MEMOIRS OF THE FACULTY OF SCIENCE, KYOTO UNIVERSITY, SERIES OF BIOLOGY
Vol. V, pp. 57-65, December 1971

The Effect of Buffer Solutions on the Feeding Activity of the Silk-worm, *Bombyx mori* L.*

By

Kiryo LIN, Masaru KATO and Hiromi YAMADA

Department of Zoology, Faculty of Science
Kyoto University

(Received September 10, 1971)

ABSTRACT First of all, the authors would like to point out the fact that the newly hatched larvae of the silk-worm, *Bombyx mori* L. were really fond of feeding on the certain given alkaline substances 0.05M-(KOH+NaOH), pH 12.4, if the diet is simply composed of sucrose, inositol, cellulose and agar. On the contrary, those acids including H_3PO_4, H_2SO_4, HCl, HNO_3, $HClO_4$ and H_3BO_3 from the range of 0.01M to 0.32M were found to have caused repellence on larval feeding thoroughly. When changed with complex diet which is a usual one in our lab. that contained soybean powder, vitamin mix., Wesson's Salt, etc. for feeding assay, it was found that 0.05M-(KOH+NaOH)+M-H_3PO_4, pH 7.0 increased larval feeding to the utmost. On the bioassay of organic buffer solutions to the complex diet contained casein, vitamin mix., etc, we found phthalic acid caused highest synergistical feeding promotion on the newly hatched larvae of the silk-worm.

Introduction

Studies on the feeding behavior of the silk-worm, *Bombyx mori* L. have been carrying out for some years in this particular research field, for the development and improvement of the artificial synthetic diet to a better best. Based on both the fundamental study—focused upon the promotion of larvae's feeding activity, and advanced study—for the well growth of the whole larval stages. The former's experiments were executed methodically using biting assay diet (BAD), while the latter only directly applied complex diet consisted of a variety of indispensable components.

By the application of new augmentative method and BAD, those of feeding stimulating substances such as β-sitosterol (Hamamura), isoquercitrin (Hayashiya), n- & neo-chlorogenic acids, and free fatty acids (Lin et al) were found synergetically and complementally increased larval feeding.

On the other hand, it has been reported that an inorganic substance—potassium

* This study was supported partly by a grant from Takeda Science Foundation.

phosphate dibasic also caused feeding and growth promotive effects by the addition of it to the artificial diet (Hamamura et al, Horie et al). But when it was tested using our BAD, no significant feeding activity could be found. Meanwhile, when tested with complex diet (Kato et al), the feeding activity was also found lower than adding extra volume of M-KOH. In such case, the pH of the complex diet was raised from 5.0 to 7.0 which is nearly equal to the water fraction of the ML powder (pH 6.8).

Therefore, the fundamental study of various kinds of inorganic acids solutions consistently with pH series and graded doses were brought into minute experiments. In addition, the effect of organic acids buffer solutions on the feeding activity of the silk-worm larvae was also assayed in details in this paper for those organic acids on the influence of feeding action still have not yet been clarified.

Materials and Methods

Egg and Larva The eggs were kindly provided by Gunze Silk Co. periodically. They were incubated at 27±1°C every time in a certain amount for hatching. Those 10 individuals of the newly hatched larvae in the same batch (F_1 of Gunko x Manri) were used respectively as aliquid for bioassays.

Diet for Bioassay of Feeding Activity Three kinds of diet were used: BAD, and complex diets (CD-1 and CD-2) as Table 1 shows.

Table 1. Components of BAD, CD-1 and CD-2

BAD (dry wt. g)		CD-1 (dry wt. g)		CD-2(dry wt. g)	
sucrose	0.1	cellulose	1.6	〃	2.0
inositol	0.02	starch	0.5	〃	0.6
cellulose	0.8	β-sitosterol	0.1	〃	0.05
agar	0.2	soybean meal	1.8	casein	1.7
buffer	10 ml.	sucrose	0.3	〃	0.1
		oleic acid	0.1	7V**	0.01
		ascorbic acid	0.075	〃	0.075
		n-chlorogenic acid	0.05	〃	0.05
		vitamin mix.*	0.025	〃	0.025
		inositol	0.025	〃	0.02
		choline	0.005	〃	0.005
		sorbic acid	0.03	〃	0.03
		Wesson's Salt	0.01	〃	0.01
		buffer	12ml	〃	10ml.

Note : * see Kato et al (1966)
** see Lin et al (1971)

Preparation of Inorganic Buffer Solutions Those alkaline series : LiOH, NaOH, KOH, $Ca(OH)_2$, $Ba(OH)_2$ and $C_4H_{11}NO_3$ were made from 0.01M to 0.32M, and assayed with simplex diet (BAD).

Preparation of pH series of Potassium Phosphate The complex diet (CD-1) series

were made from pH 2.0 to 12.0 by titrating M-H_3PO_4 or M-KOH. The total volume of the diet in each petri-dish must be well adjusted to control no more than 12 ml..

Preparation of Neutral Inorganic Buffer Solutions 0.05M-(KOH+NaOH) was separately titrated with M-H_3PO_4, H_2SO_4, HNO_3, HCl, $HClO_4$ and H_3BO_3 to form neutral solutions. One more buffer-$KNaCO_3$ was prepared with 0.05M-($KHCO_3$+$NaHCO_3$), pH 7.8. Those neutral solutions were abbreviated as K-Na-PO_4, K-Na-SO_4, K-Na-NO_3, K-Na-Cl, K-Na-ClO_4, K-Na-BO_3 and K-Na-CO_3 and tested with BAD and CD-2.

Preparation of Neutral Organic Solutions Thirty-four kinds of commercially obtained organic acids were separately dissolved in 0.05M-(KOH+NaOH) to constitute neutral buffers, then tested with CD-2.

Check of pH Value Each buffer or assayed diet was checked carefully using both Horiba M-3 pH Meter and Toyo pH Test Paper.

Check of Feeding Activity Each diet menu of Table 1 is the appropriative amount for one 6 cm petri-dish and 10 larvae fetching. The number of feces was counted after the given hours (20 or 24 hrs) administration of diet to the same lot of the newly hatched larvae.

Results and Discussion

Inorganic Buffer

In Table 2 (a), we found that the graded doses from the range of 0.01M to 0.1M of the lithium hydroxide solution caused very low feeding activity. In those conditions, agar and agarose were found no any significant difference for feeding activity. Thus it was proved that the impurities contained in agar did not influence larval feeding under alkaline condition. In Table 2 (b), 0.05M (pH 12.2) of sodium hydroxide showed rather high activity than any other doses. Here, we found that if pH value exceed 13.0, the biting action became inactive perfectly. The inactive reason was clarified by the augmentative method. It was found that the sucrose and inositol were decomposed and changed their color into dark brown in

Table 2. Effect of inorganic alkaline buffer solutions on larval feeding (used BAD)
(a) Graded doses of lithium hydroxide

Mole	pH	Feces no. per 20 hrs	
		agar	agarose
0.005	8.6	29	25
0.01	9.5	18	18
0.05	10.0	2	6
0.1	10.4	4	3
0.5	12.2	0	0
H_2O	—	5	5

(b) Graded doses of sodium hydroxide

Mole	pH	Feces no. per 24 hrs
0.005	11.0	16
0.01	11.6	15
0.05	12.2	27
0.1	13.0	0
0.5	13.4	0
H_2O	—	0

(c) Graded doses of potassium hydroxide

Mole	pH	Feces no. per 20 hrs
0.01	11.6	66
0.02	11.8	39
0.05	12.4	109
0.16	13.0	77
0.32	13.4	11
H_2O	—	4

(d) Graded doses of calcium hydroxide

Mole	pH	Feces no. per 20 hrs
0.005	11.3	44
0.01	12.2	28
0.05	12.5	8
0.1	12.5	0
0.5	12.5	0
H_2O	—	18

Ntoe : calcium hydroxide caused suspension

(e) Graded doses of barium hydroxide

Mole	pH	Feces no. per 24 hrs
0.005	11.6	0
0.01	12.5	0
0.05	12.7	0
0.1	12.7	0
0.5	12.7	0
H_2O	—	5

Note : Barium hydroxide also caused suspension

(f) Graded doses of tris-amino methane

Mole	pH	Feces no. per 24 hrs
0.005	8.7	0
0.01	9.0	10
0.05	10.2	19
0.1	11.2	14
H_2O	—	0

overdose solution. In Table 2 (c), the result of potassium hydroxide solutions showed that the most effective dose was 0.05M (pH 12.4). Excessive dose of this solution also caused inhibition gradually. In Table 2 (d), it was confirmed that the effective quantity of the calcium hydroxide was from 0.005M to 0.01M. Overdose also happened to be adversely on larval feeding. This hydroxide as we know is incompletely soluble in water, therefore, it goes without saying that the pH of the suspension surely caused deflection. Another buffer, barium hydroxide was so disappointed to be found that caused lethal toxicity to the silk-worm larvae. The lethal action was nothing dealing with pH value although it could be possible caused deviation owing to the incompletely solubility in water. Therefore, anyway this alkaline substance is a toxic factor and not allow to be used as buffer solution for the newly hatched larvae. Table 2 (f) shows that the tris-amino methane also caused rather low activity on the feeding promotion of the larvae. Conclude above inorganic alkaline solutions, the arrangement of their specific activations to the BAD on the increase of larval feeding are thus : $KOH > NaOH > C_4H_{11}NO_3 > LiOH > Ca(OH)_2$.

It has been reported in 1963 (Ishikawa et al) that the evidence of the contact chemoreceptors associated with two sensilla styloconica on the maxillary palpus of the silk-worm were proved applying electrophysiological method. They reported that the activity of sucrose cell and inositol cell in sensilla stylocconica hairs could be evoked by sodium hydroxide solution at pH 11.0. Now here we should point out the result given in Table 2 (b) surely coincides with the result of their electrophysiological experiment. The feeding of larvae is brought about owing to the excitation of inositol and sucrose receptors. But we should further indicate that suppose Ishikawa raised sodium pH value to 13.0 or used other alkaline solutions such as barium hydroxide as electroassayed material, the result might be caused reversely. Ishikawa also reported that the threshold of the sucrose receptor of the silkworm *Bombyx mori* L. was at 10^{-4}M, and at 1.5×10^{-4}M to inositol receptor. In our experiments, the amount of sucrose and insitol as shown in Table 1 were far more exceed their ranges. Another counterpart report was described by a senior research worker—Schoonhoven in 1967. He also made a description of the

Table 3. Graded doses of phosphate on the promotion of larval feeding

Mole	pH	Feces no. per 20 hrs
0.16	2.4	0
0.08	2.8	0
0.04	3.8	0
0.02	6.3	8
0.01	8.9	50
0.05 (KOH)	12.4	169
0.05 (KOH+NaOH)	12.4	192
0.05 (KOH+NaOH+$Ca(OH)_2$)	12.4	66
H_2O	—	27

Note : Moles of phosphoric acid were neutralized by 0.05M-KOH of the equal volume and tested with simplex diet (BAD)

lepidopteran larvae, *Pieris brassicae* on its specific feeding behavior to sucrose (Hanson).

Using BAD for the graded doses assay of the potassium phosphate from the range of 0.16M to 0.01M (pH 2.4 to 8.9). The resultant data of Table 3 show that below pH 3.8, the feeding activity was zero. The buffer at pH 8.9 which is equivalent to the potassium phosphate dibasic (K_2HPO_4) also caused fairly low activity. In the same Table, potassium hydroxide and sodium hydroxide mixture —0.05M-(KOH+NaOH) at the ratio of 1:1 showed higher activity than 0.05M-KOH on the feeeding promotion. Therefore, it is clearly proved that among inorganic buffers, KOH and NaOH are able to induce synergistical effect. But if added with calcium hydroxide at the ratio of 1 : 1 : 1, the activity was found greatly decreased. The reason as we speculated might be due to the antagonistical action between K and Ca, Na and Ca, or K-Na and Ca, and diminish the irritant excitability of the sensilla styloconica.

Table 4. Effect of potassium phosphate pH series on larval feeding (tested with CD-1)

pH series	Feces no. per 20 hrs
2.0	0
4.0	25
5.0	72
6.0	220
7.0	291
8.0	81
10.0	62
12.0	0

Note : CD-1 is at pH 5.0, then added with extra volume of M-H_3PO_4 or M-KOH

It was found in Table 4 that the alkaline solution as well as acidified solution decreased larval feeding to a large extent, especially at pH 2.0 and pH 12.0 are distinctly on two thresholds of larval feeding, and pH 7.0 was found effectively caused the highest activity when tested with complex diet (CD-1). We doubted that since 0.05M-KOH or 0.05M-(KOH+NaOH) highly increased larval feeding in BAD (Table 2 (b), (c), and Table 3) why it affected reversely in complex diet (Table 4). Thus the inhibiting reason was put to the test by using augmentative method, and clarified that the starch, casein, Wesson's Salt used as indispensable components in complex diet repelled larval feeding under pH 12.4 condition. The result is given in Table 5.

In Table 6 (a), the neutral inorganic buffer solutions were assayed with two kinds of diet; the BAD and CD-2. The resultant data show that the former caused no significant activity on feeding, while the latter was found greatly activated by 0.05M-(KOH+NaOH)+M-H_3PO_4 abb. K-Na-PO_4, pH 7.0. Those K-Na-Cl and K-Na-SO_4 also showed rather high activities on the promotion of larval feeding. And those K-Na-CO_3 and K-Na-BO_3 were found entirely caused repellence. Thus the order of those neutral inorganic buffer solutions mentioned above on feeding action

Table 5.. Check of inhibiting component of the CD-2 in 0.05M-(KOH +NaOH) buffer solution (pH 12.4)

BAD	Casein	Starch	Vit. mix	Ascorbic acid	7V	Wesson's salt	Choline	Feces no. per 20 hrs
+	−	−	−	−	−	−	−	80
+	+	−	−	−	−	−	−	0
+	−	+	−	−	−	−	−	0
+	−	−	+	−	−	−	−	78
+	−	−	−	+	−	−	−	82
+	−	−	−	−	+	−	−	19
+	−	−	−	−	−	+	−	0
+	−	−	−	−	−	−	+	35
+	+	+	−	−	−	−	−	0
+	+	+	+	−	−	−	−	0
+	+	+	+	+	−	−	−	0
+	+	+	+	+	+	−	−	0
+	+	+	+	+	+	+	−	0
+	+	+	+	+	+	+	+	0

can be : $K\text{-}Na\text{-}PO_4 > K\text{-}Na\text{-}Cl > K\text{-}Na\text{-}SO_4 > K\text{-}Na\text{-}ClO_4 > K\text{-}Na\text{-}NO_3$. In addition, the comparative effect of $K\text{-}PO_4$ and $Na\text{-}PO_4$ using complex diet (CD-2) was clearly made. The experimental result shows that simply 0.05M-NaOH+M-H_3PO_4, pH 7.0 was found lower than 0.05M-KOH+M-H_3PO_4, pH 7.0 on the promotion of larval feeding. Moreover, the combination of 0.05M-(KOH+NaOH)+M-H_3PO_4, pH 7.0 was found synergistically increased larval feeding (see Table 6 (b)).

Table 6a. Effect of neutral inorganic buffer solutions on larval feeding

Buffer (pH 7.0)	Feces no. per 24 hrs	
	simplex diet (BAD)	complex diet (CD-2)
$K\text{-}Na\text{-}PO_4$	14	452
$K\text{-}Na\text{-}SO_4$	47	197
$K\text{-}Na\text{-}NO_3$	3	119
K-Na-Cl	34	220
$K\text{-}Na\text{-}BO_3$	—	0
$K\text{-}Na\text{-}CO_3$	—	0
$K\text{-}Na\text{-}ClO_4$	—	188
H_2O	27	90

Table 6b. Comparative effect of sodium phosphate, potassium phosphate and sodium-potassium phosphate on larval feeding (tested with CD-2)

Buffer	pH value	Feces no. per 20 hrs
$Na\text{-}PO_4$	7.0	17
$K\text{-}PO_4$	7.0	31
$Na\text{-}K\text{-}PO_4$	7.0	87

Note : Feeding activity was found respectively decreased due to the vitality of larvae

Organic Buffer

Each acid of commercially obtained was titrated by 0.05M-(KOH+NaOH) to form a neutral solution. Some hard dissolved organic acids such as tannic acid or sorbic acid, etc. have got to be heated.

Among organic acids, the well-known n-chlorogenic acid was previously confirmed as existed one per cent in mulberry leaves (ML), and was proved having both feeding and growth promotive effects (Kato et al, Lin et al). Protocatechuic acid also demonstrated its necessity on the growth of the newly hatched larvae (Kato et al). Gallic acid has also been used for the growth from 3rd to 5th instars larvae (Hamamura et al). Benzoic acid was reported of its appreciable existence in most berries of ca. 0.005% (Schwab et al). Fumaric acid was also confirmed to be existed in ML (Hamamura). The effects of those acids on feeding action still remained to be elucidated. Therefore, in this particular field, 34 kinds of commercially obtained free forms of organic acids including above ML-existed, were carried out for the bioassay of their feeding activities. The result is given in Table 7.

Table 7. Effect of neutral organic buffer solutions on larval feeding (tested with CD-2)

Buffer (pH 7.0)	Diet color	Feces no. per 24 hrs	Buffer (pH 7.0)	Diet color	Feces no. per 24 hrs
K-Na-acetate	yellow	307	K-Na-nicotinate	do.	108
K-Na-formate	do.	0	K-Na-styphnate	do.	0
K-Na-protocatechuate	grey	177	K-Na-resorcylate	light brown	10
K-Na-caffeate	brown	173	K-Na-trichloroacetate	yellow	59
K-Na-quinate	yellow	222	K-Na-salicylate	do.	11
K-Na-sorbate	do.	127	K-Na-pyromellitate	do.	142
K-Na-gallate	brown	184	K-Na-sulfamate	do.	12
K-Na-vanillate	yollow	97	K-Na-levulinate	do.	73
K-Na-benzoate	do.	28	K-Na-cyanoacetate	do.	112
K-Na-phthalate	do.	366	K-Na-cumarillate	do.	40
K-Na-citrate	do.	0	K-Na-adipate	do.	19
K-Na-malate	do.	334	K-Na-tannate	brownish yellow	0
K-Na-fumarate	do.	176	K-Na-tropate	yellow	20
K-Na-succinate	do.	34	K-Na-picrate	do.	0
K-Na-cis-aconitate	do.	0	K-Na-itaconate	do.	0
K-Na-pyruvate	do.	7	K-Na (pH 12.4)	do.	0
K-Na-tartarate	do.	183	K-Na-PO_4	do.	90
K-Na-oxalate	do.	29	H_2O	do.	40
K-Na-DNA	do.	48			

In Table 7, phthalic acid, malic acid and acetic acid solutions were found greatly promoted larval feeding. Gallic acid, protocatechuic acid also doubled stimulated larval feeding than controlled diet. Quinic acid, sorbic acid, caffeic acid, ML-existed fumaric acid, and tartaric acid were also more or less caused positive activities. Those formic acid, citric acid, styphnic acid, tannic acid, picric acid and itaconic acid were found completely inhibited larval feeding. The repellent reasons might be due to overdoses of those organic acids, or the occurrence

of antipathic actions among those organic acids and K-Na solutions. The synergistical activities of organic acids on the increase of larval feeding can be arranged as : phthalic acid>malic acid>acetic acid>gallic acid>protocatechuic acid>quinic acid>caffeic acid>fumaric acid>tartaric acid>pyromellitic acid>sorbic acid.

It is obviously proved that the organic acid can act synergetically with inorganic ions to a fairly large extent, but the quite interesting interaction of their chemical behaviors and the physiological mechanisms to the silk-worm larval feeding are still required to be elucidated.

References

1) HAMAMURA, Y., Food Selection by silkworm larvae. Nature, **183**, 1746-1747, 1959
2) HAMAMURA, Y., K, HAYASHIYA, K. NAITO, K. MATSUURA and J. NISHIDA, Food selection by silkworm larvae, *Bombyx mori* L.. Bull. Kyoto Univ. Ind. & Tech., **3** (3), 567-582, 1962
3) HAYASHIYA, K., Requirements of the feeding stimulating substances for insect. Shokubutsu Bôeki, **23** (8), 344-348, 1969
4) LIN K., H. YAMADA and M. KATO, A comparative study of the effect of chlorogenic acid and its isomers on the growth of the silkworm, *Bombyx mori* L.. J. Sericult. sci. Japan, **39** (3), 153-157, 1970
5) LIN, K., H. YAMADA and M. KATO, Free fatty acids promote feeding behavior of the silk-worm, *Bombyx mori* L.. Mem. Fac. Sci., Kyoto Univ. Ser. Biol., **4** (2), 108-115, 1971
6) HORIE, Y., K. WATANABE and T. ITO, Nutrition of the silkworm, *Bombyx mori* L.. XVIII Quantitative requirements for potassium, phosphorus, magnesium and zinc. Bull. Sericult. Expt. Sta., **22**, 181, 1967
7) KATO, M. and H. YAMADA, Silkworm requires 3, 4-dihydroxybenzene structure of chlorogenic acid as a growth factor. Life Sciences, **5** (8), 717-722, 1966
8) ISHIKAWA, S. and T. HIRAO, Electrophysiological studies of the taste sensation in the larvae of the silkworm, *Bombyx mori* L.,-Responsiveness of sensilla styloconica on the maxilla. Bull. Sericult. Expt. Sta., **18**(5), 297-339, 1963
9) HANSON, F. E., Sensory responses of phytophagus Lepidoptera to chemical and tactile stimuli. Control of insect behavior by natural product. Academic Press, New York, 81-91, 1970
10) HAMAMURA, Y., K. KUWATA and H. MASUDA, Effect of gallic acid on the growth of the silkworm larvae, *Bombyx mori* L.. Nature, **212**, 1386-1387, 1966
11) SCHWAB, F. W. and E. WICHERS, Preparation of benzoic acid in high purity. J. Natl. Bur. Std., **25**, 624-628, 1940