needles. mp 232—235°. Mixed mp on admixture with the authentic sample[3a] showed no depression and IR spectra of two samples were entirely identical. The adsorbent corresponding to the spot (^3Rf 0.29) was eluted with hot $CHCl_3$–acetone and the eluate was recrystallized from MeOH to give methyl (3-benzyloxy-16β-hydroxyestra-1,3,5(10)-trien-17β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (VII) (68 mg) as colorless needles. mp 256—257.5°. Mixed mp on admixture with the authentic sample[3a] showed no depression and IR spectra of two samples were entirely identical.

Methyl (3-Benzyloxy-16-oxoestra-1,3,5(10)-trien-17β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (VIII)——To a solution of VII (9 mg) in acetone (0.3 ml) was added CrO_3 reagent (CrO_3 (1.33 g), H_2SO_4 (1.15 ml) diluted to 5 ml with H_2O) (0.01 ml) and stirred for 1.5 hr at 0—5°. The resulting solution was diluted with ether, washed with 5% $NaHCO_3$, H_2O and dried over anhydrous Na_2SO_4. After usual work-up the crystalline product obtained was recrystallized from MeOH to give VIII (3 mg) as colorless needles. mp 158—159°. Mixed mp on admixture with the authentic sample[3a] showed no depression and IR spectra of two samples were entirely identical.

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Studies on the Glucaric Acid Pathway in the Metabolism of D-Glucuronic Acid in Mammals. IV.[1] Fluorometric Method for the Determination of D-Glucaric Acid in Serum[2]

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In connection with the biochemical studies on D-glucaric acid, a normal constituent of human urine which is derived from D-glucuronolactone,[4] we developed a colorimetric method for the quantitative determination of this acid.[5] Thus, D-glucaric acid separated from mammalian urines using ion-exchange column chromatography was oxidized with periodic acid to give glyoxylic acid, which was further converted into the intensely colored 1,5-diphenylformazan[6] and determined colorimetrically. During the course of our studies on D-glucaric acid a much more highly sensitive method was required for the accurate estimation of normal serum level of D-glucaric acid in mammals.[1] The present paper deals with the fluorometric determination of D-glucaric acid which is based on the condensation of glyoxylic acid derived from D-glucaric acid with 4'-hydrazino-2-stilbazole to yield a highly fluorescent product using a modification of the procedure for the determination of α-oxo acids originally reported by Mizutani, *et al.* (Fig. 1).[7]

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3) Location: *Takada 3-Chome, Toshima-ku, Tokyo.*
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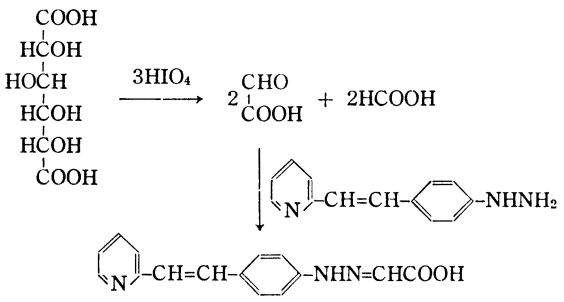

Fig. 1. Reaction Sequence for Fluorometric Determination of D-Glucaric Acid

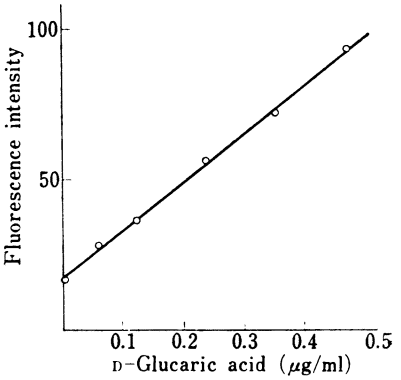

Fig. 2. Calibration Curve for Determination of D-Glucaric Acid

Experimental

Chemicals——Potassium hydrogen D-glucarate was prepared as described previously.[5] 4′-Hydrazino-2-stilbazole was obtained from Tokyo Kasei Co., Tokyo. All other reagents used in this study were of analytical grade.

Ion-exchange Resin——Dowex 1X-8, 200—400 mesh, borate was prepared as described earlier.[5]

Reagents——A. Solvents for ion-exchange column chromatography[5]: 1) 0.1M sodium borate; 2) 0.05M sodium borate–0.02M sodium sulfate; 3) 0.05M sodium borate–0.1M sodium sulfate. B. Reagents for quantitative determination: 1) 0.5M aqueous acetic acid; 2) 5 mM periodic acid; 3) 12.5 mM thioacetamide; 4) 35 μM 4′-hydrazino-2-stilbazole hydrochloride; 5) 5M hydrochloric acid; 6) methanol.

Instruments——Beckman Model 772 Ratio Fluorometer with double-beam mercury vapor lamp was used. Uranium rods, No. 4 and 5, were employed as supplementary standards for setting the sensitivity of the instrument. The following filters were selected as primary filters: UG 11 in the supplementary standard path and two filters, 5961 and 5978, in the sample path. Two filters, 97610 and 97613, were used as the secondary filters. Excitation and emission light of fluorescence were 450 mμ and 550 mμ respectively. Hitachi Fluorescence Spectrophotometer MPF-2 was used for the measurement of excitation and emission spectra.

Serum Samples——Serum samples were prepared as described previously.[1]

Separation of Serum D-Glucaric Acid——The procedure used for the separation of serum D-glucaric acid was reported earlier.[1]

Fluorometric Determination——Into a 25 ml brown-colored glass-stoppered graduated test tubes were placed 2.0 ml of the sample solutions (effluent with 0.05M sodium borate–0.1M sodium salfate; D-glucaric acid fraction[5]) and 1.0 ml of 0.5M aqueous acetic acid with mixing. To the mixture solution was added 0.10 ml of 5 mM periodic acid and mixed. After standing for 15 min at 20°, 0.10 ml of 12.5 mM thioacetamide was added to the solution with mixing and left for 15 min at 20°. Subsequently, 2.0 ml of 35 μM 4′-hydrazino-2-stilbazole was added to the resultant solution followed by mixing and kept in the dark for 30 min at 20°. Each 2.0 ml of 5M hydrochloric acid and methanol was added to the solution. They were mixed and the contents of each tube were transfered to a fluorometer cell with 1.0 cm light path and the fluorescence was read at 550 mμ with an excitation wavelength of 450 mμ. The blank solution consisted of the test reagents with 2.0 ml of 0.05M sodium borate–0.1M sodium sulfate. Calibration curves were prepared as follows: About 1.7 mg of potassium hydrogen D-glucarate was weighed accurately and dissolved in 0.05M sodium borate–0.1M sodium sulfate to make 100 ml of stock solution. Just before use, 3.0 ml of the stock solution was further diluted to 100 ml with the same solvent to prepare working solution. To 0.5 to 2.0 ml of the working solution was added enough 0.05M sodium borate–0.1M sodium sulfate to make a volume of 2.0 ml. These standards, containing various known amounts of potassium hydrogen D-glucarate, were treated as described above to afford calibration curves. It is essential to assay standards along with every determination of samples. All the samples were run in triplicate. The content of D-glucaric acid was calculated by subtracting the fluorescence intensity of the blank from that of the sample. Linear proportionality between fluorescence intensity and concentration was obtained for D-glucaric acid (calculated from potassium hydrogen D-glucarate) at 0.05 to 0.50 μg/ml of the standard solution (Fig. 2).

Result

Oxidation of D-glucaric acid with periodic acid was carried out in slightly acidic condition (pH 4.4) by adding acetic acid to 0.05M sodium borate–0.1M sodium sulfate solution. After the oxidation, excess periodic acid was removed by reduction with thioacetamide.[8] Glyoxylic acid thus formed was condensed with 4′-hydrazino-2-stilbazole, which was used for the fluorometric determination of α-oxo acids by Mizutani, *et al.*[7] Thirty minutes was chosen as the standard condensation time, since the maximum fluorescence intensity was obtained in 30 to 60 min. The effect of the concentration of 4′-hydrazino-2-stilbazole on the fluorescence intensity is shown in Fig. 3. Higher concentration of the reagent gave higher blank value. Therefore, 35 μM 4′-hydrazino-2-stilbazole solution was used as the standard concentration of the reagent. The stability of the hydrazone formed was examined after addition of 5M hydrochloric acid and methanol. The fluorescence was found to be stable at least for one hour.

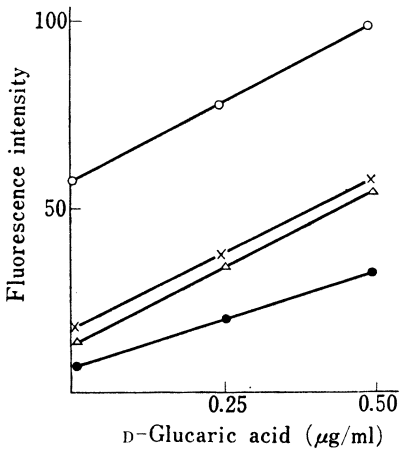

Fig. 3. Effect of 4′-Hydrazino-2-stilbazole Concentration on the Fluorescence Intensity

—○—: 500 μM —×—: 125 μM
—△—: 35 μM —●—: 17.5 μM

Since the excitation and emission spectra of the fluorescence obtained by the above procedure were quite similar to those reported by Mizutani, *et al.*,[7] their excitation and emission wavelengths, 450 mμ and 550 mμ respectively, were employed for the determination of the fluorescence intensity by using the appropriate filters described above. Recoveries of potassium hydrogen D-glucarate added to human and rat sera were satisfactory as shown in Table I. Their recoveries were calculated as described previously.[5]

Table I. Recovery of D-Glucaric Acid after Addition of Potassium Hydrogen D-Glucarate to Human Serum (3.0 ml) and Rat Serum (1.0 ml)

Species	Serum number	D-Glucaric acid (μg)[a]				% recovery
		Added	Serum	Calc.	Found	
Man	1	8.32	0.40	8.72	7.73	89
	2	2.60	0.60	3.20	3.40	103
	3	1.67	0.86	2.53	2.64	104
	4	1.30	1.50	2.80	2.80	100
Rat	5	2.50	1.11	3.61	3.32	92
	6	3.75	0.97	4.72	4.65	98
	7	3.00	3.40	6.40	6.00	94
	8	13.40	0.60	14.00	14.80	106

a) Values were obtained by the Procedure I reported previously.[5]

Discussion

Quantitative determination of D-glucaric acid in biological fluids, especially in urine and serum, might be a useful test for alterations in the glucuronic acid pathways in man lacking

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in the glucuronic acid–L-ascorbic acid cycle.[9-11] Although a colorimetric method was developed earlier in this laboratory[5] and was successfully applied to the determination of D-glucaric acid in normal mammalian urines as well as in human serum after loading of its precursors,[1] it was not applicable to the estimation of the normal serum level of the acid in man because of its insufficient sensitivity.

The normal serum levels of D-glucaric acid in man and the rat determined by the fluorometric method described above are given in Table II. Actually, a comparison between the colorimetric and the fluorometric methods with normal mammalian sera was unattainable, while a satisfactory coincidence between the two methods was observed with normal mammalian urines.[12]

TABLE II. Serum Level of D-Glucaric Acid in Man and the Rat

Species	D-Glucaric acid (μg/dl)[a]	Mean (±S.E.)
Man	50, 48, 47, 42, 40, 37, 33, 29, 24, 20, 17, 13, 10	32±14
Rat	216, 200, 150, 121, 111, 97, 83, 55, 40	119±60

a) Values were obtained by the Procedure I reported earlier.[5]

Concerning the drug-induced stimulation of the glucuronic acid pathways in man, the serum levels of D-glucaric acid in normal human adults and epilepsy patients chronically under treatment with diphenylhydantoin and phenobarbital, both are known as potential stimulator of drug metabolism, were determined by the presented fluorometric method and the results were reported previously.[9]

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C-Glycosyl Nucleoside. III.[1] Ethynylation of Glucosyl Bromide with Ethynylmagnesium Bromide

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In the reaction of sodium acetylide with tetra-*o*-acetyl-α-D-glucosyl bromide in liquid ammonia, Zelinski and Meyer[3] obtained an unknown compound in about 4% yield having mp 183—185.5°, $[\alpha]_D^{25}$ −68.8°, and an uncharacterized syrup in about 9% yield. On the other hand, Hurd and Holysz[4,5] reported the reaction of phenyllithium with tetra-*o*-acetyl-α-

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