IDENTIFICATION OF DONAGGIO REACTION POSITIVE SUBSTANCES AND INCREASE OF α_1 -ACID GLYCOPROTEIN IN URINE AFTER EXHAUSTIVE EXERCISE

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The Donaggio reaction positive substances from the urine after hard work were identified.

These substances obtained from deproteinized urine by heat at pH 5.0 and having the high titer of Donaggio reaction were composed of α -glycoproteins, which were shown to be α_1 -globulin and small amounts of α_2 -globulin by means of cellulose-acetate electrophoresis.

This α_1 -globulin nearly composed of serum α_1 -acid glycoprotein and one of the α_2 -globulin was composed of serum α_2 -HS globulin, both of which were shown by immuno double diffusion and by single radial immunodiffusion. The results of immunodiffusion indicated that the other α_1 -glycoproteins were not contained, such as tryptophan-poor α_1 -glycoprotein, α_1 -antitrypsin, and α_1 -antichymotrypsin.

The levels of α_1 -acid glycoprotein in urine before and after running, which were measured by single radial immunodiffusion, paralleled the Donaggio-titer.

Serum mucoprotein purified by perchloric acid from human serum contained Donaggio positive substances.

From these results, we can conclude that Donaggio positive substances were composed of at least two sorts of mucoprotein, α_1 -acid glycoprotein as a major part and α_2 -HS globulin as a minor.

It is well known Donaggio titer keeps high level in the workers' urine working in the industry after hard work, especially under high temperature and high humidity circumstances.¹⁾ On the other hand, it has been also well known that amounts of urinary protein are increased by hard physical work as reported by Poortmans²⁾ and Suganami.³⁾ Tanabe⁴⁾ reported that urinary Donaggio titer was derived from high blood pressure, acidosis and excretion of adrenal hormone.

Many reports have been published on Donaggio reaction positive substances which inhibited the precipitation yielded by mixing thionine with ammonium molybdate⁵⁾. Tayeau⁶⁾ and Yamazoe⁷⁾ purified Donaggio reaction positive substances by using ethanol, and proved that the Donaggio reaction positive substances contained sugar and amino acid derivatives. Shimokawa⁸⁾ firstly reported that Donaggio reaction positive substances were mucoprotein

because of having non-dialysabiltic and heat stabiltic properties.

Ogata *et al*⁹⁾ in 1958 demonstrated that these substances have similar characteristics to α -globlin in human serum which was shown by paper-electrophoresis at pH 8.5, and pH 3.4. Kamiyama *et al*¹⁰⁾ in 1972 further suggested that this substance was α_1 -acid glycoprotein by immuno-electrophoresis.

Many investigations have been reported that urinary protein (albumin, α_1 -AG, Zn- α_2 -glycoprotein, transferrin, and IgG) markedly increased by hard work as described by Poortmans.¹¹⁾

The present study was undertaken to identify the Donaggio positive substances by single radial immuno diffusion with specific antibody of human serum of α_1 and α_2 glycoprotein fractions.

Further, we measured titer of Donaggio reaction of purified serum mucoprotein obtained from the supernatant after addition of perchloric acid.

MATERIALS AND METHODS

Anti human serum-rabbit serum was obtained from Behring-werke (Western Germany).

Urine of atheletic marathon runners of 17 or 18 years of age were collected before and after hard work. The condition of physical exercise was the running of 15,000 meter for 2hr.

Then, urine of the other five high school students (17 years of age), exercised training running machine (Run-Run, National electric Co.) 1hr at a speed rate of 210 step/min (Calculated RMR is about 6) at 17°C, were also collected.

Collection of urine

The runners were instructed to collect a half hour urine after the end of exercise. One ml of 5% sodium azide was added into the each urine to conserve.

Donaggio reaction

Both the above mentioned urine and control urine (rest urine) were boiled in water bath at 100°C over a flame for 10 min, acidified as to pH 5.0 by the addition of acetic acid. Details of the Donaggio reaction had been reported by Sato¹²⁾ in 1949.

Total protein

Determination of urinary total protein was performed by Kingsbury-Clark method.¹³⁾ Some of the samples were also determined by Lowry's method¹⁴⁾ to confirm.

Concentration and lyophilization

Urine was dialysed and concentrated to about 12-fold by single and multiple hollow fiber concentrator (MDA SCIENTIFIC. INC). Then, the urine samples were lyophilized.

Cellulose-acetate membrane electrophoresis

The dialized, condensed and lyophilized urine was dissolved and diluted to 40-fold

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with pure water. Then, it was spotted on a cellulose acetate membrane and applied for electrophoresis¹⁵⁾ at 0.8 mA/cm, for 45 min. After strained with Ponso-3R, it was analysed by densitometry (Jōkō Sangyo Co DENSITRON 20 M). Simultaneously standard sample of human serum (Behring standard human serum Batch-B 975) was treated by the same method to compare urine with serum.

Double diffusion method

Qualitative determination of the protein in concentrated urines was performed according to the original technique of Ouchterlony¹⁶⁾. Anti subfraction of serumprotein rabbit serum, such as albumin, α_1 -acid glycoprotein (α_1 AG), α_1 -antitrypsin (α_1 AT), α_1 -anti-chymotrypsin (α_1 X), Zn binding α_2 -glycoprotein (Zn α_2), α_2 -macroglobulin, α_2 -HS globulin (α_2 HS), immunoglobulin G (IgG) were determined. These antiserum were poured into peripheral well on agar slide. And urine samples deproteinized by heat at pH 5.0, into central well. After 12 hr at room temperature these precipitated lines are observed.

Single radial immuno diffusion technique (SRID)

Quantitative determination of the Donaggio reaction positive mucoproteins in concentrated urine was performed according to the original technique of Mancini *et al.*¹⁷⁾

2% agar-gel (Difco) is prepared by melting in a boiling water-bath and cooling down to 60°C. The antiserum made in barbiturate buffer, is brought to 55°C, after which equal volumes of both solutions are mixed. 5 ml of antiserum-agar mixture were overlaid on microscopic slideglass, 7.5 by 5.5 cm. The antiserum-agar solution set almost immediately and the slides were removed to a moist chamber at 4°C to firm slowly. Eight circular wells were punched in a line along the long axis of the slide, and the agar in the wells was removed by gentle suction. Dissolved urines (2 μ l) were placed on the wells by filling them to flatness. These agar slides were removed to a moist chamber at room temperature (20°~25°C) and after about 12 hr ring shaped precipitates were measured. A standard curve was constructed the diameter of each standard serum to the second was plotted on Y axis, against its concentration on X axis. And these concentration could be determined by the length of the diameter.

Fractionation of human α -mucoproteins (Winzler's mucoprotein¹⁸⁾)

To remove the other proteins, 200 ml of serum were diluted with an equal volume of water, and 200 ml of 1.8 M perchloric acid were added, admixing. Filtrating procedure through a Whatman No. 5 filter paper was started within 2 or 5 min after protein precipitation by perchloric acid. The filtrate was dialyzed against water until acid free state. The mucoprotein in the dialysate were precipitated by saturating with ammonium sulfate at a pH of 4.0. The precipitated materials were exhaustively dialyzed against distilled water.

The dialyzed solution was used as materials for the determination of Donaggio reaction by Sato's method. α_1 -AG concentration was measured by SRID.

RESULTS

Changes of Donaggio titer and protein concentration after deproteinization by heat and acid treatments

Titer of Donaggio reaction is decreased from 50 dilution unit to 22 dilution unit (44 %) by deproteinization procedure. Total protein concentration is decreased much more than that of 57 mg/dl to 17 mg/dl (30 %) by the same procedure, suggesting that glycoprotein still remained after deproteinization.

Cellulose-acetate electrophoresis

The urine was condensed to 40-fold by deproteinization, then it was analysed by the same method as serum. It was observed that densitometric curve of untreated urine after exercise on oxide paper was almost similar to that of blood serum. On the other

Fig. 1. Densitometric curve after cellulose-acetate electrophoresis.

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hand, main components of exercise urine after being treated were observed located above α_1 and α_2 globulin regions as shown in Fig. 1.

Double diffusion

Using anti α_1 -AG or α_2 -HS serum, it was observed that precipitine line existed between the treated urine at 100°C for 10 min at pH 5.0 and the antiserum as shown in Fig. 2. No precipitine line due to the other anti α_1 -globulin serum, such as α_1 -T, α_1 -AT or α_1 -X in urine was observed.

Single radial immuno-diffusion (SRID)

SRID was performed to measure the quantity of α_1 -AG and α_2 -HS in the deproteinized

Fig. 2. Schema of double immunodiffusion of exercised urine (EXU), and boiled acidified urine (BAU), were allowed to precipitate with human α_1 -AG and α_2 -HS specific antisera. $\times 10C$; concentrated 10 times, $\times 20C$; concentrated 20 times.

Table 1. Analytical results of Donaggio positive substances purified from urine.

	Method	Unit	NU ¹⁾	EXU ²⁾	BAU (pH 5) ³⁾
Donaggio reaction	Sato-standard method	D.U. ⁴⁾	4	50	22
Total protein	Kingsbury-Clark method	mg/dl	10	57	17
Albumin			n.d. ⁵⁾	45	n.d.
α_1 -AG			n.d.	6.8	4.2
Globulin α_2 -HS	SRID ⁶⁾	mg/dl	n.d.	1.5	0.7
IgG			n.d.	4.6	n.d.
Donaggio titer/ α_1 AG ⁷⁾		D.U./mg	—	7.34	5.24

¹⁾ NU: Normal urine, ²⁾ EXU: exercised urine, ³⁾ BAU: being boiled with acid treated exercised urine, ⁴⁾ D.U.: Dilution Unit, ⁵⁾ n.d.: non detectable, ⁶⁾ SRID: single radial immuno-diffusion, ⁷⁾ Donaggio titer/ α_1 -AG: ratio of Donaggio titer to α_1 -acid glycoprotein concentration.

urine as shown in Fig. 3. The levels of α_1 -AG and α_2 -HS were slightly decreased by deproteinization, comparing with untreated urine. The level of α_1 -AG was as much as 5~6 times that of α_2 -HS.

The concentration of α_1 -acid glycoprotein in urine was slightly increased after exercise in measuring its quantity by SRID.

Albumin and IgG could not be found in deproteinized urine. Perhaps these might have been obliterated by the heat treatment at pH 5.0.

The results are summarized in Table 1.

Change in Donaggio titer and concentration of urinary α_1 -acid glycoprotein after exercise

Increase in the titer of Donaggio reaction on urine after marathon running paralleled

Fig. 3. Schema of diffusion rings obtained in the human α_1 -AG and α_2 -HS antibody-agar plate with normal urine, exercised urine and boiled exercised urine.

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Table 2. Change in the levels of Donaggio-titer and urinary α_1 -acid glycoprotein after marathon running.

	No. of subject unit	1		2		3		4	
		bef. ¹⁾	aft. ²⁾	bef.	aft.	bef.	aft.	bef.	aft.
Donaggio-titer	D.U. ³⁾	—	20	5	12	2	20	2	38
α_1 -AG	mg/dl	—	3.4	n.d.	2.1	n.d.	5.8	n.d.	10.0
Donaggio titer/ α_1 AG	D.U./mg	—	5.9 ⁵⁾	—	5.7 ⁵⁾	—	3.5 ⁵⁾	—	3.8 ⁵⁾

¹⁾ bef.: before exercise, ²⁾ aft.: after exercise, ³⁾ D.U.: Dilution unit by Donaggio-reaction.

⁴⁾ n.d.: non detectable, ⁵⁾ Donaggio titer/ α_1 -AG=4.7 $\pm$ 1.1 (m $\pm$ SD).

with the concentration of urinary acid glycoprotein as shown in Table. 2.

Donaggio titer and α_1 -acid glycoprotein concentration of Wintzler's serum glycoprotein

Purified Wintzler's serum glycoprotein showed 24 dilution units of Donaggio titer, 46.0 mg/dl of protein concentration by Lowry's method¹⁴⁾ and 11.6 mg/dl of α_1 -AG by SRID. The results suggest that the titer of Donaggio reaction is 2.07 unit/ α_1 -AG mg.

Serum albumin showed positive Donaggio reaction¹⁹⁾ but the serum albumin was obliterated by the heat deproteinization at pH 5.0 as shown in electrophoretic pattern Fig. 1. Therefore, the serum albumin was not involved in Donaggio reaction (Table 1). IgG was almost negative in Donaggio reaction¹⁹⁾ and was also obliterated by the heat deproteinization.

These data indicate that α_1 -AG seems to be the major component and α_2 -HS the minor component of Donaggio reaction positive substances.

DISCUSSION

The results of the present experiment showed that urine was deproteinized by heat at pH 5.0 and titer could be measured by Satō's standard method.

Data of the present experiment also indicate that the increasing of the titer of Donaggio reaction in the urine after exercise parallels with the increase in the amount of α_1 -acid glycoprotein. The results also agree with our assumption that α_1 -acid glycoprotein is the major Donaggio reaction positive substances by immunochemical method.

The results that the serum mucoprotein purified by Wintzler's method yields a high titer of Donaggio reaction indicate that α_1 -acid glycoprotein might be also the main Donaggio reaction positive components.

It may be added that this mucoprotein is composed of 3 subfractions as demonstrated by electrophoresis at pH 4.5¹⁸⁾. The relationship between Donaggio reaction positive protein and these three subfractions should be clarified by future experiments. From the results that urinary α_1 -AG increased after exercise in parallel with Donaggio titer in this experiment. It is inferred that the determination of urinary α_1 -AG can be done by Donaggio reaction.

REFERENCE

- 1) Satō, S. (1956). *J. Sci. Labour*, **32**, 135. (in Japanese)
- 2) Poortmans, J. (1969). *Med. Sport*, **3**, 312.
- 3) Suganami, S. (1976). *J. Phys. Fitness Jap.*, **25**, 64. (in Japanese)
- 4) Tanabe, S. (1959). *Okayama Med. J.*, **71**, 6463. (in Japanese)
- 5) A. Donaggio, D. (1933). *Bull. Soc. Ital. Biol. Sper.*, **8**, 1456.
- 6) Tayeau, F. (1954). *Exposes Annuels de Biochimie Medicale*, **16**, 215.
- 7) Yamazoe, S. (1956). *Igaku no Ayumi*, **21**, 303. (in Japanese)
- 8) Shimokawa, S. (1953). *J. Physiol. Soc. Jap.*, **15**, 636. (in Japanese)
- 9) Ogata, M. and Mochizuki, Y. (1958). *Jap. J. Hyg.* **13**, 488. (in Japanese)
- 10) Kamiyama, S. and Michioka, O. (1972). *Jap. J. Ind. Health* **19**, 488. (in Japanese)
- 11) Poortmans, J. and Jeanloz, R.W. (1968). *J. Clin. Invest.*, **47**, 386.
- 12) Satō, T. (1945). *Seitai no Kagaku*, **1**, 37. (in Japanese)
- 13) Kingsbury, F.B., Clark, C.P., Williams, G. and Post, A.L. (1926). *J. Lab. Clin. Med.*, **11**, 981.
- 14) Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). *J. Biol. Chem.*, **193**, 265.
- 15) Kohn, J. (1957). *Clin. Chem. Acta.* **2**, 297.
- 16) Ouchterlony, O. (1949). *The Lancet*, 346.
- 17) Mancini, G., Carbonare, A.O. and Heremans, J.F. (1965). *Immunochemistry*, **2**, 325.
- 18) Winzler, R.J., Doror, A.W., Mehl, J.W. and Smyth, I.M. (1948). *J. Clin. Invest.*, **27**, 609.
- 19) Ogata, M. and Mochizuki, Y. (1958). *Jap. J. Hyg.*, **13**, 188. (in Japanese)