Bull. Org. mond. Santé } 1967, **36**, 590-593
Bull. Wld Hlth Org.

Feeding Stimuli and Artificial Feeding

R. GALUN [1]

The blood-feeding process of mosquitos can be divided into four successive steps: (1) attraction to the host and settling, (2) probing with the fascicle, (3) sucking or maintained feeding, and (4) withdrawal of the stylets.

The response of the first two components is known to be elicited by visual, thermal, humidity, and chemical stimuli, operating separately or in combination (Laarman, 1955; see also review by Dethier, 1957). In the last decade, the factors involved in the transition from probing to sucking have been studied. So far, it is not known what determines the fourth step; that is, the cessation of feeding in mosquitos.

A blood meal taken by a mosquito passes directly to the midgut, while sugar solutions are generally dispatched to the diverticula. Day (1954) suggested that the epipharyngeal sensilla of *Aedes aegypti* acted as monitors to control the distribution of ingested fluids between the diverticula and the midgut. The observations of Owen (1963) support Day's suggestion that the cibarial taste receptors are stimulated by blood and supply the sensory output to control sucking. Hosoi (1959) identified erythrocyte adenosine 5′-phosphates as the main stimulus for gorging and dispatching the blood meal into the midgut in *Culex pipiens*. In *Culex*, adenosine 5′-monophosphate (AMP) is the most active nucleotide (Hosoi, 1959); in *A. aegypti*, adenosine triphosphate (ATP) is the most active stimulant among blood nucleotides (Galun, Avi-Dor & Bar-Zeev, 1963). Many other mosquito species are stimulated by ATP (Rutledge, Ward & Gould, 1964).

The aim of this work was to elucidate the specific molecular mechanism of stimulation of the taste receptors by adenosine nucleotides. Preliminary results of this study have been published previously.

The technique employed in our experiments was as follows. In each test, 70 or more female mosquitos were kept in cylinders covered with " silverlight " membranes through which they could probe and suck blood or other stimulant-containing solutions. All solutions offered were at 38°C and contained a small amount of red dye (Safranin O) so that the feeding response (i.e., the percentage of mosquitos fed on the solutions through the membrane) could be determined by the presence of the dye in the midgut.

The membrane feeding technique has often been utilized in studies on transmission of bacterial, viral and protozoan pathogens by mosquitos. It is a useful tool in nutritional and behavioural studies and has also been employed for the collection of oral secretions from mosquitos (Allen & West, 1962). Tarshis (1958) reviewed the literature on this subject and tested a great variety of membranes in detail. He found that the best membrane for use in studies with mosquitos is " silverlight ", which is obtained from the outermost layer of the caecum of the ox. However, this material is no longer available commercially, and more recently several investigators (Collins, Harrison & Skinner, 1964; Rutledge, Ward & Gould, 1964) had satisfactory results with Baudruche membranes made from the intestine of the ox.[2]

In general, the literature indicates that animal-derived membranes are superior to those of vegetable or synthetic origin. Recently, Parafilm " M " membrane, which is made of wax, was reported to be satisfactory for feeding *Anopheles stephensi* but very poor for *Aedes* (Rutledge, Ward & Gould, 1964). The author believes that the inferiority of this membrane for *Aedes* is due to its impermeability to water and water-soluble materials. " Silverlight " and Baudruche membranes are highly permeable, and perhaps *Aedes* is stimulated to probe by some material that diffuses through the membrane so that it can be detected by the tarsal chemoreceptors.

Among the adenine nucleotides tested in stimulating the feeding response, effectiveness increased in the following order: adenosine 5′-monophosphate (AMP) < adenosine diphosphate (ADP) < adenosine triphosphate (ATP) < adenosine tetraphosphate. Diphosphopyridine nucleotide (DPN) and flavin adenine dinucleotide (FAD), which include adeno-

---

[1] Head, Department of Entomology, Israel Institute for Biological Research, Ness-Ziona, Israel.

[2] Supplied by Long & Long Co., Roosevelt Avenue, Belleville, N.J., USA.

sine 5′-diphosphoric acid as a part of their molecule (even though the phosphate groups occupy an " intermediate " position), also exerted marked feeding stimulation. When the phosphate group was in the 3′ instead of the 5′ position on the ribose, the activity was reduced and, when it was at the 2′ position, the stimulatory effect was almost nil.

The monophosphates and triphosphates of inosine, guanosine and cytidine were ineffective in eliciting response (Table 1).

The results indicate that, while the presence of the adenine moiety is a prerequisite for stimulation of the feeding response, the location and number of the phosphate groups is also of importance.

The optimal range of pH for feeding on adenine nucleotides was found to be between 8 and 9; i.e., higher than that of blood. Of the various buffers tested, $NaHCO_3$ was always superior to phosphate or Tris buffer. At 10^{-2} M $NaHCO_3$, most of the mosquitos were seen to feed to completion, while on the other buffers many fed only partially. At this concentration of $NaHCO_3$ (pH 8-9), the concentration which induces 50% feeding was found to be about 10^{-3} M AMP, 5×10^{-4} M ADP and 10^{-4} M ATP.

TABLE 1

FEEDING RESPONSE OF MOSQUITOS EXPOSED TO NUCLEOTIDES, RELATED COMPOUNDS, AND CHELATING AGENTS

Compound [a]	No. exposed	Percentage feeding 10^{-2} M	No. exposed	Percentage feeding 10^{-3} M
Adenosine	343	4	119	0
Ribose-5-phosphate	106	1		
Riboflavin-5′-phosphate	187	1		
Adenosine 5′-phosphate	1 066	74	574	17
Adenosine 3′-phosphate	89	59		
Adenosine 2′-phosphate	100	8		
ADP	580	70	392	31
ATP	631	85	1 037	58
Adenosine tetraphosphate	322	85	504	67
DPN	125	57		
FAD			63	39
Inosine monophosphate	134	1		
Inosine triphosphate	165	1	268	0
Guanosine monophosphate	154	2		
Guanosine triphosphate	195	2	209	1
Cytidine monophosphate	94	0	25	1
Cytidine triphosphate	192	0	217	0
EDTA	612	20	479	3
Dipicolinic acid	562	11	294	3
None	264	3		

[a] All compounds were offered in 0.15 M NaCl.

ATP is known to participate in many metabolic processes of the cell. In order to determine whether the stimulation of the mosquito by ATP is due to interaction directly with a receptor enzyme, metabolic inhibitors were added to the stimulatory solution. None of these compounds—iodoacetate, 5,5′-dithiobis,-2-nitrobenzoic acid, potassium fluoride or ouabain—obliterated the feeding response due to ATP (Table 2).

It was therefore concluded that the ATP receptor surface is not that of an enzyme and that ATP does not stimulate the chemoreceptor *via* the glycolytic cycle. Failure of metabolic inhibitors to block stimulation of sugar receptors of the blowfly has also been reported, and it was also concluded that the glycolytic cycle was not involved (Dethier, 1956).

Hosoi (1959) found that osmotic pressure isotonic to blood was essential to obtain stimulation by AMP. The author investigated the role of osmotic pressure and of various ions by offering the mosquitos AMP or ATP in increasing concentrations of NaCl or other salts. The lowest concentration of NaCl that gave maximum results (more than 70% feeding) was 0.15 M. When the saline was replaced by an isotonic solution of a non-electrolyte, never more than 25% feeding was observed. With distilled water (containing AMP), there was practically no feeding. With NaCl at 0.037 M, the percentage of mosquitos

TABLE 2

FEEDING RESPONSE OF *AEDES AEGYPTI* IN THE PRESENCE OF INHIBITORS

Compound [a]	No. exposed	Percentage feeding
None	89	76
10^{-3} M iodoacetate	263	78
10^{-3} M′-dithiobis-2-nitrobenzoic acid	156	61
10^{-3} KF	255	57
10^{-3} Ouabain	277	51

[a] Added to 10^{-3} M ATP 10^{-2} M $NaHCO_3$ 0.15 M NaCl.

TABLE 3

EFFECT OF VARIOUS IONS AND NON-ELECTROLYTES ON THE FEEDING RESPONSE OF MOSQUITOS

Composition of medium	No. exposed	Percentage feeding
Distilled water	273	2
0.037 M NaCl	373	28
0.075 M NaCl	333	41
0.15 M NaCl	537	76
0.30 M NaCl	429	71
0.15 M KCl	363	15
0.10 M $CaCl_2$	305	6
0.10 M $MgCl_2$	299	10
0.15 M NaCl+0.15 M KCl	259	18
0.15 M NaCl+0.10 M $CaCl_2$	298	6
0.15 M NaCl+0.10 $MgCl_3$	341	6
0.30 M glucose	288	25
0.30 M sucrose	476	22
0.30 M sucrose (without AMP)	379	1
0.30 M lactose	393	21
0.037 M NaCl+0.225 M lactose (isotonic)	380	27

feeding (Table 3) could not be further increased by adjusting the tonicity with lactose. When Na^+ was replaced by K^+, Ca^{++}, or Mg^{++}, the percentage of mosquitos feeding was low, and when these ions were added to AMP in NaCl, a strong inhibitory effect was exerted (Table 3). Thus it seems that in addition to the need for osmotic pressure, Na^+ is specifically required for the response to be optimum.

The dependence of the system on Na^+ and the inhibition of K^+ recall the now well-known Hodgkin-Huxley-Katz theory of excitation which ascribes depolarization to a sudden increase in Na^+ conductance, followed by increase in K^+ conductance. It was therefore supposed that ATP, which is known to be a good chelator, acts upon divalent ions of the dendrite membrane of the chemoreceptor. This assumption was supported by our observations on the relative stimulating activity of the various adenine nucleotides, which seems to correlate very well with their chelating properties. Thus, with increased length of the phosphate chain, the stability of the complex with divalent ions increases in the order AMP<ADP<ATP (Walaas, 1958; Khan & Martell, 1962a). Complexes of metals are also formed with nucleotides containing two " intermediate " phosphate groups such as FAD and DPN (Walaas, 1958). The complexes of adenosine-3′-phosphoric acid with divalent ions are less stable than those of the 5′-phosphoric acid, due to the proximity of the phosphoric group to the adenine residue (Khan & Martell, 1962b).

The effect of some chelating agents such as ethylenediamine-tetra-acetic acid (EDTA) and dipicolinic acid, which could affect membrane-bound divalent ions, was studied. Both had some stimulatory effect on feeding (Table 1).

There are numerous data establishing the importance of divalent ions, especially Ca^{++}, in determining membrane permeability (Edelman, 1961). By offering the mosquitos metal chelates of ATP of increasing stability constants, an attempt was made to determine which ion is involved in the change of permeability of the membrane of the ATP receptor. The stability increases in the sequence Ca<Mg<Co<Mn<Zn<Ni<Cu (Khan & Martell, 1962a, 1962b). Chelating of ATP with Ca, Mg, Co or Mn did not interfere with the stimulatory effect of ATP. Chelates of zinc or of metals with higher stability constants such as Ni or Cu provoked practically no feeding response (Table 4). If zinc is given in a 2×10^{-3} M solution in the presence of 10^{-2} M ATP there is no inhibition in the feeding response (Table 4); i.e., Zn-ATP chelate does not inhibit feeding as long as there is free ATP in the solutions.

The role of zinc in processes of biological interest has commanded increasing attention in recent years

TABLE 4

EFFECT OF ADDITION OF DIVALENT IONS ON THE STIMULATION OF FEEDING RESPONSE IN MOSQUITOS BY ATP

Compound [a]	No. exposed	Percentage feeding
$CaCl_2$	517	72
$MgCl_2$	291	84
$CoCl_2$	193	67
$MnCl_2$	260	64
$ZnCl_2$	173	8
$ZnCl_2$ (+10^{-2} M ATP)	116	84
$NiCl_2$	207	1
$CuCl_2$	106	1

[a] Added in 2×10^{-3} M to 10^{-3} M ATP in 0.15 M NaCl and 10^{-2} $NaHCO_3$.

(Myers, 1963). Edman (1960) has shown that zinc is present in the skeletal muscle in appreciable amounts and may play a role in muscular contraction. Complexers of Zn^{++} such as ATP and EDTA reduce the amount of free Zn^{++} in the muscle and cause the system to contract. Edman postulated that the ATP of the muscle chelates the Zn^{++}. When the Zn^{++} is released from the chelate after hydrolysis of ATP to AMP, muscular relaxation takes place again.

On the basis of our results, the following hypothesis is suggested. Depolarization of the dendrite membrane depends on the removal of Zn^{++} ions from the site to which they are bound on the membrane (and not Ca^{++} as previously suggested). ATP could act at this stage because of its chelating properties. In about one quarter of the mosquito population, Zn^{++} removal is sufficient to depolarize the nerve membrane, as shown by the results obtained with non-electrolyte solutions. In the bulk of the population, however, an influx of Na^{++} made possible by the removal of Zn^{++} is necessary in order to lower the potential and depolarize the membrane. It could be assumed that the nucleotide is bound through the adenine moiety to a specific receptor site on the surface of the membrane. The geometry of the ATP molecule may be such that the terminal phosphate groups of the bound nucleotide are brought into juxtaposition with a second site on the surface where the Zn^{++} ion is located. This may explain why ATP is superior to other polyphosphates as a stimulant and also to stronger chelating agents such as EDTA, despite its lower complex formation constant with Zn^{++} ions.

REFERENCES

Allen, J. R. & West, A. S. (1962) *Mosquito News*, **22**, 157-159

Collins, W. E., Harrison, A. J. & Skinner, J. C. (1964) *Mosquito News*, **24**, 25-27

Day, M. F. (1954) *Aust. J. Biol. Sci.*, **7**, 515-524

Dethier, V. G. (1956) *Amer. Inst. Biol. Sci. Publ.*, **1**, 1-30

Dethier, V. G. (1957) *Exp. Parasit.*, **6**, 68-122

Edelman, I. S. (1961) *Ann. Rev. Physiol.*, **23**, 37-70

Edman, K. A. P. (1960) *Acta physiol. scand.*, **49**, 330-342

Galun, R., Avi-Dor, Y. & Bar-Zeev, M. (1963) *Science*, **142**, 1674-1675

Hosoi, T. (1959) *J. Insect Physiol.*, **3**, 191-218

Khan, M. M. T. & Martell, A. E. (1962a) *J. Amer. chem. Soc.*, **84**, 3037-3041

Khan, M. M. T. & Martell, A. E. (1962b) *J. physiol. Chem.*, **66**, 10-15

Laarman, J. J. (1955) *The host seeking behaviour of the malaria mosquito* Anopheles maculipennis atroparvus, Utrecht, Kemink en Zoon (Thesis, University of Leiden), 1-44

Myers, H. M. (1963) *Ann. Rev. Pharmacol.*, **3**, 307-334

Owen, W. B. (1963) *J. Insect Physiol.*, **9**, 73-87

Rutledge, L. C., Ward, R. A. & Gould, D. J. (1964) *Mosquito News*, **24**, 407-419

Tarshis, I. B. (1958) *Feeding techniques for bloodsucking arthropods.* In: *Xth International Congress of Entomology, Montreal, Aug. 17-25, 1956 (Proceedings)*, pp. 767-783

Walaas, E. (1958) *Acta chem. scand.*, **12**, 528-536
