Biochemical Studies on "Miso", Fermented Soy-been Paste. Part IV.

On the Effect of Cystine upon the Nutritive Value of "Miso-protein" when fed to Albino Rats as a Supplement to Rice.

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INTRODUCTION

The author⁽¹⁾ has recently found that an adequate amount of cystine increases the supplementary nutritive value of "Miso-protein", but the amount to be given largely depends upon the age of the animal, namely, for young rats, until sexual maturity, the most satisfactory amount was found to about 0.1% of the diet.

According to A. C. Curtis, L. H. Newbrough and F. H. Thomas⁽²⁾, rats fed on 18% casein diet which supplied 0.047% free cystine grew well. As the cystine content of casein was found to be 0.17%⁽³⁾, the diet contained 0.078% cystine and this diet was favorable to healthy growth. Similarly, cystine accounting to 0.43% of food protein may favorably influence the growth and nitrogen retention of the rat. According to the experiment of U. Suzuki, Y. Okuda and Y. Matsuyama⁽⁴⁾ the rats receiving a 10~18% cod-fish protein diet, exhibited very good growth. As the cystine content of the protein was reported to be 0.67%⁽⁵⁾, an adequate amount of cystine was calculated to be 0.06~0.121% of the diet, or 0.61~0.67% of the protein.

In the preceeding experiment, however, young rats fed on the "Miso" diet have improved in rate of growth, so it may be held that "Miso" contains some cystine. Since the animals were restricted to the diets containing about 10% protein, in order to reduce the amount of NaCl, it is probable that the diets were deficient in cystine, but it is uncertain whether or not the "Miso-protein" is qualitatively poor in cystine.

From this point of view, it is very important to estimate the cystine in "Miso", owing to the caramelized brown colour, which developed during the fermentation of "Miso", its presence remains to be proved. Fortunately, the present author observed that the characteristic brown color is hydrolysed with concentrated hydrochloric acid and then can be easily decolorized with a small amount of charcoal. Hereupon, Y. Okuda's method⁽⁶⁾ for the estimation of free cystine was applied to this case and the result was compared with Folin and Looney's method⁽⁷⁾⁽⁸⁾.

During the feeding experiment it was found that the urine of the rats receiving cystine diet gave a positive Folin and looney's reaction, which seemed to indicate the presence of a large amount of cystine.

The purpose of the present investigation was to ascertain the absolute amount of cystine in the "Miso" diets as well as the adequacy of cystin in the "Miso-protein". Moreover, in order to determine whether any of the primary constituents were responsible for the amount of cystine administration, cystine in urine, kidneys and liver has been colorimetrically estimated.

EXPERIMENTAL.

I. Determination of Cystine in "Miso".

(A) Y. Okuda's method: —

For acid hydrolysis, 25 g of "Miso" freshly drawn were boiled for 12 ~ 20 hours with 125 c.c. of concentrated hydrochloric acid. This was then filtered by a nutsche, washed with hot water, made up to 250 c.c. and used for determination. The following reagents were used in the experiment.

- (1) 5% of KI.
- (2) exactly 4% HCl.
- (3) 20% NaOH.
- (4) M/300 KIO_3 ; 2.14 g of potassium iodate were dissolved in 3 l. of exactly 2% HCl.
- (5) Cystine solution in 2% HCl; 1 c.c. of the solution should be contain 1 mg. of 1-cystine.

To 25 c.c. (or 35 ~ 40 c.c.) of the sample, were added 10 c.c. of a standard cystine solution and 20% NaOH, and brought up to 50 c.c. with water. After ascertaining the solution to be 4.0% HCl, 0.5 g of Merck's pure animal charcoal were added. The solution was then boiled for exactly one minute and allowed to stand for 10 minutes. Then it was filtered by suction, washed with about 5 c.c. of hot water, and after cooling to 20, the solution was reduced by 0.5 g of zine powder, and shaken several times during a period of 30 minutes. The filtrate was filled up to 100 c.c., well mixed and after confirming the fact that the solution was exactly 2% HCl, 20 c.c. of the hydrolysate was poured into a dry Erlenmeyer flask and to the mixture was added 5 c.c. of both (1) and (2). Immediately, it was titrated by M/300 of potassium iodate until the yellow color fades in exactly one minute. The filtration should place in exactly two minutes, as the color starts to increase with the lapse of time.

It is important to note that cystine is more or less adsorbed by charcoal. Accordingly, the reading was revised by subtracting the standard from unknown plus standard. All steps in the determination should be carried out in the same manner both standard and unknown plus standard. As a differ-

ence of the temperature of the solution on titration considerably influences the amount of potassium iodate which acts on cystine, c.c. of M/300 of potassium iodate was obtained as follows :

0.0098 g of cystine is equivalent to 4.55 c.c. of M/300 of KIO_3 at 23° , then
 cystine (g) = $0.0098/4.55 \times Q$
 = $0.00216 \times Q$

Similarly, 0.0098 g of cystine is equivalent to 5.10 c.c. of M/300 of KIO_3 at 25° , and then cystine (g) = $0.0098/5.1 \times Q$
 = $0.0019 \times Q$ $Q \dots \dots$ Reading

(B) Folin and Looney's Method : —

In the presence of sodium sulfite, cystine produces a deep blue color with phospho-tungstic acid. Though other reducing substances give the same color with tungstic acid, with sodium sulfite the color is not changed. Therefore, the amount of cystine was colorimetrically estimated by the increase of the color intensity. For estimation the following reagents were used. : —

(1) Standard cystine solution ; in 1 c.c. of 5% sulphuric acid contains 0.5 mg cystine.

(2) Standard sodium carbonate solution.

(3) 20% of sodium sulfite.

(4) 20% of lithium sulfate.

(5) Folin and Deni's uric acid reagent ; to 80 c.c. of 85% phosphoric acid (S.G.=1.17) were added 100 g of sodium phospho-tungstate and 700 c.c. of water. This was boiled in a water-bath for 20 hours with a reversible color, and after cooling, filled up to 1 L.

For the determination, three 100 c.c. volumetric flask were required. In the first volumetric flask 1 c.c. of standard solution containing 0.5 mg of cystine, 20 c.c. of standard sodium carbonate, 10 c.c. of 20% sodium sulfite, and 1 c.c. of 20% lithium sulfate were added and the solution was well mixed. In the second and in the third flask 1~10 c.c. of the sample were placed and then treated in the same manner as above, but in the third flask the sodium sulfite solution was not added. Now to each flask was added 3 c.c. of uric reagent, mixed by shaking vigorously, and let to stand for exactly five minutes. Subsequently, these solutions were filled up to the mark with water, and the readings were taken, within 8 minutes (room temperature at $10 \sim 16$) after adding uric reagent and keeping the standard at 20 mm of Duboscq colorimeter. It was clear that the reading of the unknown should be taken within the range of from $13 \sim 30$ mm, Since the greater difference of reading between the standard and the unknown the greater the discrepancy. The determination adopted for 25 c.c. of "Shiromiso" hydrolysate was as follows :

(1) 0.25 mg of standard cystine set at 20 mm.

(2) Reading of 2.5 c.c. of the sample with sodium sulfite was 13.9 mm,

then cystine (mg) = $20 \times 0.25/13.9 = 0.36$.

(3) Reading of 2.5 c.c. of the sample was 26.9 mm. then cystine (mg) = $20 \times 0.25/26.0 = 0.192$, therefore, the cystine in 2.5 c.c. of the sample = $0.36 - 0.192 = 0.168$ mg. The cystine content of a different kind of "Miso" is as follows :

TABLE I. Cystine Content in "Miso"

"Miso"	Time required for hydrolysis	Water	Cystine by Okuda's method	Cystine by Folin-Looney's method
"Shiromiso"*	12	50.26	67.4	71.5
	20	50.26	61.0	68.7
	Average	50.26	64.2	70.1
"Yedomiso"*	12	45.20	42.7	85.6
	20	45.20	48.0	84.8
	Average	45.20	45.4	85.2
"Sendaimiso"*	12	50.13	49.4	86.6
	20	50.13	52.3	87.0
	Average	50.13	50.9	86.8
"Hatchomiso"***	12	41.85	98.3	103.2
	20	41.85	88.5	115.5
	Average	41.85	93.4	109.9

(Cystine mg in 100 g of fresh substance.)

*.....Preparation of the Suehiro-miso Manufacturing Co.

***.....Preparation of the Iseya-miso Manufacturing Co.

II. Determination of Cystine in Polished Rice and Soy-beans.

Both to 25 g of polished rice and to 10 g of soy-beans were added six times the amount of concentrated hydrochloric acid. This was boiled for 20 hours on a sand-bath, filtered by suction, washed with hot water, and the former filled up to 500 c.c. the latter to 250 c.c.

The amount of cystine determined by Okuda's and by Folin and Looney's methods is then shown in the following table :

TABLE II. Cystine Content of Polished Rice and Soy-beans.

(Cystine mg in 100 g of substance)

	Water	Cystine by Okuda's method	Cystine by Folin-Looney's method
Polished rice used for Preparation of "Miso"	9.84	50.4	66.0
Polished rice used for the experiment of Vitamin B	10.64	37.8	59.3

Polished rice used for the feeding exp. of "Miso"	8.25	55.2	83.6
Soy-beans used for preparation of "Miso"	10.5	271.5	277.8
25 g Polished rice + 25 g Soy-beans	—	80.7	100.4

From cystine contents in "Miso" and polished rice determined by Okuda's method, the amount of cystine to be contained in the "Miso" diets, which were used in the preceeding experiment, was calculated :

TABLE III. Amount of Cystine in Diets.

	"Yedomiso" diet	"Sendaimiso" diet	"Hatchomiso" diet
"Miso" in 100 g of the diet.	40.2	39.3	22.4
Cystine (mg) in the above "Miso".	18.3	20.0	20.9
Polished rice in 100 g of the diet.	73.2	73.2	73.2
Cystine (mg) in the above Polished rice.	43.7	43.7	43.7
Total cystine (mg)	62.0	63.7	64.6

As indicated in the above table, it was found that "yedomiso", "Sendaimiso" and "hatchomiso" diets contained cystine in the proportion of 62.0, 63.7 and 64.6 mg respectively, amounting to 0.062~0.065% of the diet, or to 0.57 % of the protein. Based on the fact that the cystine content of the diet should be sufficient for the normal growth of white rats at 0.43~0.67% of the diet-protein, or that 0.037—0.121 g of cystine in 100 g of diet shows good growth, it may be justifiable conclusion that polished rice with "miso" alone gave a relatively poor growth, which may have been largely due to the impossibility of eating a sufficient amount of cystine rather than to the defectiveness of the protein constituents, since each diet consisted of 10.76% protein.

III. Excretion and Deposition of Cystine in Animals on a Cystine Diet.

Considering the fact that the urine of rats fed on the "Miso" diet supplemented by free cystine were strongly positive on Folin-Looney's reaction, the urine was collected by putting an enamelled plate covering with copper net, in the cage of the young rats. After 24 hours the urine was weighed, filtered, made up to 100 c.c. with water, after which 10 c.c. were used for Folin-Looney's colorimetric determination. Although this reaction is not specific for free cystine it is probable that dialanyl-cystine, glutathion⁽¹⁰⁾, insulin⁽¹¹⁾, ergothioneine⁽¹²⁾, and a solution of wool in HCl⁽¹³⁾, the presence of an appreciable amount of cystine or allied substances. Hence, the results of the determi-

nation were indicated as Folin-Looney's value being expressed by cystine mg. Concerning the presence of thiocyanic acid¹⁴, a number of specimens of the urine gave a negative reaction.

TABLE IV. Folin-Looney's Value of the Urine of Rats fed on the "Miso" Diets.

Experimental diet	Number of rat	Total body wts. (g)	Urine exc. per day (g)	Cystine exc. per day (mg)	Cystine exc. per 100 g of body wts. (mg)	NaCl exc. per day (g)	NaCl exc. per day per 100 g body wts (g)
"Yedomiso"	3	304	30.1	1.1	0.36	1.05	0.34
"Yedomiso" + 0.5% Cystine	3	393	35.8	1.8	0.50	1.29	0.33
"Sendaimiso"	3	286	28.6	0.9	0.31	0.91	0.32
"Sendaimiso" + 0.1% Cystine	3	401	30.8	1.4	0.35	1.38	0.34
"Sendaimiso" + 0.5% Cystine	3	445	41.6	1.5	0.34	1.44	0.32
"Sendaimiso" + 1.0% Cystine	3	333	40.9	4.5	1.35	1.19	0.36
"Hatchomiso"	3	302	35.0	1.5	0.50	1.05	0.35
"Hatchomiso" + 0.5% Cystine	3	418	45.7	2.6	0.63	1.39	0.33
A. Patrogen	2	400	25.0	0.4	0.10	0.09	0.02
B. Patrogen	2	438	28.0	0.5	0.11	0.12	0.03

As indicated in Table IV it was shown that Folin-Looney's value of the urine was much influenced by the amount of cystine administrated to the rats, their value being estimated as follows:

"Miso" diets were 0.31~0.50 (cystine mg excreted per day per 100 g of body weight), "Miso" plus 0.5% cystine 0.34~0.62, and "Miso" plus 1.0% cystine 1.35 mg while Patrogen, 8.5 g of which was added per 100 g of body weight, was 0.10~0.11 mg.

The amount of NaCl excreted per 100 g of body weight was also determined, rats placed on a "Miso" diet being found to have excreted 0.32~0.36 g per day while those rats fed on Patrogen 0.02~0.03 g.

Further it was found that in cases of cystinuria the amount of cystine in kidneys and liver was abnormally high and was proportional to the hypertrophy of the kidneys, probably due either to the increased administration of cystine or to the excess of NaCl. The result of the determination of cystine

was indicated in the following table.

TABLE V. Folin-Looney's Value of Kidneys and Livers.

Experimental diet	No. of rat	Final body wts (g)	Kidneys (g)	Cystine in Kidneys (mg)	Liver (g)	Cystine in liver (mg)
Polished rice + "Yedomiso"	53	99	1.21	—	5.25	—
	54	108	0.75	0.58	3.87	1.05
	55	122	0.80	0.66	4.16	1.38
	Average	109.7	0.78	0.62	4.42	1.22
Polished rice + "Yedomiso" + 0.5% cystine	56	127	1.15	0.70	4.92	—
	57	133	1.17	0.67	5.05	1.48
	58	142	1.63	0.81	5.31	1.50
	Average	134	1.32	0.73	5.06	1.49
Polished rice + "Sendaimiso"	59	111	0.90	—	3.98	—
	60	115	0.70	0.65	3.64	1.45
	61	131	0.91	0.72	4.12	1.60
	Average	119.0	0.85	0.69	3.91	1.53
Polished rice + "Sendaimiso" 0.1% cystine	62	124	1.25	0.70	4.80	1.39
	63	152	1.43	0.79	5.38	1.95
	64	129	1.20	0.58	5.23	1.62
	Average	135.0	1.29	0.69	5.31	1.65
Polished rice + "Sendaimiso" + 0.5% Cystine	65	134	1.38	0.75	4.99	1.69
	66	142	1.51	0.80	5.21	1.87
	67	146	1.75	0.96	5.29	2.05
	Average	140.7	1.55	0.83	5.16	1.87
Polished rice + "Sendaimiso" + 1.0% cystine	68	103	—	—	—	—
	69	107	1.39	0.97	4.86	1.78
	70	134	1.64	0.98	4.97	2.35
	Average	114.7	1.52	0.98	4.92	2.07
Polished rice + "Hatchomiso"	71	122	1.05	—	5.12	—
	72	112	0.82	0.81	4.05	1.40
	73	129	1.01	0.85	4.92	1.56
	Average	121.0	0.92	0.83	4.70	1.48
Polished rice + "Hatchomiso" + 0.5% cystine	74	137	1.43	0.86	5.10	1.97
	75	134	—	—	—	—
	76	152	1.65	1.20	5.39	2.60
	Average	141.1	1.54	1.03	5.24	2.15

The weight of the kidneys differs according to the cystine administered, varying from 0.78 to 1.55 g with a corresponding rise in Folin-Looney's value which made obvious variation from 0.62 to 1.03. On the contrary the weight

of the liver was slightly less than normal. The deposition of cystine in the liver differed according to the amount of cystine administered, ranging from 1.22 to 2.15 mg.

In connection with the cystinuric investigation several specimens of human urine were examined but no suggestive evidence in cystine metabolism was obtained, but it was found that Folin-Looney's value in the urine is closely related to the sex, age, diet and physiological condition of the subject.

TABLE VI. Folin-Looney's Value of Human Urine.
(Cystine mg in 100 c.c. of Urine)

Kind of urine	Time of excretion	Color tone	Folin-Looney's value (mg)	Okuda's method cystine (mg)	c.c. of AgNO ₃ titrating 5 c.c. of Urine
A	10 a.m.	##	18.4	—	12.1
B	10 a.m.	###	15.6	—	18.8
C	3 p.m.	+	4.8	—	12.0
D	4 p.m.	##	5.6	—	13.3
E	2 p.m.	+	7.1	3.86	10.6
F	10 a.m.	+	11.8	4.54	12.4
G	3 p.m.	+	12.0	3.56	9.5
H	5 month old child, 8 a.m. colorless		0	—	0.8
I	Maternity case, 8 a.m. almost colorless		0	—	7.9

The administration of "miso", consisting of polished rice, soy beans and salt to an animal, produces an accumulation of a large amount of potassium salts in the body, as may be reduced by a corresponding amount of sodium salt.

TABLE VII. Potassium, Sodium and Calcium salts in Rice⁽¹⁵⁾ and in Soy-beans.
(g in 1000 g of dry matter)

	K ₂ O	Na ₂ O	CaO
Rice	2.0	0.4	0.3
Polished rice	1.4	0.1	0.2
Soy-beans	12.6	0.3	1.7

In order to determine whether these salts in a large amount are responsible for hypertrophy of the kidneys, or whether the animal is not affected by an excess of NaCl, the next experiment was carried out.

Now two groups of white rats, each consisting of two rats, were used. For 35 days the first group was placed on the "Sendaimiso" diet supplemented with 1.0% cystine, and the second was placed on the same diet supplemented with *d*-glutamic acid (m p 196~198 uncor.) and containing the same amount of nitrogen. The latter group did not differ much from the first to indicate the appreciable effect of glutamic acid, although of a short duration

of the feeding. After 35 days feeding the urine of both groups was determined by Folin-Looney's test and then on the 39th day the diets were exchanged (Chart IV), the first group receiving the diet supplemented with glutamic acid, while the second received the cystine diet. On the 46th day to each

Chart IV.

diet was added 1.0% NaCl in which NaCl was increased from 4.5 to 5.5% of the diet, and the cystine value of the urine was determined as shown in the following table.

TABLE VIII. Influence of Excess of NaCl upon a Rat's Urine.

Days of feeding	Body	Weight (g)	Cystine exc. per day (mg)	c.c. of N/10 AgNO ₃ per day
(A) The first group, "Sendaimiso" diet 1.0% cystine.				
0	43	47	—	—
35	70	88	—	—
36	72	90	2.3	178
37	73	91	2.5	180
38	73	92	2.5	181
"Sendaimiso" diet plus 1.2% glutamic acid				
39	75	95	2.0	190
40	76	96	0.7	195
41	—	—	—	—
42	78	97	0.7	194
43	81	99	0.6	189
44	81	100	0.6	194

The above diet plus 1.0% NaCl.

45	89	102	1.9	125
46	79	103	1.0	214
47	80	102	0.9	225
48	81	104	0.8	220
49				
50	83	105	0.7	235

(B) The second group, "Sendaimiso" diet 1.2% glutamic acid.

0	43	46	—	—
35	91	97	—	—
36	92	102	0.6	170
37	90	103	0.5	185
38	92	105	0.6	167

"Sendaimiso" diet plus 1.0% cystine.

39	93	106	1.2	175
40	93	107	1.8	181
41				
42	98	113	2.1	188
43	99	114	2.0	170
44	100	115	1.8	175

The above diet plus 1.0% NaCl.

45	102	116	1.2	189
46	104	118	1.1	193
47	107	119	0.8	208
48	109	121	0.8	208
49				
50	112	122	0.9	213

These feeding experiments, although of short duration, contain some points of sufficient interest to be recorded. The daily cystine excretion of the animal receiving 1.0% cystine was 2.3~2.5 mg, while that of the animal receiving 1.2% glutamic acid was 0.5~0.6 mg. On the exchange of diets the former showed a rapid decrease in cystine after two days feeding while the administration of 1.0% NaCl gave a temporary rise in the cystine content. The latter showed a slow rise in quantity due to the cystine administration and no change was observed in the amount of NaCl.

SUMMARY.

(1) The content of cystine in various kinds of "Miso" in polished rice, and in soy-beans was determined by Y. Okuda's or by Folin-Looney's method, and whether or not the amount of cystine in the polished rice diet supplemented with "Miso" was sufficient for the growth of white rats was investigated in connection with the excretion of a cystine complex in the urine.

(2) It was found that cystine in "Miso" can be easily decolorized and determined by Okuda's method, after hydrolysing it with concentrated HCl for 12~20 hours. Consequently, the amount of cystine in 100 g of the fresh

"Miso" was as follows:

"Shiromiso" 64.2, "Yedomiso" 45.4, "Sendaimiso" 50.9, and "Hatchomiso" 93.4 mg

(3) The amount of cystine estimated by Folin-Looney's method was slightly larger than the above, assuming "Shiromiso" 67.2, "Yedomiso" 85.6, "Sendaimiso" 86.6, and "Hatchomiso" 103.2 mg.

(4) The cystine content in 100 g of polished rice was 37.8~55.2 mg by Okuda's method.

(5) From the above, the amount of cystine in 100 g of "Miso" diet was calculated to be 0.062~0.065 g and consequently is very slightly deficient in cystine for the growth of white rats, since the diet contains 0.57% cystine for the diet protein.

(6) It was pointed out that the cystine value of the urine, kidneys and livers of the cystine-fed animals were greater than those of the control diet animal, and that the value were probably proportional to the cystine administration. The animal fed on 0.1% cystine diet was approximately normal. The excretion of cystine by the animal placed on 1.0% cystine diet was 4.5 mg per day and was ten times that of the Patrogen fed animal.

(7) Folin-Looney's value of the urine excreted by the animal when fed on an excess of cystine, was temporarily influenced by the excess of NaCl but it gradually diminished.

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