Amino Acids (1993) 5: 341–350

Amino
Acids

A comparative study on the arginine degradation cascade for sperm maturation of *Bombyx mori* and *Drosophila melanogaster*

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Accepted May 24, 1993

Summary. The spermatophore of the silkmoth, *Bombyx mori*, is a reactor with a specific energy-yielding system for sperm maturation, the arginine degradation cascade. On mating, the highly viscous secretions from various glands in the male reproductive tract, which contain many enzymes and their substrates, are transferred to the female bursa (b.) copulatrix to form the spermatophore. In the spermatophore, transferred arginine-rich proteins are digested by initiatorin, an Arg-C endopeptidase of serine-protease type, and a carboxypeptidase. The produced free arginine is then hydrolyzed to urea and ornithine by arginase. Ornithine is metabolized to glutamate, follwed by forming alanine and 2-oxoglutarate. The latter, as a member of TCA-cycle, is a preferred respiratory substrate for spermatozoa and accelerates the post-testicular sperm maturation.

In contrast to *Bombyx mori*, *Drosophila melanogaster* produces only eupyrene spermatozoa and does not form the spermatophore. The sperm of this dipteran insect acquire motility in the v. seminalis of males. As reported for *Drosophila*, a high glutamate-pyruvate aminotransferase activity was found in the spermatophore as well as the v. seminalis of the silkmoth. The value in the latter organ reaches 58.3% of the whole male reproductive tract that participates in transfer of the seminal fluid.

In the male reproductive system of *Drosophila*, the concentration of arginine is low, whereas those of urea and ammonia are high. The accessory gland secretion contains much phosphoserine. Theses substances are transferred to female uterus with spermatozoa during mating. Most amino acids increase distinctly at 30 min after the termination of mating (ATM) and then decline, suggesting active degradation of transferred proteins in the uterus. As found in *Bombyx*, urea increases at the post-mating period, while ornithine shows a rather low concentration. Ornithine must be converted to glutamate. In this connection, it is notable that alanine rises markedly at 30 min following mating. As in the silkmoth, the energy metabolism of the fruit fly spermatozoa involves also

arginine, ornithine, urea, and proline. These findings suggest that the occurrence of the arginine degradation cascade or related metabolic pathway in this insect.

Keywords: Amino acids – Arginine degradation cascade – Sperm maturation – Aminotransferase – Spermatophore – *Bombyx mori* – *Drosophila melanogaster*

Abbreviations: ATM, after the termination of mating; Arg-C, arginine-carbon; b., bursa; d., ductus; g., glandula; GPA, L-glutamate-pyruvate aminotransferase; $NADH_2$, reduced nicotinamide-adenine dinucleotide; TCA, tricarbonic acid; v., vesicula.

Introduction

Osanai et al. (1987a) have demonstrated that, in the male silkmoth *Bombyx mori* anucleated, apyrene spermatozoa are immotile, and nucleated, eupyrene spermaozoa that fertilize eggs occur as bundles in the vesicula (v.) seminalis before ejaculation. These two types of spermatozoa mature in the spermatophore which is formed with secretions of the male reproductive glands following transfer to females on mating. Apyrene spermatozoa acquire motility and eupyrene sperm bundles become dissociated (Katsuno, 1977). In the course of this functional maturation process, a series of important metabolic reactions for energy supply, the arginine degradation cascade, take place (Osanai et al., 1987a). Thus, the spermatophore is a site of sperm maturation as well as a reactor for the supply of energy needed.

Arginine is formed from male secretion proteins by initiatorin derived from the glandula (g.) prostatica, a specific endopeptidase of serine-protease type (Aigaki et al., 1987). The latter enzyme cleaves protein on the C-side of arginine residue, and followed by an active arginine carboxypeptidase from the g. prostatica and v. seminalis which is also activated by initiatorin. Owing to the presence of arginase derived from the v. seminalis, the produced free arginine is hydrolyzed to urea and ornithine (Osanai et al., 1986; 1987b).

Compared with the rapid increase of urea, the concentration of ornithine is rather low (Osanai et al., 1986). Parallel to the increase of urea, the concentration of alanine rises also rapidly. It can be anticipated that this ornithine is converted to glutamate which forms 2-oxoglutarate by coupling with the production of alanine from pyruvate derived from glycolysis (Osanai et al., 1987b). Thus, the activity of L-glutamate-pyruvate aminotransferase (GPA) in the male reproductive system of the silkmoth has been analyzed in this paper.

On the other hand, as in most dipteran insects, adult males of the fruit fly, *Drosophila melanogaster* do not use a spermatophore for sperm transfer. This insect produces only eupyrene spermatozoa. *Drosophila* spermatozoa acquire their motility in the v. seminalis and are transferred directly with the semen to the female genital tract during mating. In spite of such a difference in their mode of sperm transfer, it would be of interest to know, whether corresponding metabolic alternations in the reproductive systems between these two insects exist. In the fruit fly there is so far no information on the reproductive significance and the physiological-biochemical basis of these reactions. To our knowledge, no data on the metabolic changes following deposit of the semen in the female

genital tract of this insect are available. Thus, changes of amino acids and related compounds in females at the initial phase after copulation has been studied in this paper.

Materials and methods

Silkworm and fruit fly

J02 X C02, a hybrid of the silkworm *Bombyx mori* was reared on artificial diet. The temperature was kept at 25°C throughout all developmental stages. Selection of sexes and maintenance of adults were as described previously (Osanai, 1978). The testis, ductus (d.) deferens, ampulla d. deferentis, g. pellucida, g. lacteola, v. seminalis, g. spermatophorae + g. alba and g. prostatica were each excised from unmated male moths one day after emergence (Osanai et al., 1986). The b. copulatrix including the spermatophore was excised from newly emerged and mated female moths 20 min after the beginning of copulation (Osanai et al., 1987b).

A wild type of *Drosophila melanogaster* was reared at 20°C. Fifty pairs of the whole accessory glands excised from 5-day-old virgin male flies were collected in an Eppendorf tube containing 20 μl ice-cold Ringer. The secretion contained in the the glandular lumen was lightly squeezed out into Ringer solution. Following brief centrifugation, the samples were used for analysis of free amino acids and related compounds.

Virgin females and male flies at 3–6 days after eclosion were immobilized on ice, and individual pairs were transferred to a glass vial with food. At room temperature the flies usually became active within less than 1 min. Mating was observed continuously, and those flies which copulated shorter than 10 min were eliminated. Copulation was usually terminated at 20 min after its beginning. At intervals of 0, 0.5, 1, 3, 4 and 5 hr ATM, the flies were immediately cooled on ice. The uterus of each female fly was then dissected out, but without removal of the parovaria, spermathecae and seminal receptacles. Following dissection in a drop of cold Ringer solution, twenty uteri were collected individually in an Eppendorf tube containing 20 μl Ringer.

After homogenization the sample was adjusted to a total volume of 100 μl and centrifuged at 10,000 rpm for 15 min at 2°C. The extaction procedure was repeated twice. The three supernatants were pooled, dried in a vacuum evaporater and preserved at −30°C until analysis of free amino acids.

Analysis of free amino acids

Each dried sample was taken up in 200 μl buffer solution, filtered through a syringe with a teflon filter and examined in an amino acid autoanalyzer, JEOL JLC-300 (Japan Electron Laboratory, Akishima, Japan) by a continuous develpment with six lithium citrate buffers (Osanai and Yonezawa, 1986). Parallel samples of the accessory glands and v. seminalis from isolated unmated male flies and the uteri from virgin females were also prepared by identical procedures. Amino acids were analyzed using at least three replicate samples.

Assay of aminotransferase

Chen et al. (1984) revealed a high activity of GPA in *Drosophila*. This is especially true in *D. nigromelanica*, since its male accessory glands accumulate a large quantity of glutamate (Chen and Oechslin, 1976). In the male reproductive tract and spermatophore of *Bombyx*, GPA activity was assayed in the direction of pyruvate formation which was coupled to $NADH_2$ oxidation via lactate dehydrogenase (Chen and Baker, 1976). The oxiation of $NADH_2$ was monitored at 340 nm by maintaining the temperature at 25°C. After homogenization of each gland in cold 50 mM Tris-HCl buffer (pH 7.8) containing 0.1% cetyltrimethylammonium bromide, the sample was centrifuged at 10,000 rpm for 15 min at 2°C. The supernatant was used for assay of the enzyme activity, of which one unit was defined

as the activity producing one micromole of $NADH_2$ per minute. Enzyme activity was determined at least in triplicate samples.

Results

Aminotransferase in the male reproductive system of the silkmoth

In one whole male reproductive system of the 1-day-old male silkmoth, GPA has a total activity of 458 mU before ejaculation (Table 1). The sum of GPA activities of three male reproductive organs, the testis, d. deferens and ampulla d. deferentis represents 40.8% of the total GPA activity of the whole male reproductive system. However, there is no difference before and after ejaculation, because fluids of these organs are not transferred to females by ejaculation (Osanai and Nagaoka, 1992). Male secretions transferred are derived from the male reproductive tract, namely the g. pellucida, g. lacteola, v. seminalis, g. spermatophorae, g. alba and g. prostatica (Ômura, 1938; Osanai et al., 1986). Among these the v. seminalis shows by far the highest enzyme activity (58.3% of total activity). Significant activities are also localized in the g. lacteola (25.2%) and the g. spermatophorae + g. alba (10.4%). The GPA activity of each gland without its content revealed from the GPA activity after ejaculation is similar to that of its secreted material. About one half of whole GPA activity contained in the male reproductive tract is transferred, which corresponds to that of the b. copulatrix after mating. Prior to mating GAP activity is hardly found in this organ.

Table 1. L-Glutamate-pyruvate aminotransferase activity of the male reproductive system and the female bursa copulatrix of *Bombyx mori* ($n = 5$)

Organ	L-Glutamate-pyruvate aminotransferase activity (mU/organ)		
	before ejaculation (A)	after ejaculation (B)	A–B
Testis	52.3 ± 12.8	56.8 ± 14.2	
D. deferens	66.7 ± 18.8	70.2 ± 20.3	
Ampulla d. deferentis	67.9 ± 15.0	60.4 ± 15.6	
Male reproductive tract			
G. pellucida	4.5 ± 1.0	3.5 ± 1.1	1.0 (0.7%)
G. lacteola	80.5 ± 17.6	46.7 ± 14.3	33.8 (25.2%)
V. seminalis	150.9 ± 34.6	72.6 ± 16.7	78.3 (58.3%)
G. spermatophorae	24.8 ± 6.2	10.8 ± 5.1	14.0 (10.4%)
G. prostatica	10.1 ± 3.5	2.9 ± 0.9	7.2 (5.4%)
Total	270.8	136.5	134.3 (100%)
B. copulatrix	0.4 ± 0.3	169.8 ± 33.8	169.4 (126.1%)

Activity difference of aminotransferase before (*A*) and after ejaculation (*B*) is presented as *A*–*B*. Total activity of A–B of all glands of the male reproductive tract is expressed as 100 percent and A–B activity of each gland and that of b. copulatrix are shown in parentheses.

Amino acids in the male reproductive tract of fruit fly

Concentrations of total amino acids in the v. seminalis, whole accessory gland and its secretion per body weight of *Drosophila* approximately correspond to those of *B. mori*. Compared to the v. seminalis, whole accessory gland contains about twice more total amino acids of which about 90% are stored in the secretion in the lumen (Table 2). It has been known that about one third concentrations of the accessory gland secretion is transferred to the female uterus during copulation (Chen, 1984). The concentration of each amino acid in the secretion differs from that in the epithelial cells. Phosphoserine is more concentrated in the whole accessory gland than its secretion, while the reverse is true

Table 2. Contents of free ninhydrin-positive compounds in the male reproductive system of *Drosophila melanogaster* aged 5 days (nmole/50 insects)

Compound	Semianl vesicle	Whole accessory gland	Secretion of accessory gland
P-serine	1.2 ± 0.2	131.7 ± 2.9	105.4 ± 0.1
PEA	—	—	—
Urea	17.5 ± 4.2	14.6 ± 3.7	21.5 ± 6.0
Asp	1.3 ± 0.3	3.0 ± 0.3	1.1 ± 0.1
Thr	0.9 ± 0.3	1.8 ± 0.3	0.8 ± 0.0
Ser	4.1 ± 1.5	3.9 ± 1.2	2.0 ± 0.4
Asn	0.1 ± 0.1	1.2 ± 0.2	0.4 ± 0.2
Glu	0.6 ± 0.1	11.6 ± 0.2	7.4 ± 3.8
Gln	0.5 ± 0.0	2.7 ± 0.0	0.8 ± 0.2
Gly	2.8 ± 1.1	3.2 ± 0.6	1.6 ± 0.1
Ala	1.3 ± 0.7	4.4 ± 0.5	1.5 ± 0.1
Citrulline	0.6 ± 0.0	0.9 ± 0.3	0.5 ± 0.0
Val	0.6 ± 0.2	0.7 ± 0.2	0.2 ± 0.2
Cys	—	0.3 ± 0.2	—
Cystathionine	—	0.1 ± 0.0	—
Ile	0.4 ± 0.2	0.7 ± 0.1	0.2 ± 0.2
Leu	0.8 ± 0.2	1.4 ± 0.2	0.6 ± 0.1
Tyr	0.2 ± 0.1	0.5 ± 0.3	—
β-Ala	—	—	—
Phe	0.3 ± 0.1	0.3 ± 0.1	—
GABA	—	—	—
NH_3	85.1 ± 4.9	72.3 ± 3.5	97.3 ± 7.0
Ornithine	1.6 ± 0.5	1.2 ± 0.4	0.5 ± 0.2
His	0.6 ± 0.3	2.4 ± 0.5	0.5 ± 0.1
Lys	0.4 ± 0.2	7.9 ± 0.4	1.0 ± 0.3
3M-His	—	—	—
Try	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.0
Arg	1.1 ± 0.1	2.9 ± 0.3	0.8 ± 0.1
Pro	—	6.9 ± 0.9	2.6 ± 0.2
Sum	123.4 ± 4.8	278.0 ± 4.5	248.0 ± 3.7

Whole accessory glands consist of accessory glandlar tissues and the secreted contents in the lumen. Secretion of the accessory gland was collected by lightly squeezing the excised accessory gland (see the text).

for urea. Of amino acids contained in both the whole accessory gland and its secretion, concentration of phosphoserine is extremely high, followed by ammonia, urea, and glutamate. In the v. seminalis, urea and ammonia showed similar high levels to those in the accessory gland and its secretion, but the concentrations of glutamate and phosphoserine are low. These two compounds must be produced in the accessory gland. The concentrations of most amino acids including glutamine, arginine, ornithine and alanine are low in all the three organs analyzed.

Amino acids in the uterus of female fruit fly

Differences of amino acid patterns between virgin and mated females are shown in Fig. 1. An extremely high concentration of phosphoserine was found in the uterus of mated females (Figs. 1 and 2). Since this substance has been shown to be present in the male accessory glands (47.3% of total amino acids) and the virgin females contain only a trace of it (1.3% of total amino acids), its great part must be transferred to females at copulation.

In general, most amino acids increase distinctly at 30 min ATM and decline in the uterus (Figs. 3 and 4). The increase of free amino acids shortly after mating indicates the degradation of proteins transferred from the male accessory gland. Glutamine level declines slightly 1 hr ATM, but thereafter rises gradually. Levels of arginine, proline and glutamate change almost in parallel. They decrease until 3 hr ATM and then increase slightly. Unlike these amino acids, alanine and urea

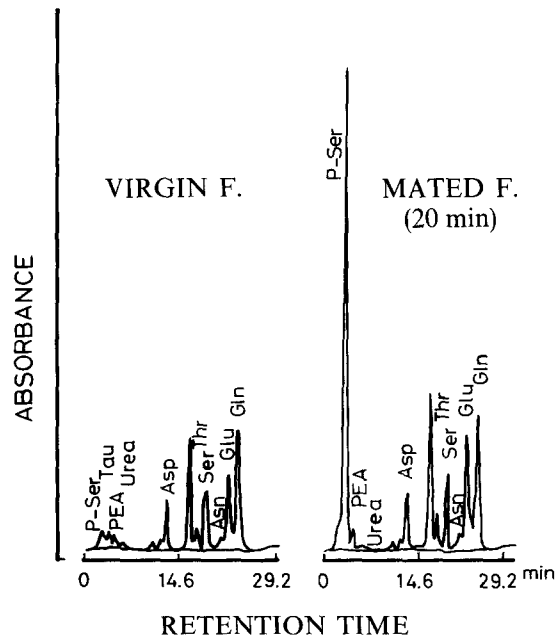

Fig. 1. Transfer of phosphoserine (*P-Ser*) and several ninhydrin-positive compounds from male to female in *Drosophlla melanogaster* at mating. Abscissa shows retention time in minute. *Tau* taurine; *PEA* phosphoethanolamine; *Asp* aspartate; *Thr* threonine; *Ser* serine; *Asn* asparagine; *Glu* glutamate, *Gln* glutamine

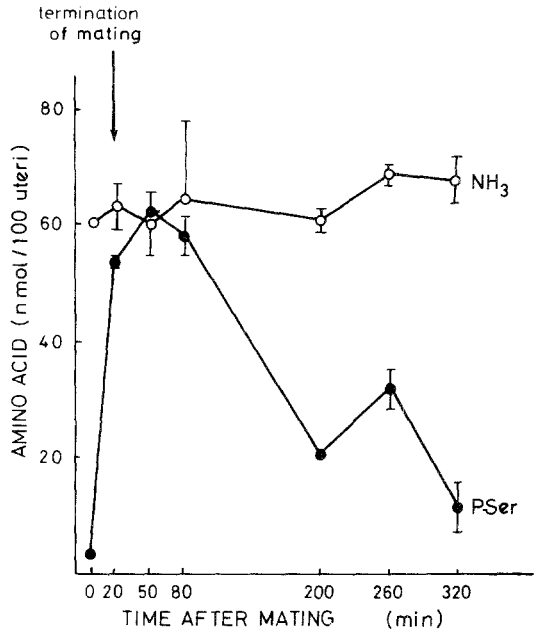

Fig. 2. Quantitative changes of phosphoserine (*P-Ser*) and ammonia (NH_3) in the uterus of mated female of *Drosophila melanogaster*. Time 0 means the initiation time of mating

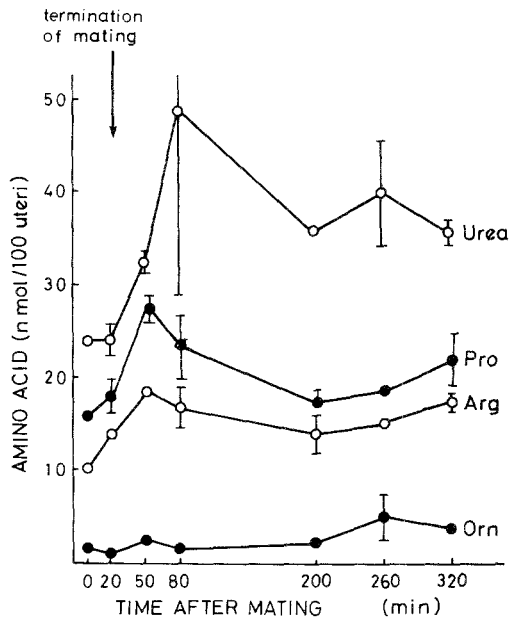

Fig. 3. Quantitative changes of urea, proline (*Pro*), arginine (*Arg*) and ornithine (*Orn*) in the uterus of mated female of *Drosophila melanogaster*. Time 0 means the initiation time of mating

increase until 1 hr ATM, and then decrease until 3 hr. Thereafter urea keeps a constant level, while alanine increases slightly. Although urea is contained in the uterus at a rather high concentration prior to mating, it exhibits a steep increase

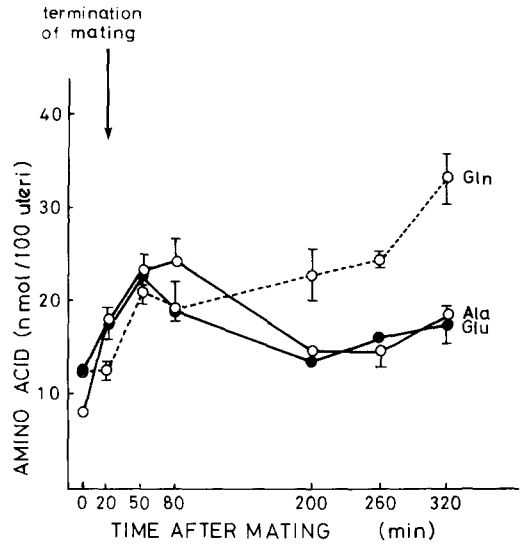

Fig. 4. Quantitative changes of glutamine (*Gln*), glutamate (*Glu*) and alanine (*Ala*) in the uterus of mated female of *Drosophila melanogaster*. Time 0 means the initiation time of mating

immediately following its termination. Ornithine shows almost no distinct level differences before and after copulation. Ammonia behaves similarly as ornithine, but its concentration is high.

Discussion

In the spermatophore of *B. mori*, GPA catalyzes the conversion of glutamate derived from ornithine to 2-oxoglutarate. This is coupled by the production of alanine from pyruvate as a consequence of the associated glycolytic process. These results yield an explanation of the low concentration of ornithine and the rapid rise of alanine at sperm maturation in this insect (Osanai et al., 1987b). High GPA activities have been reported in the male reproductive system of *D. hydei, D. nigromelanica* and *D. melanogaster* (Chen et al., 1985). On the basis of these results, it seems that GPA is closely involved in the supply of energy substrate for insect spermatozoa.

The increase in most amino acids including arginine in the *Drosophila* uterus until 30 min ATM showed that they are derived from the degradation of proteins that are transferred from males (Figs. 2–4). Initially arginine has probably its origin in the female, since its concentration in the male accessory secretion is low (Table 2 and Fig. 3). As in the spermatophore of the silkmoth, ornithine exhibits a rather low concentration in the uterus of the fruit fly. On the other hand, there is a definite increase of urea at the post mating period (Fig. 4). Ornithine is apparently in part metabolized to glutamate. In this connection it is worthy to note that, like urea, alanine rises distinctly at 30 min ATM. Level changes in alanine and urea are also parallel after the termination of mating.

As in the silkmoth, protein degradation, energy metabolism and high GPA activity of the fruit fly spermatozoa involves also arginine, ornithine, urea, alanine

in the female reproductive tract on mating. It may be regarded as the arginine degradation cascade or related pathway for energy supply for spermatozoa.

There is an additional possibility that also proline is utilized in fruit fly for 2-oxoglutarate production through the post-pathway of the arginine degradation cascade. *Drosophila* differs from *Bombyx* by maintaining a relatively high concentration of proline (Fig. 3). In a blood-sucking fly, *Glossina*, this amino acid has been reported to serve as an essential energy source (Bursell, 1963). Proline can be easily converted by proline oxidase to glutamate which is then deaminated to 2-oxoglutarate and thus enters the TCA-cycle. There is yet no convincing evidence for such a metabolic pathway in other dipterans.

The interactions of these metabolites proceed more rapidly and occur at a shorter period (0.5–1 hr subsequent to mating) in *Drosophila* than in *Bombyx*. This is obvious due to the fact that *Drosophila* sperm are motile at the time of transfer, whereas *Bombyx* sperm become functionally mature only in the spermatophore which is formed after their transfer to the female genital tract.

A closer examination of the amino acid patterns depicted in Fig. 4 reveals further a steady increase of glutamine relative to the concentration of glutamate. In view of the high concentration of ammonia (Fig. 2), it is not surprising that this amide is formed through the activity of glutamine synthetase.

A prominent deviation is the occurrence of highly concentrated phosphoserine in the genital tract of the fruit fly, and its absence in the silkmoth. The spermatozoa of *Drosophila* are immotile in the testis, but acquire motility during their passage through the testiculo-deferential valve to the v. seminalis. It seems that this compound is not related to sperm maturation in the fruit fly. The reproductive significance of this phosphate ester is unknown. It could be involved in the insemination reaction (Fowler, 1973) or the formation of the so-called waxy-plug (Bairati, 1968). It has been known that this substance acts as a precursor for formation of serine and glycine and an essential component of phospholipids.

Acknowledgements

Authors thank Dr. Hideo Sugita, Tokyo Metropolitan Sericultural Station, Akigawa, Japan for supply of the silkworm. The excellent technical assistence and useful discussions of Dr. Johannes Balmer, Zoological Institute, University of Zürich, Switzerland and Drs. Toshiro Aigaki and Hiroko Kasuga, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan, are gratefully acknowledged. This work was supported in part by research grant (Nos. 02304020 and 03455026) from the Ministry of Education, Science and Culture of Japan, and in part by the Georges und Antoine Claraz-Schenkung and the Karl Hescheler-Stiftung at the University of Zürich, Switzerland.

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Received April 22, 1993