Journal of Research of the National Bureau of Standards Vol. 50, No. 3, March 1953 Research Paper 2400

Synthesis of Lactose-1-C^{14} and Lactobionic-1-C^{14} Delta Lactone From 3-β-D-Galactopyranosyl-α-D-Arabinose[1]

Harriet L. Frush and Horace S. Isbell

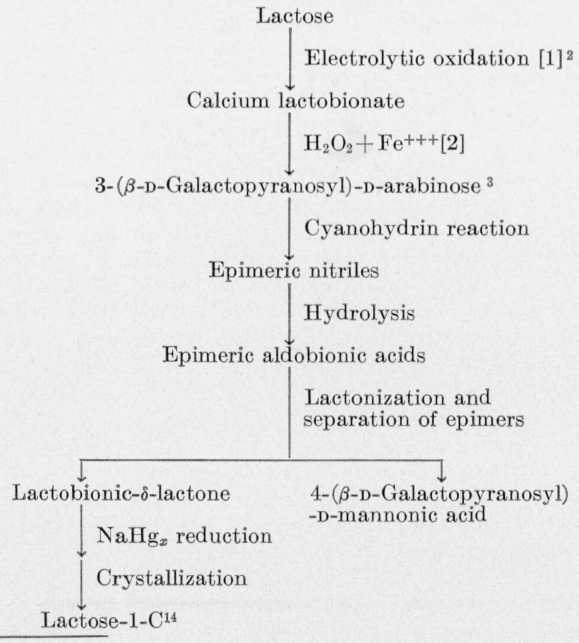
Lactose-1-C^{14}, which has been prepared for the first time, was obtained in 38-percent radiochemical yield by the cyanohydrin synthesis with 3-(β-D-galactopyranosyl)-D-arabinose. The latter substance was prepared in crystalline form and its reaction with $NaC^{14}N$ was studied under a variety of conditions. Acid catalysts favor production of the epimer having the mannose configuration. An improved procedure is given for the lactonization of lactobionic acid; by slow crystallization from methyl cellosolve, large crystals of high-purity lactobionic-1-C^{14} delta lactone were obtained. The lactone was reduced to lactose by sodium amalgam in the presence of sodium acid oxalate with a yield (by analysis) of 84 percent. A modification of the process in which the separation of the epimeric acids or lactones is omitted, was also found to be practicable for the production of lactose-1-C^{14}.

1. Introduction

There are many unsolved problems in human and animal nutrition that can be attacked by means of certain position-labeled carbohydrates. Lactose, one of the most important of these carbohydrates, has not heretofore been available in radioactive form. This paper reports the preparation of high-activity 1-C^{14}-labeled lactose in good yield by means of a cyanohydrin synthesis on 3-galactosyl-arabinose.

2. Discussion of the Experimental Method

The over-all plan for the production of labeled lactose consisted of the following steps:



To provide a basis for directing the cyanohydrin reaction to lactobionic nitrile rather than to 4-galactosyl-mannonic nitrile, the condensation of 3-galactosyl-arabinose with cyanide was studied by means of a tracer technique. This involved condensation of the sugar with labeled cyanide in buffered solutions, hydrolysis of the cyanohydrins, and separation of the labeled lactobionic acid as the calcium lactobionate calcium bromide double salt [4]. It was found that sodium bicarbonate and presumably other general acid catalysts in the cyanohydrin mixture favor formation of the 4-galactosyl-mannonic epimer; calcium chloride or sodium carbonate, however, favor formation of the 4-galactosyl-gluconic (lactobionic) epimer. These results obtained by tracer methods, correspond qualitatively to those obtained by optical rotation and isolation methods in the study of the cyanohydrin synthesis for the production of D-glucose-1-C^{14} and D-mannose-1-C^{14} from D-arabinose [5]. In the preparation of labeled lactose, both the calcium chloride method [6], and the sodium carbonate method for conducting the cyanide condensation were used, but the latter is preferable because it avoids the introduction and subsequent troublesome removal of the chloride ion. In early preparations by the calcium chloride method, lactobionic acid was isolated from the cyanohydrin hydrolyzate as the crystalline calcium lactobionate calcium chloride double salt [7]. The procedure involved several steps later eliminated by condensation in the presence of sodium carbonate, and crystallization of lactobionic lactone from the hydrolyzate. The latter step was made practicable by the development of a procedure for the complete conversion of lactobionic acid to the lactone.

To ascertain favorable conditions for reduction of the lactone to lactose, numerous samples of nonradioactive lactobionic delta lactone were reduced with various quantities of sodium amalgam in the presence of sodium acid oxalate. The reduction was conducted in the apparatus described previously [5], but modified by a sidearm that permitted addition of amalgam while the solution was being stirred. It was found by analysis that, under suitable conditions, 84 percent of the lactone is converted to the sugar.

[1] Part of a project on the development of methods for the synthesis of radioactive carbohydrates, sponsored by the Atomic Energy Commission.

[2] Figures in brackets indicate the literature references at the end of this paper.

[3] The alpha modification of this sugar, 3-(β-D-galactopyranosyl)-α-D-arabinose had been crystallized several years ago by Zemplén [3], but no seed crystals were available in this laboratory. However, after about 2 months, crystals were obtained from a sirup containing methyl cellosolve and isopropyl ether. Thereafter, crystallization of other preparations was readily effected by use of seed crystals.

After removal of sodium salts, the lactose was separated by crystallization from aqueous methanol with the addition of isopropanol. In radioactive preparations, additional labeled sugar was recovered from the mother liquor by the carrier technique. In a preparation starting with 3.5 mc of C^{14}-labeled sodium cyanide the yield of labeled lactobionic delta lactone was 54 percent. In the reduction step 68 percent of the lactone was isolated as crystalline lactose. The over-all radiochemical yield of lactose-1-C^{14} was thus 37 percent.

It was also found practicable to prepare lactose without the separation and purification of any intermediate, by reducing the crude lactone mixture prepared from the cyanohydrin reaction. The resulting solution gave one crop of crystalline lactose without carrier; additional crops, obtained by use of carriers, raised the radiochemical yield to 38 percent.

3. Experimental Details

3.1. Preparation of 3-(β-D-Galactopyranosyl)-α-D-Arabinose

A mixture of 375 g of calcium lactobionate pentahydrate, 20 g of barium acetate monohydrate, and 10 g of ferric sulfate (with about $6H_2O$), was added to 3 liters of boiling water [2]. The mixture was cooled to 35° C, and 120 ml of 30-percent hydrogen peroxide was added. During the ensuing reaction period, the solution was cooled to maintain a temperature of less than 50° C. After about 1 hr the solution, which had turned dark brown, was cooled to 40° C, and a second 120-ml portion of hydrogen peroxide was added. When the second reaction was complete, about 25 g of a decolorizing carbon and 10 g of calcium carbonate were added. The mixture was filtered, and the filtrate was evaporated under reduced pressure to a sirup of about 80-percent total solids. The sirup was mixed first with 300 ml of methanol, and then 600 ml of a 1:1 mixture of methanol and ethanol was added. The resulting precipitate was separated by filtration, and was washed, first on the filter, then in a beaker, and finally on the filter with a total of 500 ml of ethanol. The residue was discarded. The filtrate and wash liquor deposited some gummy material from which the clear liquid was decanted. The residue was triturated with 100 ml of ethanol, and the ethanol extract was filtered. The solid was discarded and the filtrate, combined with the alcoholic solution previously separated by decantation, was evaporated under reduced pressure to a sirup of about 90-percent total solids. The sirup was then extracted by trituration successively with two 200-ml portions of methanol and two 200-ml portions of isopropanol; the residue was discarded. The alcoholic extracts were combined and allowed to stand at room temperature for further separation of gummy impurities. When the supernatant liquid had become clear, it was decanted from the residue and evaporated under reduced pressure to a thick sirup. This was dissolved in water, and the solution was passed over a column (2 by 30 cm) containing a mixture of cation and anion exchange resins.[4] The combined effluent and wash liquor was evaporated under reduced pressure to a thick sirup, which was dissolved in about 50 ml of methyl cellosolve. From this solution several fractions of sirupy 3-galactosyl-arabinose were precipitated by the addition of isopropyl ether. Each of the fractions was dissolved in methyl cellosolve and the solutions, brought to incipient turbidity by means of isopropyl ether, were stored in a desiccator over calcium chloride. After 2 months, the sirupy residue of one fraction crystallized. The remaining fractions crystallized when seeded, and a total of 50 g of crude crystalline 3-(β-D-galactopyranosyl)-α-D-arabinose was obtained. To recrystallize, the material was dissolved in a minimum of hot water, and the solution was filtered with the aid of decolorizing carbon and treated with isopropanol almost to the point of turbidity. It was also recrystallized from a thick aqueous sirup (80° Brix) by the addition of methyl cellosolve. $[\alpha]_D^{20}$ of the purified product was −62.5° at equilibrium (water, $c=2$) in approximate agreement with the value previously reported by Zemplén [3] ($[\alpha]_D^{20}=-50.3°\rightarrow-63.1°$).

3.2. Epimeric Proportions of the Products Formed by Condensation of 3-(β-D-Galactopyranosyl)-D-arabinose with Cyanide

The experiments outlined in table 1 were conducted to determine optimum conditions for the formation of the lactobionic epimer. In each experiment 2 ml of a solution containing 0.0636 millimole of C^{14}-labeled cyanide with 5.12 μc of activity was placed in a glass-stoppered tube. The solution was frozen in a carbon dioxide-acetone freezing mixture to prevent loss of cyanide, after which 2 ml of a solution, containing 0.0705 millimole of 3-galactosyl-arabinose and the various substances listed in table 1, was introduced. The mixture was shaken gently until the ice dissolved and was then allowed to stand at room temperature. After 48 hr, the mixture was heated at 80° C for 5 hr to effect hydrolysis, and then was evaporated to dryness in an air stream. The residue in each tube was dissolved in a few drops of water, and the solution was neutralized to the end point of phenolphthalein by the addition of dilute hydrobromic acid. Nonradioactive calcium lactobionate calcium bromide (531.3 mg of Ca $(C_{12}H_{21}O_{12})_2.CaBr_2.6H_2O$) [4] was dissolved in each hydrolyzate, and the solutions were concentrated to thick sirups in a stream of air. Each of the sirups was diluted with 50-percent aqueous isopropanol to a volume of about 2 ml. Any turbidity that developed during the dilution was discharged by the dropwise addition of water, and the solutions were finally treated with isopropanol to incipient turbidity, and were seeded with the double salt. As crystallization proceeded, additional isopropanol was added. After 72 hr, the mother liquor was removed with a capillary pipette, and the crystals were washed with 50-percent aqueous isopropanol. The labeled

[4] The cation exchange resin was Amberlite IR100, analytical grade, Resinous Products Division of Rohm & Haas Co., Philadelpnia, Pa.; the anion exchange resin was Duolite A-4, Chemical Process Co., Redwood City, Calif.

compound from each experiment was recrystallized from concentrated aqueous solution by the method described above. Samples of the recrystallized calcium lactobionate calcium bromide were dried over calcium chloride, and their carbon-14 content was determined by direct count in formamide solution [8]. A 22.2-mg sample of the product from experiment 1, dissolved in 1 ml of formamide gave 41.1 c/s in a proportional counter, in which 1 c/s is equivalent to 0.00241 μc. A 20.6-mg sample of the product from experiment 2 gave 20.5 c/s. These values correspond to 0.00446 and 0.00240 μc/mg, respectively.

Table 1. *Epimeric proportions in the condensation of 3-galactosyl-D-arabinose with cyanide*

Experiment	Reaction mixture		Activity of purified carrier $Ca(C_{12}H_{21}O_{12})_2 CaBr_2.6H_2O$ [c]		Lactobionic epimer [d]
		Millimoles	*μc/mg*	*μc/milliequivalent of lactobionate*	*Percent*
1	NaC^*N	[a] 0.0636	0.00446	2.37	47.7
	Na_2CO_3	.06			
	3-Galactosyl-arabinose	.0705			
2	NaC^*N	[a] .0636	0.00240	1.28	25.3
	$NaHCO_3$	.06			
	CO_2	[b] Saturated			
	3-Galactosyl-arabinose	0.0705			

[a] Contained 5.12 μc of carbon-14, or 80.5 μc/millimole.
[b] A piece of CO_2 ice, the size of a pea, was added after the cyanide solution had been frozen.
[c] 531.5 mg of $Ca(C_{12}H_{21}O_{12})_2.CaBr_2.6H_2O$ (1 milliequivalent of lactobionate) was added to the reaction mixture.
[d] Percentage of lactobionic epimer$=(A_2/(A_1-A_2))(100/0.0636)$, where A_1 and A_2 are, respectively, the activities per milliequivalent of the lactobionate formed and the lactobionate isolated.

3.3. Lactonization of Lactobionic Acid, and Crystallization of the Delta Lactone [9]

Because complete conversion of lactobionic acid to the lactone is difficult, a series of experiments was conducted to ascertain suitable conditions for the quantitative production of the lactone on a millimole scale. The following procedure was found to be satisfactory: An aqueous solution of the acid was mixed with an equal volume of methyl cellosolve and was lactonized by gradual evaporation of the solvent under a stream of dry air at room temperature, in the presence of seed crystals of lactobionic delta lactone. After the material had been concentrated to a semisolid mass, it was stored in a desiccator over calcium chloride, with the addition from time to time of sufficient methyl cellosolve to keep it moist. At the end of a week, the addition of methyl cellosolve was discontinued, and the material was stored at room temperature over calcium chloride for a week or longer. The product was recrystallized by dissolving it in 50 parts of methyl cellosolve at 95° to 100° C. The solution was filtered and concentrated under reduced pressure in the presence of seed crystals. When a satisfactory crystallization had occurred, the mother liquor was separated by use of a capillary pipette, and the crystals were washed with methyl cellosolve. By concentration of the mother liquors nearly all of the material was recovered. The melting point of the product, 199° to 205° C. is somewhat dependent upon the rate of heating. The value found in the present study is noticeably higher than the melting point (196° to 197° C.) previously reported [9].

3.4. Reduction of the Lactobionic Lactone with Sodium Amalgam in the Presence of Sodium Acid Oxalate

To provide a basis for the efficient reduction of lactobionic lactone, the reductions outlined in table 2 were conducted in the apparatus of figure 1. In each case 0.5 millimole of crystalline lactone was placed in the apparatus together with the oxalic acid and sodium oxalate listed. Ten milliliters of ice water was then added and, after the stirrer had been started,

TABLE 2. *Reduction of lactobionic delta lactone [a] by sodium amalgam in the presence of oxalate buffers*

Experiment	Sodium oxalate	Oxalic acid	$NaHg_x$ [b]	Yield of lactose (by analysis)
	g	*g*	*g*	*Percent*
1	0.10	0.09	0.6	80.3
2	.20	.18	1.2	84.2
3	.40	.35	2.3	82.7
4	.80	.70	4.6	80.0
5 [c]	.80	.70	4.6	79.3
	.80	.60	4.6	
6 [d]	.80	.70	4.6	78.9
	.80	.60	4.6	
	.80	.60	4.6	

[a] 0.5 millimole in 10 ml of water.
[b] 5-percent amalgam.
[c] The second quantity of amalgam and buffer were added after the first had completely reacted.
[d] Three successive additions of amalgam and buffer were used.

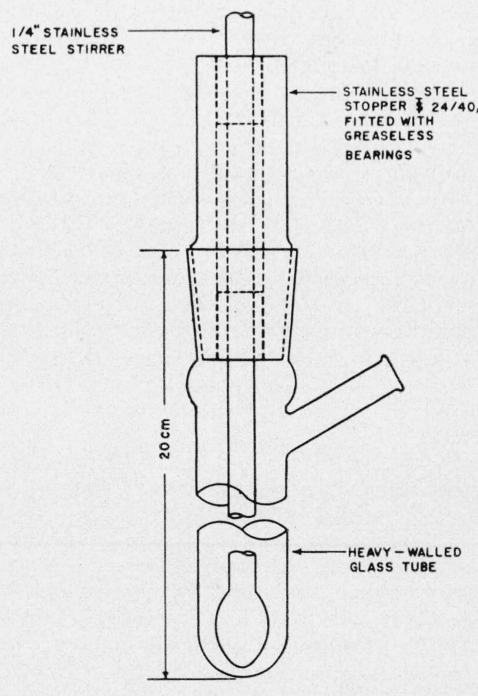


FIGURE 1. *Sodium amalgam reduction apparatus.*

the indicated quantity of sodium amalgam pellets was added through the sidearm. The mixture, cooled in an ice bath, was stirred vigorously until the amalgam was spent (about 2 hr). The mercury was removed, and the solution was neutralized by the addition of sufficient sodium hydroxide solution to give a faint permanent pink color with phenolphthalein; this was discharged by dropwise addition of a solution of oxalic acid. A fivefold quantity of methanol was added, and the crystalline salts were removed by filtration, washed with methanol, and discarded. The mother liquor was diluted with water to exactly 100 ml, and 10-ml aliquots were transferred to flasks for analysis by the modified Scales method [10]. Before analysis, the alcohol was removed from each sample by evaporation in an air stream, and the residue was dissolved in the requisite amount of water (10 ml).

For the isolation of lactose, the reduction was carried out as described in the preceding paragraph, and with the proportions of materials given in experiment 2 of table 2. After removal of the salts by filtration, the alcoholic filtrate was concentrated to a thin sirup under reduced pressure, a fourfold quantity of methanol was added, and the small crop of salts thus precipitated was immediately separated.[5] The methanol in the filtrate was removed by evaporation, the product was diluted with water, and the solution was passed over a column of mixed cation and anion exchange resins (about 20 ml). The resin was washed with water until the activity of the effluent was negligible, and the combined solution and washings were concentrated under reduced pressure, and finally were lyophilized (freeze-dried). The residue, dissolved in a few drops of water, was immediately transferred to a tared test tube, and the solution was seeded, and treated with methanol to incipient turbidity. Isopropanol was added as crystallization proceeded, and the material was finally stored for several days in a refrigerator. The mother liquor was then removed by means of a capillary pipette, and the crystals were washed with a methanol-isopropanol mixture (2:1). To recrystallize, the lactose was dissolved in a minimum amount of hot water; the solution was filtered with the aid of a decolorizing carbon, and the filtrate was concentrated, if necessary, and treated with methanol and isopropanol in the presence of seed crystals. When crystallization appeared to be complete, the mother liquor was removed, and the crystals were washed with methanol-isopropanol mixtures, and finally dried in a vacuum desiccator over calcium chloride.

3.5. Preparation of Lactobionic-1-C^{14} delta Lactone and Lactose-1-C^{14}

To a small flask containing 343 mg (1 millimole) of 3-galactosyl-arabinose and 84 mg of sodium bicarbonate, there was added at ice temperature 5 ml of a solution containing 1 millimole each of sodium hydroxide, and of C^{14}-labeled sodium cyanide with 3.5 mc of activity. The flask was sealed and allowed to stand at room temperature for 3 days. The resulting cyanohydrins were then hydrolyzed by heating the mixture at 70° C for 6 hr in the presence of an air stream. Water was replaced from time to time, and at the end of the period the solution was allowed to evaporate to dryness. The residue, dissolved in 50 ml of water, was passed through a column of cation exchange resin, which was then washed until no appreciable activity remained.

The combined solution and washings were concentrated under reduced pressure to a sirup. The material was transferred by means of 2 ml of water to a test tube, lactonized by the method described in section 3.3, and recrystallized from hot methyl cellosolve. In order to remove the residual labeled lactone from the mother liquor by the carrier technique, several quantities of nonradioactive lactobionic lactone were employed. These were successively crystallized from the mother liquor in the manner previously described, and recrystallized before analysis. The quantities and radiochemical yields are given in table 3.

The crops of lactobionic lactone listed in table 3 were reduced in three portions. Before reduction, the 53-mg quantity of lactone obtained without carrier was combined with the first two carrier crops.

TABLE 3. *Summary of radiochemical yield of lactobionic-1-C^{14} delta lactone from 3.5 millicuries of* $NaC^{14}N$

Crop	Lactone added as carrier	Lactone isolated	Activity
	mg	*mg*	*μc*
1	None	52.5	486
2	200	165	379
3	200	120	217
4	500	338	248
5	500	630	567
Total			1,897
Radiochemical yield			54.2%

TABLE 4. *Summary of radiochemical yield of lactose-1-C^{14} from lactobionic-1-C^{14} delta lactone*

Lactone reduced		Lactose added as carrier	Lactose-1-C^{14} isolated
mg	*μc*	*mg*	*μc*
331.4	1,082	---	448
338.0	248	---	189
630	567	---	468
---	---	500	109
---	---	500	59
---	---	500	23
Total	1,897	---	1,296
Radiochemical yield from lactone			68.4%
Over-all radiochemical yield [a]			37.2%

[a] From 3.5 millicuries of $NaC^{14}N$.

[5] This precipitate generally contained some sodium lactobionate. It was saved for treatment with a later preparation. Delay in removal of the salts may allow crystallization of the sugar to occur.

In each case, the reduction was conducted in the manner described in section 3.4 and with quantities of material proportional to those of experiment 2 of table 2. After the first crop of lactose had been removed, three 500-mg quantities of nonradioactive lactose were used successively as carriers, by crystallization from the mother liquor. The results of this preparation are given in table 4. It will be seen that the radiochemical yield of lactose from lactobionic delta lactone was 68.4 percent. The over-all radiochemical yield from labeled sodium cyanide was 37 percent.

3.6. Preparation of Lactose-1-C^{14} without Isolation of Intermediates

A cyanohydrin synthesis was conducted by the carbonate method with 2 millimoles of 3-galactosyl-arabinose, and 2 millimoles of labeled cyanide containing 28.3 μc of activity. After hydrolysis of the cyanohydrins, cations were not removed, but sufficient oxalic acid was added to the solution to convert the sodium ion present to sodium acid oxalate. The solution was evaporated to dryness repeatedly in the presence of aqueous methyl cellosolve and seed crystals of lactobionic lactone. Finally, the material was stored in a desiccator and moistened from time to time with methyl cellosolve. At the end of 2 weeks, the amalgam reduction was carried out by the method described in section 3.4, but allowance was made for the amount of oxalate in the mixture. The first crop of crystalline lactose contained 7.5 μc of activity, and carrier crops containing 3.4 μc brought the total yield to 10.8 μc, or 38.0 percent. Although this method is less laborious than the preparation described in section 3.5, extreme care must be observed in the purification of the lactose because the crude product contains a higher proportion of radioactive contaminants than the lactose from a purified lactobionic lactone.

3.7. Preparation of Sodium Lactobionate-1-C^{14}

After the work reported in the preceding sections was completed, F. H. Stodola of the Northern Regional Research Laboratory, United States Department of Agriculture, Peoria, Ill., kindly supplied a sample of crystalline sodium lactobionate [11].

To provide a supply of sodium lactobionate-1-C^{14}, 1 millimole of 3-galactosyl-arabinose was reacted with C^{14}-labeled cyanide, as described in section 3.5. The reaction mixture from the cation exchange resin was neutralized with sodium hydroxide, to a phenolphthalein end point. A small quantity of a decolorizing carbon was added, and the solution was filtered and evaporated to a sirup. Methanol was added to the point of incipient turbidity, and the mixture was seeded with crystalline sodium lactobionate. After several days labeled sodium lactobionate was separated. The product was recrystallized from water by the addition of methanol. The salt crystallizes well and is easily purified. The yield without carrier was 40 percent of the theoretical; by use of carrier a radiochemical yield of 50 percent was obtained.

4. References

[1] H. S. Isbell and H. L. Frush, BS J. Research **6,** 1145 (1931) RP328.
[2] O. Ruff and G. Ollendorff, Ber. deut. chem. Ges. **33,** 1798 (1900); R. C. Hockett and C. S. Hudson, J. Am. Chem. Soc. **56,** 1632 (1934).
[3] G. Zemplén, Ber. deut. chem. Ges. **59,** 2402 (1926); **60,** 1309 (1927).
[4] H. S. Isbell, J. Research NBS **17,** 331 (1936) RP914.
[5] H. S. Isbell, J. V. Karabinos, H. L. Frush, N. B. Holt, A. Schwebel, and T. T. Galkowski, J. Research NBS **48,** 163 (1952) RP2301.
[6] C. S. Hudson, O. Hartley, and C. B. Purves, J. Am. Chem. Soc. **56,** 1248 (1934).
[7] A. Stoll and W. Kussmaul, U. S. Patent 1,648,368 (1927).
[8] A. Schwebel, H. S. Isbell, and J. V. Karabinos, Science **113,** 465 (1951).
[9] H. S. Isbell, BS J. Research **11,** 713 (1933) RP618.
[10] H. S. Isbell, W. W. Pigman, and H. L. Frush, J. Research NBS **24,** 241 (1940) RP1282.
[11] G. E. N. Nelson and F. H. Stodola, J. Am. Chem. Soc. **75,** 1748 (1953).

WASHINGTON, November 6, 1952.