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Studies on the Glucaric Acid Pathway in the Metabolism of D-Glucuronic Acid in Mammals. III.[1] Determination of D-Glucaric Acid in Serum, with Special Reference to Its Level after Administration of D-Glucuronolactone and D-Glucarolactones to Man

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Serum D-glucaric acid (V) level was determined by the colorimetric method, especially after oral administration of D-glucuronolactone (I) or D-glucarolactones (II, III) to man. The highest level was observed at one hour after administration simultaneously with the lowest level of serum β-glucuronidase activity as well as the maximal β-glucuronidase inhibition of serum. *In vivo* conversion of I into β-glucuronidase inhibitor was thus demonstrated.

In the previous paper[3] a chemical (colorimetric) method for quantitative determination of D-glucaric acid (V) was reported, which was identified as normal constituent of human urine.[4a] D-Glucuronolactone (I) was found to be a direct precursor of biosynthetic V, and a new oxidative metabolic pathway of I to V in mammalian systems was demonstrated,[4] whose tentative reaction mechanism is indicated briefly in Chart 1. This paper deals with the application of the chemical assay for V to serum, particularly after administration of I and D-glucarolactones (II, III) to man which were suggested[4b,d] being involved in the conversion of I into V.

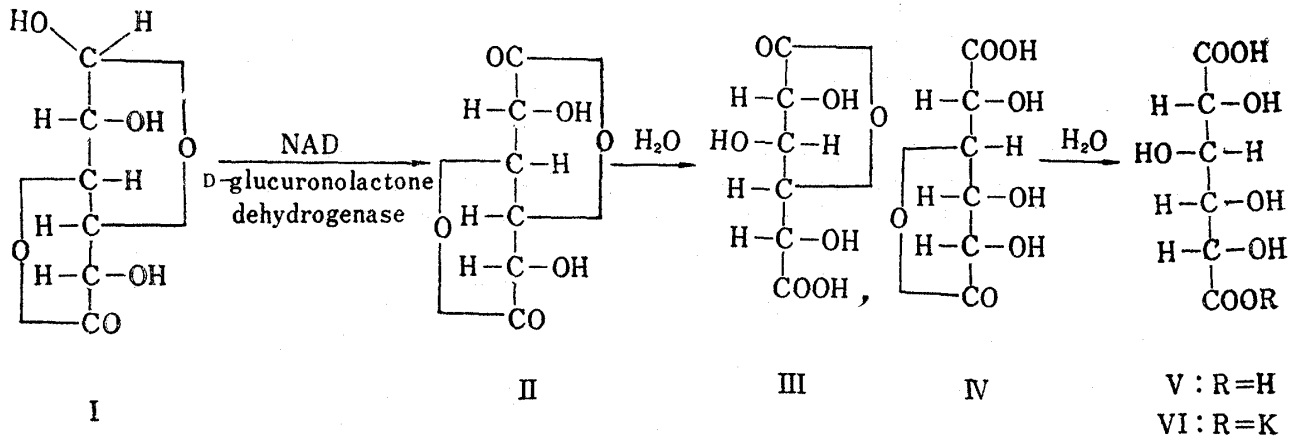

Chart 1

It has been found that the method for separation and determination of V reported earlier[3] with urine is sufficiently applicable to serum without any substantial modification. Thus, good separation of V in human or rat serum was achieved by ion-exchange column chromato-

1) Part II: M. Matsui, M. Okada, and M. Ishidate, *Chem. Pharm. Bull.* (Tokyo), **17**, 1064 (1969).
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3) M. Ishidate, M. Matsui, and M. Okada, *Anal. Biochem.*, **11**, 176 (1965).
4) *a*) C.A. Marsh, *Biochem. J.*, **86**, 77 (1963); *b*) *Idem, ibid.*, **87**, 82 (1963); *c*) *Idem, ibid.*, **89**, 108 (1963); *d*) *Idem, ibid.*, **99**, 22 (1966).

graphy using Dowex 1 X-8 borate. Subsequent colorimetric procedure for the determination of V was carried out in the same manner as described previously.[3] A chromatogram of normal human serum is shown in Fig. 1,[5] together with those of human sera obtained after oral administration of I or II. As indicated in Tables I and II, satisfactory recoveries of potassium hydrogen D-glucarate (VI), V, D-glucaro-(1→4)-lactone (III), or D-glucaro-(1→4)(6→3)-dilactone (II) added to human or rat sera were obtained with procedure I or II described below.

When a serum sample contained fairly large amounts of L-ascorbic acid or I which were known[3] to interfere with the chemical determination of V in urine, Procedure II (pretreatment of the serum sample with 2,6-dichlorophenolindophenol and sodium borohydride) should be employed, as demonstrated in Table III. In this case, however, qrocedure II using slight modification of the original one[3] was used, in which a longer

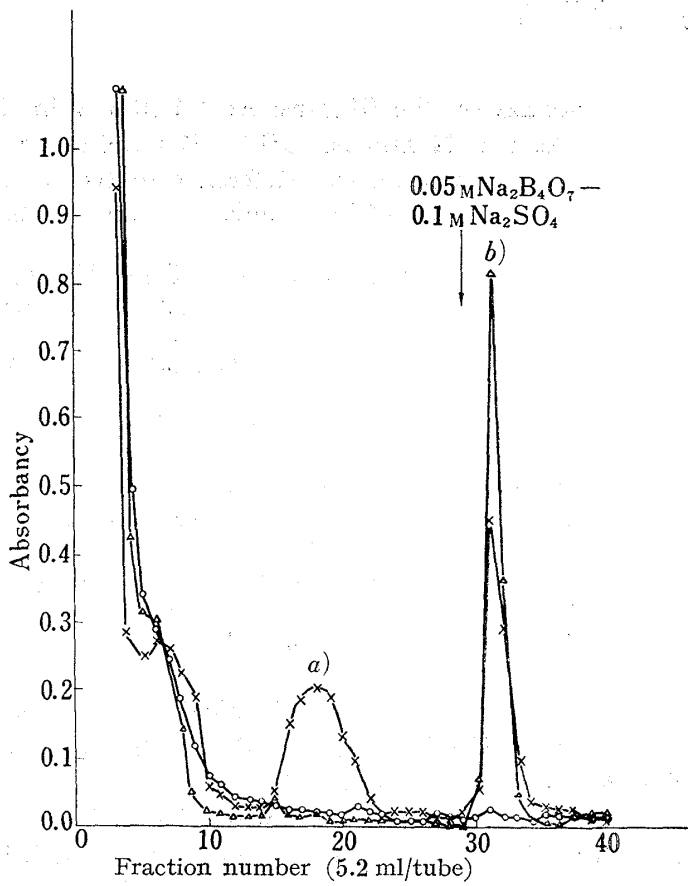

Fig. 1. Chromatography of Human Serum on Dowex 1X-8 Borate (Procedure I)

○ : normal serum (3.0 ml)
× : serum (2.0 ml) obtained at one hour after oral administration of 5g of D-glucuronolactone (I) to man
△ : serum (2.0 ml) obtained at one hour after oral administration of 1g of D-glucaro-(1→4)(6→3)-dilactone (II) to man
a) D-glucuronic acid *b*) D-glucaric acid (V)

Table I. Recovery of D-Glucaric Acid (V) after Addition of Potassium Hydrogen D-Glucarate (VI), V, D-Glucaro-(1→4)-lactone (III), or D-Glucaro-(1→4)(6→3)-dilactone (II) to Human Serum

Procedure	Compound added	D-Glucaric acid (μg/3ml) Serum	Added	Calc.	Found	Recovery (%)
I	VI	*ca.* 1.6	19.6	21.2	20.3	96
I	VI	*ca.* 3.0	84.0	87.0	85.8	99
I	V	*ca.* 1.6	490	492	457	93
I	III	*ca.* 2.0	108	110	113	103
I	II	0	101	101	96.8	96
II	VI	*ca.* 1.0	22.3	23.3	23.8	102
II	VI	*ca.* 2.4	44.6	47.0	48.1	102
II	VI	*ca.* 2.3	107	109	103	95
II	VI	*ca.* 1.0	213	214	203	94
II	III	*ca.* 1.7	28.4	30.1	27.9	93
II	III	0	121	121	117	97
II	II	0	36.4	36.4	34.4	95
II	II	0	146	146	143	98

5) An entirely similar chromatogram was obtained with rat serum, which is therefore not given in Fig. 1.

Table II. Recovery of D-Glucaric Acid (V) after Addition of Potassium Hydrogen D-Glucarate (VI) or V to Rat Serum (Procedure I)

Compound added	D-Glucaric acid (μg/ml) Serum	Added	Calc.	Found	Recovery (%)
VI	*ca.* 5.9	23.2	29.1	29.6	102
VI	*ca.* 2.9	84.0	86.9	85.1	97
V	*ca.* 5.9	92.8	98.7	98.8	100
V	*ca.* 6	464	470	437	93
V	*ca.* 2	433	435	415	96
V	*ca.* 2	865	867	766	89

time was required for the treatment of serum sample with 2,6-dichlorophenolindophenol and sodium borohydride as compared with urine sample. This could probably be due to the presence of large amounts of protein, since serum sample was used without prior deproteinization.

A comparison of two procedures, I and II, for determination of V in sera obtained after oral administration of I, III, or II to man is made in Table IV. Although no marked difference between these determinations was observed, procedure II was used exclusively for determining serum level of V in the loading experiment described below.

Table III. Effect of L-Ascorbic Acid (A) or D-Glucuronolactone (I) for Determination of D-Glucaric Acid in Human Serum

Procedure	Compound added (mg) A	I	D-Glucaric acid (mg/dl) Serum	Found
I	8.0	—	*ca.* 0.07	1.46
I	18.3	—	*ca.* 0.07	3.67
I	—	17.0	*ca.* 0.03	0.24
I	—	33.3	*ca.* 0.03	0.47
II	22.0	—	*ca.* 0.06	*ca.* 0.10
II	—	20.0	*ca.* 0.06	*ca.* 0.08
II	36.7	—	*ca.* 0.03	0.22
II	—	34.0	*ca.* 0.03	*ca.* 0.09
II	20.0	—	2.44[a]	2.57
II	—	18.3	2.44[a]	2.52

a) D-glucaric acid added to serum

Table IV. Comparison of the Two Procedures for Determination of D-Glucaric Acid in Serum after Oral Administration of D-Glucuronolactone (I), D-Glucaro-(1→4)-lactone (III), or D-Glucaro-(1→4)(6→3)-dilactone (II) to Man

Compound administered (g)	Time[a] (hr)	D-Glucaric acid (mg/dl) Procedure I	Procedure II
I (5.0)	1.5	1.66	1.38
III (5.0)	1	1.70	1.56
II (1.0)	1	2.17	2.04

a) time after oral administration of the compound to human subject

As indicated in Tables I, III, or V, as well as in Fig. 1, normal serum level of V in man (<100 μg/dl) was so low that accurate estimation of it by the colorimetric method seemed to

be almost impossible.[6] It was evident, however, that administration of I, II, or III to man resulted in a marked elevation of the serum level of V, which could be estimated by the colorimetric method without any difficulty (Fig. 1, Table IV). Then, time-course determination of the serum level of V in man was made after oral administration of these compounds.

Thus venous blood was taken from the antecubital vein from fasting normal man immediately before oral administration of I, III, or II, and blood was drawn at one, two, three, and four hour intervals thereafter. The V content of these sera was determined and is shown in Table V, together with that of 24hour urines. The serum level of V in normal man receiving I, III, or II was maximal at one hour after loading, being highest in the case of II. Incidentally, it should be noticed that the amount (1 g) of II loaded was only one fifth of that (5 g) of I or III. These results correspond very well to the previous findings[1] as to urinary excretion of V after administration of these compounds.

Table V. Serum and Urinary D-Glucaric Acid (V) Levels after Oral Administration of D-Glucuronolactone (I), D-Glucaro-(1→4)-lactone (III), or D-Glucaro-(1→4)(6→3)-dilactone (II) to Man

Subjects (sex, age)	Compound administered (g)	V in serum (mg/dl) Hours after administration 0 hr	1 hr	2 hr	3 hr	4 hr	V in urine (mg/24 hr)
M (M. 30)	I (5.0)	*ca.* 0.03	1.52	1.20	1.01	0.59	507
M (M. 30)	I (5.0)	*ca.* 0.02	1.57	1.31	1.07	0.76	526
K (M. 26)	I (5.0)	*ca.* 0.03	1.40	1.23	1.19	0.93	—
O (M. 40)	I (5.0)	*ca.* 0.03	1.57	1.70	1.24	0.93	—
M (M. 30)	III (1.0)	*ca.* 0.03	0.46	—	—	—	—
M (M. 30)	III (5.0)	*ca.* 0.02	1.56	1.20	0.78	0.46	462
O (M. 40)	III (5.0)	*ca.* 0.02	2.08	2.02	1.72	1.19	677
O (M. 40)	II (1.0)	*ca.* 0.04	3.90	2.18	1.31	0.85	844
M (M. 30)	II (1.0)	*ca.* 0.09	3.12	1.80	1.07	0.73	—
K (M. 26)	II (1.0)	*ca.* 0.03	2.30	1.63	1.37	0.98	—
K (M. 26)	II (1.0)	*ca.* 0.02	2.04	1.96	1.23	0.94	487

Fishman, *et al.*[7] had observed in 1951 that human serum β-glucuronidase (β-Gase) activity fell precipitously with a simultaneous rise in blood total (free and conjugated) glucuronic acid within one hour after oral administration of I, but without any proper comment on this interesting phenomenon, since I or D-glucuronic acid did not inhibit serum β-Gase activity. The most powerful inhibitor of the enzyme, III,[8] as well as II which has been found[9,10] to be a more potent β-Gase inhibitor *in vivo* than III, were both suggested being involved in the conversion of I into V (Chart 1). Therefore, it seemed quite reasonable to presume the presence of the β-Gase inhibitor in blood of man receiving I.[4b] The above finding of Fishman, *et al.* has now been elucidated by actual formation of the β-Gase inhibitor from I in man as follows.

Thus, the inhibitory effect of I, II, III, D-glucaro-(6→3)-lactone (IV), or V on β-Gase in human serum was examined. As shown in Fig. 2, the enzyme activity fell deeply by

6) Meanwhile, a much more highly sensitive fluorimetric method for determination of V was developed in this laboratory (M. Okada, M. Matsui, Y. Watanabe, T. Wanibe, and F. Abe, *Seikagaku*, **39**, 553 (1967)). According to this assay normal serum level of V in man was estimated to be about 30 μg/dl (10—50 μg/dl).

7) W.H. Fishman, M. Smith, D.B. Thompson, C.D. Bonner, S.C. Kasdon, and F. Homburger, *J. Clin. Invest.*, **30**, 685 (1951).

8) G.A. Levvy, *Biochem. J.*, **52**, 464 (1952).

9) S. Harigaya, *J. Biochem.* (Tokyo), **56**, 400 (1964).

10) R. Iida, S. Nagata, M. Kakimoto, H. Akaike, H. Watanabe, and A. Shioya, *Jap. J. Pharmacol.*, **15**, 88 (1965).

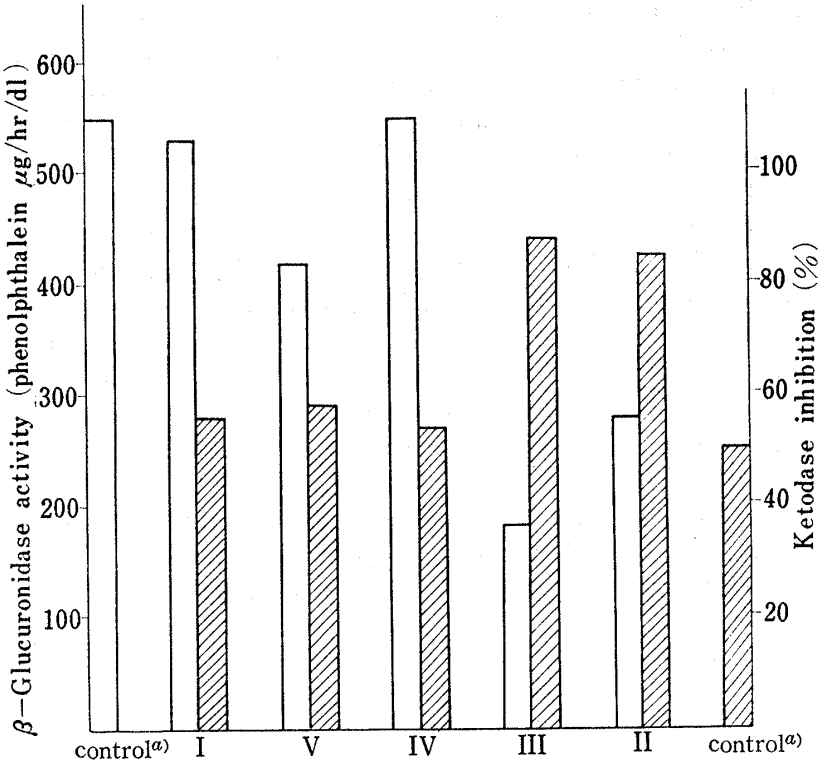

Fig. 2. β-Glucuronidase Activity and Inhibitory Effect on Ketodase of Human Serum before and after Addition of D-Glucuronolactone (I), D-Glucaric Acid (V), D-Glucaro-(6→3)-lactone (IV), D-Glucaro-(1→4)-lactone (III), or D-Glucaro-(1→4)(6→3)-dilactone (II)[b)]

□: β-glucuronidase activity ▨: ketodase inhibition
a) before addition
b) final concentration of each compound in the incubation solution: 2 μg/ml

TABLE VI. Serum D-Glucaric Acid and β-Glucuronidase Levels and Ketodase Inhibitions after Oral Administration of D-Glucuronolactone (I), D-Glucaro-(1→4)-lactone (III), or D-Glucaro-(1→4)(6→3)-dilactone (II) to Man

Subjects (sex, age)	Compound administered (g)		Hours after administration 0 hr	1 hr	2 hr	3 hr	4 hr
M (M, 30)	I (5.0)	A[a)]	*ca.* 0.02	1.57	1.31	1.07	0.76
		B[b)]	667	375	458	467	479
		C[c)]	43	75	65	62	55
K (M, 26)	I (5.0)	A	*ca.* 0.03	1.40	1.23	1.19	0.93
		B	125	42	62	78	100
		C	47	85	76	70	64
M (M, 30)	III (5.0)	A	*ca.* 0.02	1.56	1.20	0.78	0.46
		B	604	333	416	433	529
		C	59	89	82	74	72
O (M, 40)	III (5.0)	A	*ca.* 0.02	2.08	2.02	1.72	1.19
		B	750	333	520	625	708
		C	53	81	78	73	61
O (M, 40)	II (1.0)	A	*ca.* 0.04	3.90	2.18	1.31	0.85
		B	583	208	350	374	415
		C	55	81	68	63	58
K (M, 26)	II (1.0)	A	*ca.* 0.02	2.04	1.96	1.23	0.94
		B	670	241	375	479	520
		C	59	89	82	70	68

a) D-glucaric acid level (mg/dl) *b*) β-glucuronidase level (phenolphthalein, μg/hr/dl) *c*) ketodase inhibition (%)

adding III or II to the serum and to some extent by V, while almost no reduction of the activity was observed with I or IV. On the other hand, the inhibitory effect of human serum on Ketodase[11] was determined before and after addition of these compounds to the serum (Fig. 2). Human serum itself exhibited a considerable inhibitory effect on β-Gase,[12] and addition of III or II to the serum resulted in a marked intensification of the inhibitory effect, whereas addition of I, IV, or V did not practically affect it.

Following these observations β-Gase activities of the above human sera obtained before and at one, two, three, or four hour intervals after oral administration of I, III, or II were determined, while determination of the Ketodase inhibition(%) of the sera was made. These determinations are given in Table VI, together with the corresponding data of V content in the sera which were quoted from Table V. It is evident in this table that the highest level of V, the maximal Ketodase inhibition, and the lowest level of β-Gase activity are coincidentally observed at one hour after administration of these compounds, thus demonstrating the presence of the β-Gase inhibitor (III or II) in the highest concentration at this time so far examined in the loading experiment. It could be concluded further from the above result with I that the exogenous I is actually converted into the β-Gase inhibitor *in vivo*, which could play an important role in controlling glucuronide hydrolysis, although no conclusive evidence of this is yet available.

Materials and Methods

Reagents and Chemicals——Ion-exchange resin (Dowex 1 X-8 borate, 200—400 mesh) used for the separation of D-glucaric acid (V) as well as reagents for the colorimetric determination of V were prepared as reported earlier.[3] Potassium hydrogen D-glucarate (VI) was prepared according to the method described by Mehltretter.[13] D-Glucaro-(1→4)-lactone (monohydrate) (III) and D-glucaro-(6→3)-lactone (IV) were prepared as described by Bose, *et al.*[3,14] D-Glucuronolactone (I), D-glucaro-(1→4)(6→3)-dilactone (II), V, and phenolphthalein mono-β-D-glucuronic acid were obtained from Chugai Pharmaceutical Co., Ltd.

Serum and Urine Samples——Human sera were collected from normal healthy personnel in this laboratory. Venous blood was taken from the antecubital vein as described in the main text before and after oral administration of I, III, or II, and simultaneous collection of 24-hour urine samples was made after administration of these compounds. I and III (5 g) were dissolved in water (100 ml) just before administration, while II was given as powder and taken down with cold water, because it is unstable in aqueous solution.[15] From rats (Donryu) maintained on normal diets, blood was taken from the abdominal aorta after the laparotomy under ether anesthesia. Blood was allowed to clot, and the serum taken off as soon as possible. The serum was again centrifuged in a conical tube to ensure that all cells were removed.

Separation and Determination of Serum D-Glucaric Acid (V)——a) Procedure I: To 3.0 ml (/or 2.0 ml) and 1.0 ml of human or rat serum, respectively, an equal volume of 0.1M sodium borate was added. After this mixture had stood for 15 min at 20°, it was put on a column of Dowex 1 X-8 borate described earlier.[3] Subsequent elution and colorimetric procedure were the same as reported with urine.[3] A chromatogram of normal human serum is shown in Fig. 1, together with those of human sera taken after oral administration of 1 g of II or 5 g of I.

b) Proceudre II (Pretreatment of Serum with 2,6-Dichlorophenolindophenol and $NaBH_4$): To the sodium borate–added serum an excess of 0.2% aqueous solution of 2,6-dichlorophenolindophenol was added, and 1 hr later 50 mg of $NaBH_4$ was added. After the mixture had stood for 1 hr at 20°, 1 ml of acetone was added to decompose any remaining $NaBH_4$. After 20 min the serum solution was put on the column and treated as described in procedure I.

11) β-Glucuronidase preparation derived from beef liver:Warner-Chilcott Laboratories, Morris Plains, N.J., U.S.A.
12) W.H. Fishman, K.I. Altman, and B. Springer, *Fed. Proc.*, 7, 154 (1948); R.E. Dohrmann and H.J. Uhles, *Klin. Wschr.*, **41**, 527 (1963).
13) C.L. Mehltretter, "Methods in Carbohydrate Chemistry," Vol. II, ed. by R.L. Whistler and M.L. Wolfrom, Academic Press, New York, 1963, p. 46.
14) R.J. Bose, T.L. Hullar, B.A. Lewis, and F. Smith, *J. Org. Chem.*, **26**, 1300 (1961).
15) Y. Hirasaka and K. Umemoto, *Chem. Pharm. Bull.* (Tokyo), **13**, 325 (1965); Y. Hirasaka, K. Umemoto, M. Sukegawa, and I. Matsunaga, *ibid.*, **13**, 677 (1965).

Recovery of V Added to Serum——Recoveries of VI, V, III, or II added to human and rat sera were obtained with Procedure I or II as shown in Table I and II, respectively. Recoveries were calculated as reported previously with urine.[3]

Serum β-Glucuronidase Aassy——This was done according to the method of Fishman, *et al.*[16] using phenolphthalein mono-β-D-glucosiduronic acid (PMG) as a substrate, but deproteinization steps involved in the original procedure were avoided.[17] The activity was expressed in Fishman units, 1 unit being that which liberates 1 μg of phenolphthalein from 0.001M substrate per hour at 37°. All results were expressed in units/dl serum (μg/hr/dl).

Determination of the Ketodase Inhibition (%) of Serum——Two stoppered tubes containing 0.3 ml 0.2M acetate buffer (pH 4.5), 0.1 ml serum (before and after adding I, II, III, IV, or V), 0.05 ml 0.01M PMG and 0.2 ml Ketodase solution were incubated at 37° for 30 min. A blank tube containing the acetate buffer, PMG, Ketodase solution and distilled water (0.1 ml) instead of serum was also incubated. After 30 min incubation the liberated phenolphthalein was determined in a similar way as in the serum β-glucuronidase assay. The Ketodase inhibition (%) was calculated as follows:

$$\text{Ketodase inhibition } (\%) = \frac{C-I}{C} \times 100$$

C: Ketodase activity determined in control
I: Ketodase activity observed in the presence of serum or serum containing inhibitor

16) P. Talalay, W.H. Fishman, and C. Huggins, *J. Biol. Chem.*, **116**, 757 (1946); W.H. Fishman, B. Springer, and R. Brunetti, *ibid.*, **173**, 449 (1948).
17) W.H. Fishman, "Methods of Biochemical Analysis," Vol. 15, ed. by D. Glick, Interscience Publishers, New York, 1967, p. 77.