beckman DU spectrophotometer with Schreiner's modification(5) of the resorcinol method.

Gel filtration separations using Sephadex G-50 were performed with an 8 × 52 cm or 4 × 44 cm column. Inulin or the radioactively labelled derivatives were applied and eluted from the columns with distilled water. Initially, large volume cuts were collected and inulin then eluted by timed interval collection. Volumes of successive fractions, therefore, varied somewhat from one to another.

The experiment on inulin excretion was performed as follows: A 10.3 kg female dog was hydrated with 300 ml water and injected intravenously with 36 ml of a solution containing 9.1 mg inulin-$C^{14}OOH$ and 1.8 g Warner-Chilcott inulin. Twenty-four successive urine samples were collected over a 4½ hour period and analyzed for inulin and carbon[14] activity.

Results and discussion. Dialysis. Inulin and inulin-$C^{14}OOH$ were not completely dialyzable through either of the cellophane membranes, although the labelled inulin was the more freely diffusible material.

Results of a typical experiment in which inulin was dialyzed through Visking casing for 21 hours at 4°C are shown in Table I. The final inulin concentration within the sac (54.7 μg/ml) was almost 4 times greater than the concentration outside (14.2 μg/ml). The specific activity, on the other hand, was considerably higher in the outside solution

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TABLE I. Dialysis of Inulin and Inulin-$C^{14}OOH$.

	Vol (ml)	Inulin (μg/ml)	Spα (cpm/μg)
Original sol'n	5	94.2	1080
SAC	5	54.7	910
Final			
Outside sol'n	15	14.2	1270

C^{14} counting efficiency	48.7%
Inulin recovery	103 %
Inulin-$C^{14}OOH$ recovery	102 %

demonstrating the greater *relative* movement of the C^{14} labelled inulin. No inulin or inulin-$C^{14}OOH$ was bound to the cellophane membrane, for both substances were completely recovered in the solutions at the end of the dialysis.

Ultrafiltration. Owing to the fact that inulin migrated through the cellophane membrane in our ultrafiltration apparatus at a rate slower than water, the concentrations of inulin in both the ultrafiltrate and the sac contents progressively increased as ultrafiltration proceeded. Results, therefore, cannot easily be expressed on an absolute basis because the ultrafiltrate inulin concentration varied as a function of volume ultrafiltered. It was observed, however, that 4 replicate runs of the same solution gave comparable results at equivalent times since the rates of ultrafiltration with similar sacs were not widely different. In presenting our results, we employ the term: % ultrafilterable (ratio of ultrafiltrate concentration/original concentration). As shown in Table II, this factor was relatively independent of the concentration of inulin being ultrafiltered.

A variety of ultrafiltration runs in which inulin-$C^{14}OOH$ and inulin-$OCH^3{}_3$ were mixed with 2 brands of inulin are shown in Table III. In each instance, the labelled inu-

TABLE II. % Ultrafilterability of Various Concentrations of Inulin.

Original inulin solution (mg/100 ml)	0-2 hr (% UF)	2-5 hr (% UF)
488	14.2	21.9
95	13.3	20.3
18.4	14.4	21.7
3.8	17.8	24.5

Inulin: From Warner-Chilcott 10% solution.
Sac: Visking.

TABLE III. Ultrafiltration of Inulin and Its C^{14} and H^3 Labelled Derivatives.

Labelled inulin	Non-labelled inulin	% UF labelled 0-2 hr	% UF labelled 2-5 hr	% UF non-labelled 0-2 hr	% UF non-labelled 2-5 hr	Specific activity c.p.m./μg Original	UF 0-2 hr	UF 2-5 hr	SAC
C^{14}	Pfanstiel 29.6 mg%	46.2	56.8	15.5	22.8	(59.3)	177	149	56.1
C^{14}	Warner-Chilcott 42.4 mg%	43.7	50.4	16.4	21.2	(32.4)	87.3	76.2	25.2
H^3	Pfanstiel 39.6 mg%	24.0	26.7	11.8	16.4	(73.7)	150	119	36.2
H^3	Warner-Chilcott 38.7 mg%	36.4	39.8	13.7	18.5	(146)	415	316	102

Each run is based on 4 replicate ultrafiltration tubes, except figures in parentheses.

lin derivatives were found to ultrafilter more rapidly in relation to inulin, but neither material migrated at the same rate as water in that the inulin concentrations in ultrafiltrate were always considerably less than in the original solution. Specific activities of all ultrafiltrates exceeded that of the original solutions and the specific activities within the sac were always lower than the starting values.

Gel filtration. Inulin and its labelled derivatives were subjected to gel filtration using Sephadex G-50 columns with the intention of separating molecules of different sizes. The labelled material was eluted earlier than the main peak of inulin as analyzed chemically.

Inulin-$C^{14}OOH$. A typical run of 6 mg inulin-$C^{14}OOH$ (lot No. 35-92-21) on a 4 × 44 inch column is shown in Fig. 1. There was striking separation of the C^{14} radioactivity from inulin as determined chemically. Although the C^{14}-labelled material was eluted first, ultrafiltration of pooled eluates from the Sephadex column indicated that the inulin in the later fractions was more freely ultrafilterable. The specific activity of the ultrafiltrates was highest in the earlier fractions. This anomalous behavior on the Sephadex column suggested that the Sephadex separation of C^{14} labelled material from inulin was not purely on the basis of molecular size, but may have been partially due to charge effects as demonstrated by the paper electrophoresis experiments described below. If size were the important factor the labelled material should have been retained longer on the column.

Inulin-$OCH^3{}_3$. Tritium labelled material also separated from inulin upon gel filtration, with radioactivity just slightly preceding the peak as analyzed chemically. This is shown in Fig. 2, in which 6 mg of inulin-$OCH^3{}_3$ was run on Sephadex G-50. The significant qualitative difference in behavior from the inulin-$C^{14}OOH$ should be noted.

Paper electrophoresis. Inulin-$C^{14}OOH$ migrated towards the anode at pH 8.6, clearly separating from non-labelled inulin, measured after elution from paper strips, as shown in Fig. 3. Movements of the latter owing to endosmotic solvent flow was confirmed using a dextran marker. Evidently then, introduction of the carboxyl group resulted in a significantly anionic character of the resulting inulin derivative.

Renal excretion of inulin and inulin-$C^{14}OOH$. Following single intravenous injection of inulin-$C^{14}OOH$ and inulin the specific activities of 24 successive urine samples were measured over the subsequent 4½ hours. The results (Fig. 4) reveal that no urine samples demonstrated specific activities higher than the injected solution, indicating no impairment of glomerular filtration of unlabelled inulin as compared with C^{14}-labelled derivative. If this had occurred, the early samples should have exhibited specific activities higher than the administered solution, similar to the ultrafiltration experiment. The general trend, however, was towards a lower specific activity as the experiment progressed. The most likely explanation for this finding was that the labelled derivative was gradually being removed from the circulation by extrarenal processes at a greater rate than inulin itself. Unfortunately, it was not possible to measure accurately the plasma

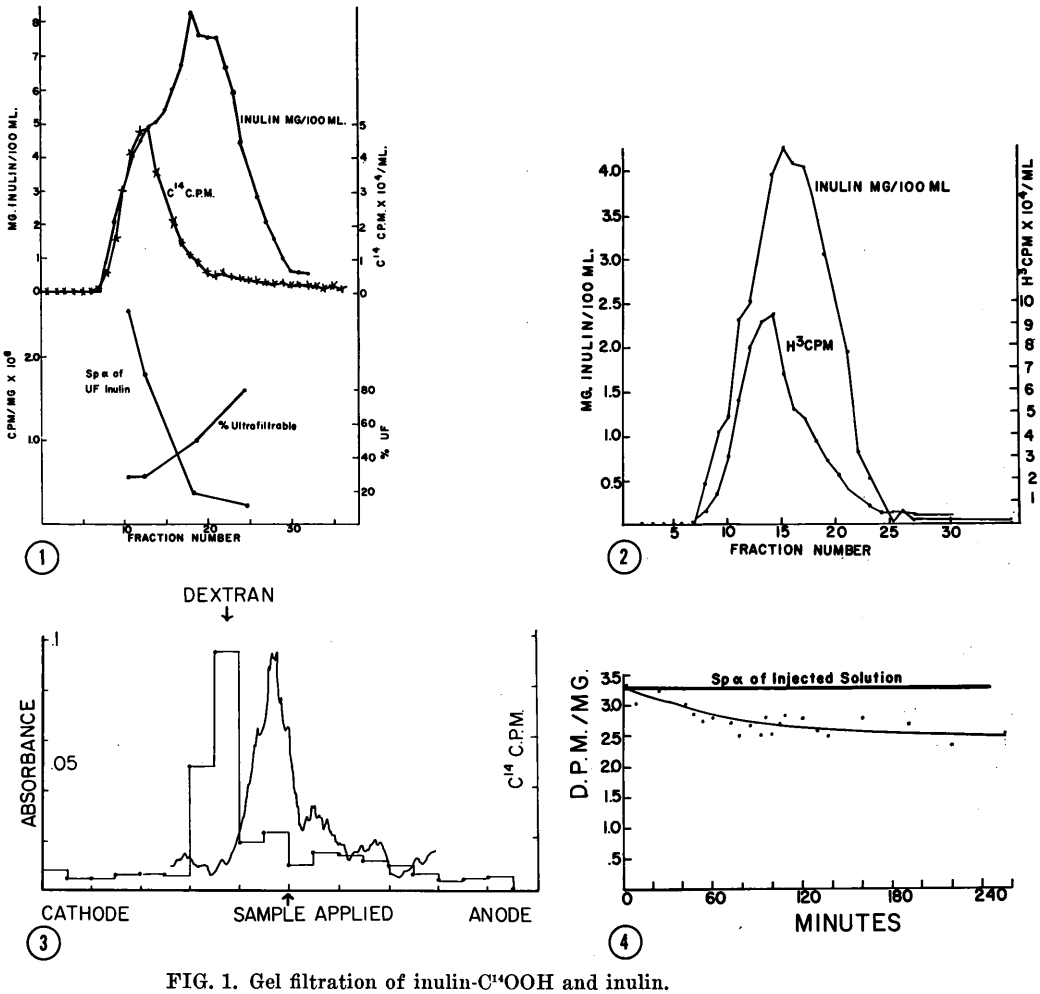

FIG. 1. Gel filtration of inulin-$C^{14}OOH$ and inulin.
FIG. 2. Gel filtration of inulin-OCH^3_3 and inulin.
FIG. 3. Paper electrophoresis of inulin-$C^{14}OOH$, inulin and dextran marker.
FIG. 4. Urine specific activity of inulin following intravenous injection.

specific activities during the experiment owing to the very low levels of C^{14} and inulin.

Discussion. Our dialysis and ultrafiltration data indicate that the labelled derivatives of inulin used in this study were more freely diffusible and of smaller molecular size than the non-labelled inulin present with the labelled material or in the commercially available non-radioactive inulin. Preliminary studies(6) in the Spinco analytical ultracentrifuge support this conclusion but indicate molecular weights of 5800-6300 for the labelled derivatives, somewhat larger figures than quoted by the supplier (see †). One point concerning inulin passage through cellophane membranes bears emphasizing. The restricted movement through this membrane consisted of at least 2 different processes; one in which the *rate* of filtration or diffusion was less than that of water, as demonstrated by re-ultrafiltering of dialysate or ultrafiltrate, and one of sieving, in which some inulin was totally incapable of passing through the cellophane pores. This latter point was most clearly demonstrated by dialyzing inulin against running water for periods up to 5 days; a semi-log plot of inulin concentration *vs* time was non-linear and had reached a plateau of 15% of the original concentration at this time.

The heterogeneity of non-labelled inulin has been reported, but renal clearance and

volume of distribution differences were not observed in the rat between the smaller "alkali-labile" inulin and the "alkali-stable" material(7), whereas clearance differences in man may have been noted(8). Strangely however, Bassir reported that such inhomogeneities were evident upon paper electrophoresis(9). Inulin molecules being uncharged should not migrate in an electric field and in the present study, the commercial inulin did not migrate, whereas the inulin-$C^{14}OOH$ was attracted to the anode. Therefore, the inulin studied by Bassir must have contained charged molecular species of some other type which gave a positive reaction with resorcinol.

The implications of our findings for physiological processes require more thorough evaluation, but it is clear that inulin is a prime example of a case where one must take special precautions to establish the validity of using a radioactively labelled derivative as a tracer for the unlabelled compound. By virtue of its polymeric and branched chain nature, various lots of the labelled inulin derivative may differ in molecular size or shape from one another and from inulin itself. The charged nature of inulin-$C^{14}OOH$ adds still another variable which may affect the physico-chemical, and hence physiological behavior of the derivatives.

It appears that glomerular pores in the dog are sufficiently large to pass inulin and the inulin-$C^{14}OOH$ used here with equal facility, but the trend of gradually declining urine specific activities from values equal to that of the injected material suggests that the smaller and more diffusible inulin-$C^{14}OOH$ may be removed more rapidly than inulin from the circulation by slow extrarenal processes. These latter processes(10, 11) as shown by various workers become obvious and measurable in experiments lasting several hours or more. For example, the apparent increased volume of distribution of inulin in nephrectomized dogs as a function of time(12) may be due to such a removal. Preliminary experiments in nephrectomized rats support the idea of faster extrarenal removal of inulin-$C^{14}OOH$ compared with inulin. Evidence is available(8,10,13) indicating that inulin may not always be freely filterable by physiological membranes and it seems entirely possible that the labelled inulin derivatives would not be valid tracers for inulin under some conditions, or with certain biological membranes.

Summary. In several *in vitro* systems it has been found that the C^{14} (inulin-$C^{14}OOH$) and H^3 (inulin-$OCH^3{}_3$) labelled derivatives behave differently from ordinary inulin. Several lots passed faster than inulin through cellophane tubing in dialysis or centrifugal-pressure ultrafiltration. Size differences were also demonstrated by gel filtration through Sephadex G-50 column. Migration of inulin-$C^{14}OOH$ from inulin occurred in paper electrophoresis because of its anionic nature. Following a single i.v. injection to a dog, urine C^{14} specific activity was not higher than administered dose but fell with time, showing that the glomerular capillaries did not restrict filtration of inulin as compared with the smaller inulin-$C^{14}OOH$. It is suggested that a more rapid rate of extrarenal removal of the radioactive inulin-$C^{14}OOH$ occurred.

1. Cotlove, E., *Fed. Proc.*, 1955, v14, 32.
2. Cooper, J. A. D., Reeb, B. B., Radin, N. S., Earle, D. P., *ibid.*, 1956, v15, 39.
3. Kinter, W. B., *Am. J. Physiol.*, 1959, v196, 1141.
4. Toribara, T. Y., *Anal. Chem.*, 1953, v25, 1286.
5. Schreiner, G. E., PROC. SOC. EXP. BIOL. AND MED., 1940, v74, 117.
6. Chen, P. S., Jr., Moore, R. A., unpublished observations.
7. Cotlove, E., *Fed. Proc.*, 1954, v13, 30.
8. Barnard, H. F., Bassir, O., Hough, J. M., *Quart. J. Exp. Physiol.*, 1955, v40, 217.
9. Bassir, O., *J. Physiol.*, 1956, v131, 586.
10. Deane, N., Smith, H. W., *J. Clin. Invest.*, 1955, v34, 681.
11. Gaudino, M., Schwartz, I. L., Levitt, M. F., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 507.
12. Finkenstaldt, J. T., O'Meara, M. P., Merrill, J. P., *J. Clin. Invest.*, 1952, v31, 627.
13. Last, J. H., McDonald, G. O., Jones, R. A., Bond, E. E., *J. Lab. Clin. Med.*, 1953, v41, 690.

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