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THE STUDY OF INSECT BLOOD-FEEDING BEHAVIOUR. 1: FEEDING EQUIPMENT, PHYSICAL AND ENDOGENOUS FACTORS, DOSE EFFECT ANALYSIS, AND DIET DESTINATION

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Experimental techniques that we have found useful during our studies of insect blood-feeding behaviour are reviewed. Some of the principal findings resulting from these techniques are discussed. Where directly applicable, the work of others is included, but no complete review of the subject has been attempted.

Since 1965, the feeding behaviour of haematophagous insects has been studied in our laboratory. The effects of nucleotide phagostimulants on *Rhodnius prolixus* have been determined (Friend & Smith, 1982), and the details of the feeding behaviour of this insect analyzed (Smith & Friend, in this volume). Subsequent to 1977, aspects of the feeding behaviour of the mosquito *Culiseta inornata* (Friend, 1985 b) and the black fly *Simulium venustum* (Smith & Friend, 1982) were investigated. The feeding behaviour of the horsefly *Tabanus nigrovittatus* was studied in collaboration with J.G. Stoffolano, Jr. (Friend & Stoffolano, 1984).

Some of the principal approaches and findings resulting from our work on blood-feeding insects are reviewed in this paper and in Smith & Friend (in this volume). Where relevant, work of others is included but no exhaustive attempt has been made to review blood-feeding behaviour in insects. Instead, we hope that a more detailed discussion of approaches and methodology that can usually be included in published works will be useful to others in the field.

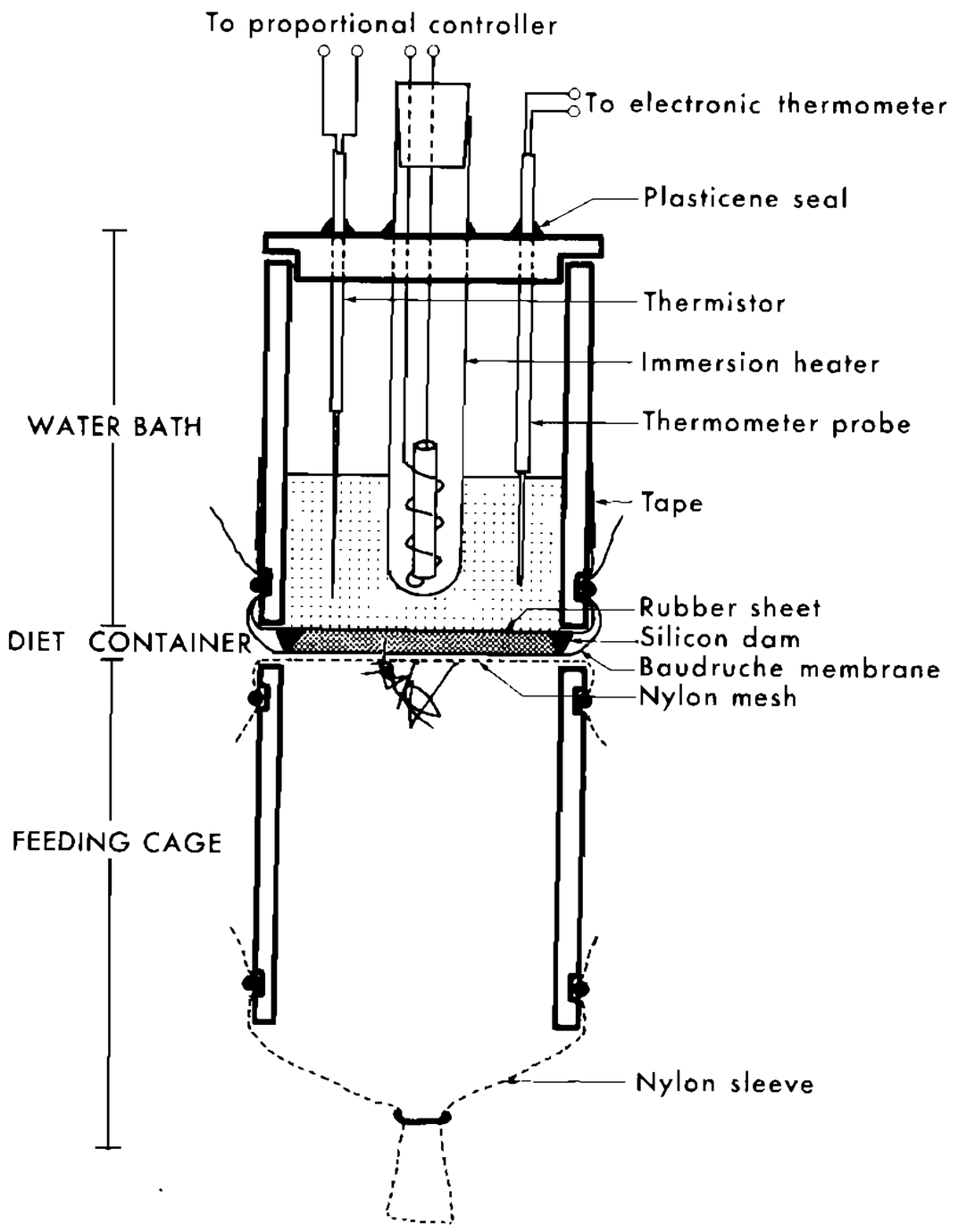

Fig. 1: Feeding apparatus for feeding small volumes of radioactive or expensive diets to mosquitoes and other blood-sucking insects (Friend & Hewson, 1978).

FEEDING APPARATUS

We have used various types of feeding apparatus to hold either artificial saline diets or blood. The basic requirements are for a container that allows the diet to be held at body temperature in contact with a suitable membrane without the interference of bubbles. An apparatus that allows detailed observations while the insect feeds has also proven to be most useful (Smith & Friend, in this volume).

Three principal methods are used to control diet temperature, which is normally maintained at $37 \pm 2^{o}C$. For the mosquito *Cu. inornata*, a micro-electrical immersion heater was developed to warm a small amount of water which, in turn, heats the diet (Figs. 1 and 2) (Friend & Hewson, 1978). For *R. prolixus*, diet chambers are heated by conduction through their bases: they are simply placed on the electrically heated surface of a commercial slide warmer (Smith & Friend, 1970), or for the "revolving restaurant" (see below and Fig. 3) on a kymograph drum heated by radiation from an infrared lamp (Smith, 1979). Radiant heating is also used for special feeding chambers designed for photographing mouthpart movements: a microscope lamp without a heat filter is focussed on a piece of black tape stuck directly on the chamber

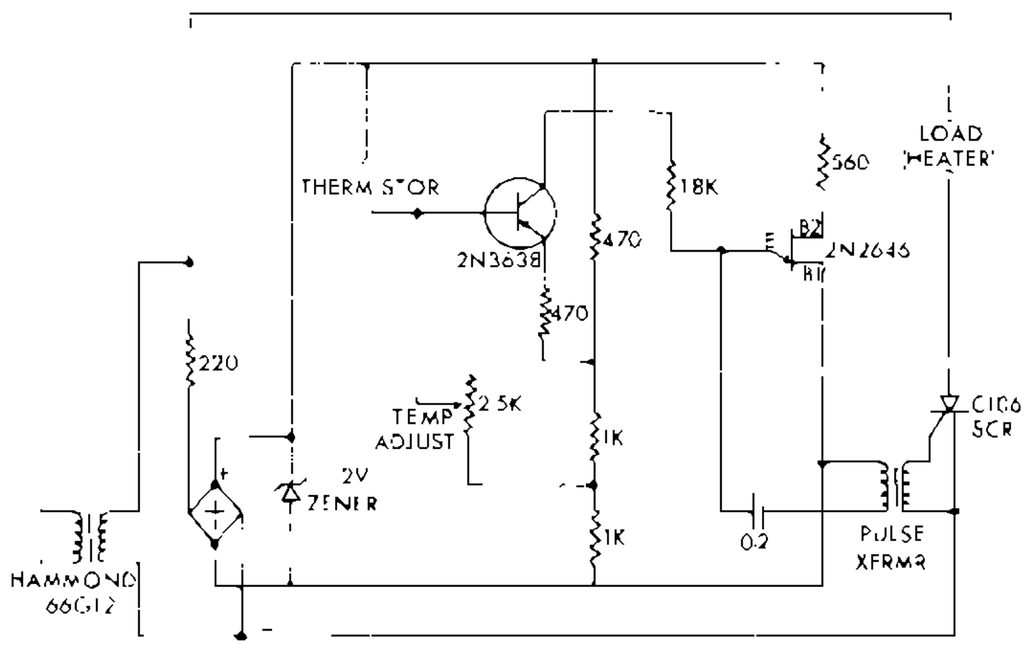

Fig. 2: Circuit diagram for proportional controller of the immersion heater in Fig. 1 (Friend & Hewson, 1978).

(Smith & Friend, 1971). For the horsefly *T. nigrovittatus*, the diets are warmed to 38°C in a water bath, then transferred to diet chambers that have been warmed on a commercial slide warmer. The diet chambers are then placed on top of the feeding cages and the temperature is monitored by means of thermistors inserted in the diet. Bright lights are directed at the diet chambers and the feeding cages to maintain very high light levels (see below), and these lights keep the diet warm. When the diet temperature drops below 35°C, the diet chamber is exchanged for one warmed to 38°C (Friend & Stoffolano, 1983).

We have not found temperature control to be very critical. Feeding occurs when there is presumably a sufficient temperature differential, sensed by the insect's heat receptors. Too high a temperature will inhibit feeding.

With the feeding apparatus shown in Fig. 1, the diet temperature can be controlled to ±0.1°C. This control is achieved by a proportional controller (Fig. 2) which supplies power to a micro-immersion heater in proportion to the difference between the actual and the desired temperature (Friend & Hewson, 1978). This apparatus has other advantages: 1 ml of diet provides about 7 cm^2 of feeding surface. The ability to use such small volumes of diet reduces the expense when using costly phagostimulants and the hazard when feeding radioisotopes. Because the diet is contained between two membranes, it can easily be removed and discarded after use. This feature minimizes

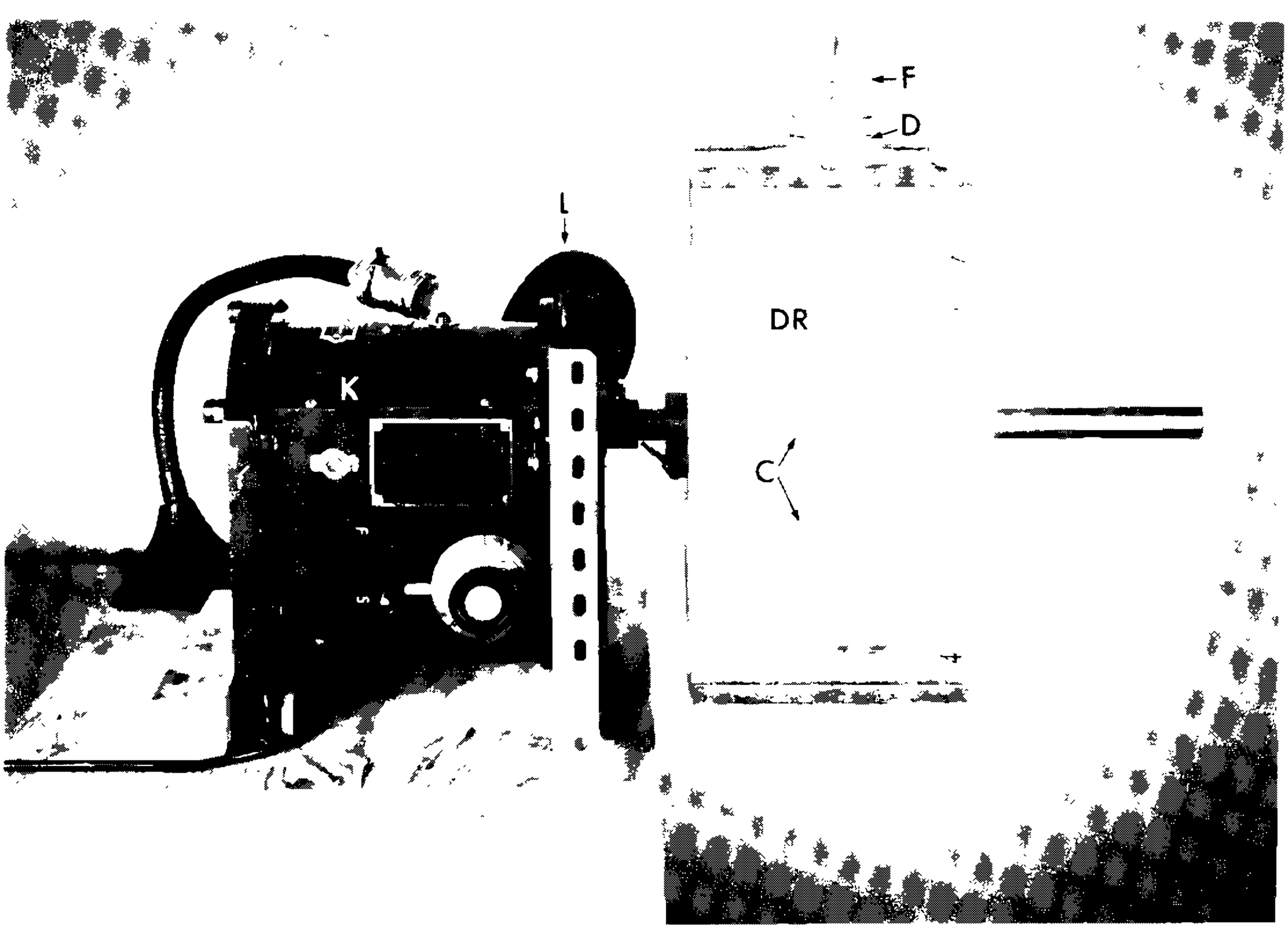

Fig. 3: "Revolving restaurant." The drum (DR) of a kymograph (K) placed on its side is warmed by an infra-red light (L) shining on the drum's inner surface. Feeding chambers (F) containing test insects are attached with Parafilm to diet chambers (D), that are slid into metal clamps (C) on the drum. The drum revolves at about 2 RPM during feeding.

contamination when using either radioactive or infectious diets (Friend & Hewson, 1978).

A special feeding apparatus nicknamed "the revolving restaurant" was developed for diets that contained red blood cells (Smith, 1979). It is important that these cells remain in suspension and evenly distributed throughout the diet during the test feeding period. Initial attempts using magnetic stirrers were not successful. We decided to attach the diet chambers to a horizontally mounted kymograph drum that rotates once every 30 s, keeping the red cells in suspension and the average concentration near the membrane equal to the overall concentration in the diet. Fig. 3 shows this apparatus. Each diet chamber consists of a Plexiglass cylinder approximately 4 cm inside diameter and 1 cm high, closed at the bottom end by a 5 cm square of Plexiglass. After being filled with warm diet, it is covered with a membrane (see below) secured by a rubber O-ring. Test insects are confined in feeding chambers consisting of Plexiglass cylinders closed with nylon mesh through which the insects can probe and feed. The feeding chambers and diet chambers are connected with tightly wrapped Parafilm, and then attached to the outer surface of the kymograph drum by sliding the base of the feeding chamber into metal clips attached to the kymograph drum. The drum is initially heated and kept warm by a 250 W infra-red lamp shining on its inner surface. No significant differences were found in the responses of *R. prolixus* to ATP-saline diets when tested on this apparatus and when tested on stationary feeding chambers (Smith & Friend, 1970).

Sometime ago we wanted to see what would happen if the gorging stimulant was removed at various times after feeding had commenced. This led to the development of a feeding apparatus that allows the rapid exchange of diets as the insect feeds (Friend & Smith, 1977a). Figs. 4 and 5 show the details of this apparatus. The basis of the design is a concentric flow system: the incoming diet enters via the inner tube and "washes" the inside surface of the feeding membrane before exiting via the outer tube. When testing earlier designs, we found that the inner tube should be kept as short as possible. It is the common path of the two diets before the membrane that is kept as short as possible: because of lower fluid velocities at the outside of the tube lumen, the longer the shared path, the more "smeared out" will be the front between the original and the new diet. It is also important to keep the inner

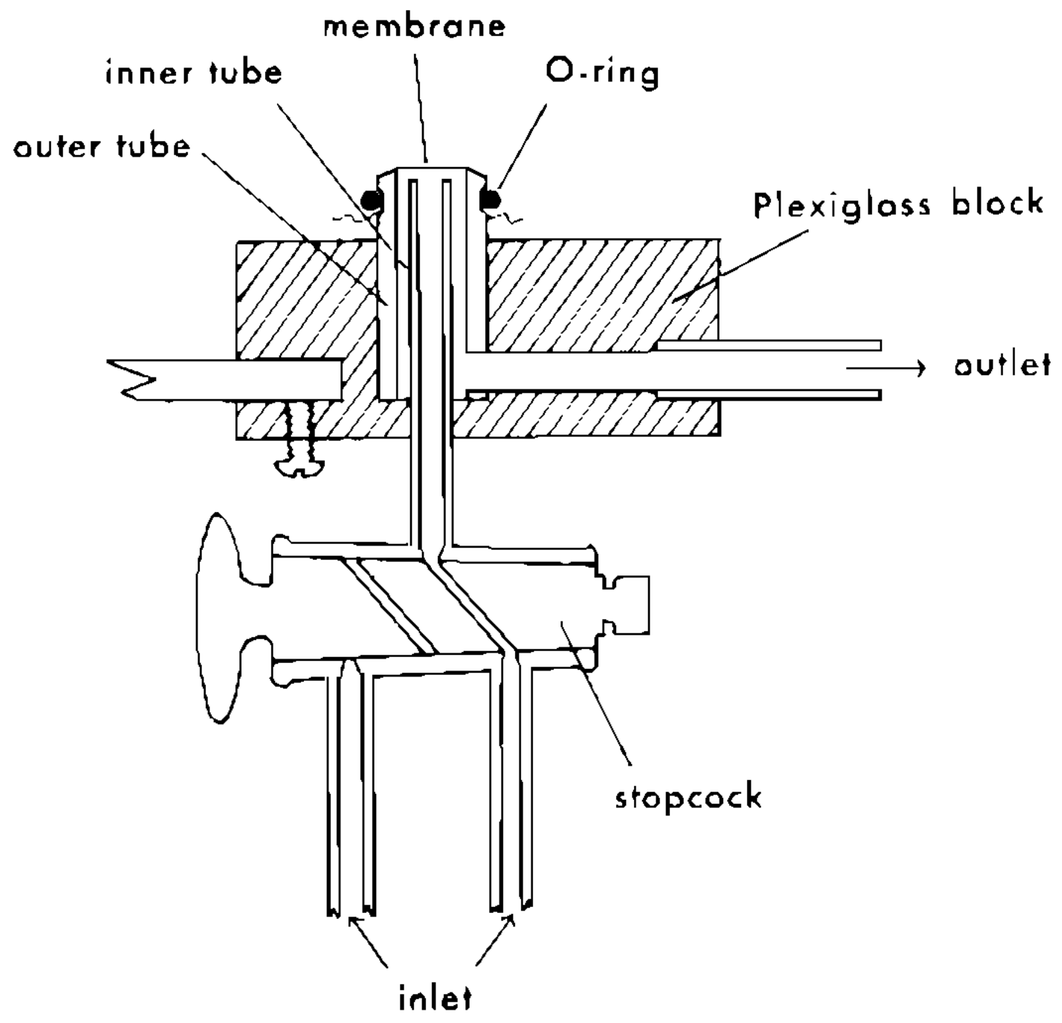

Fig. 4: Apparatus used to exchange diets during feeding of blood-sucking insects (Friend & Smith, 1977a).

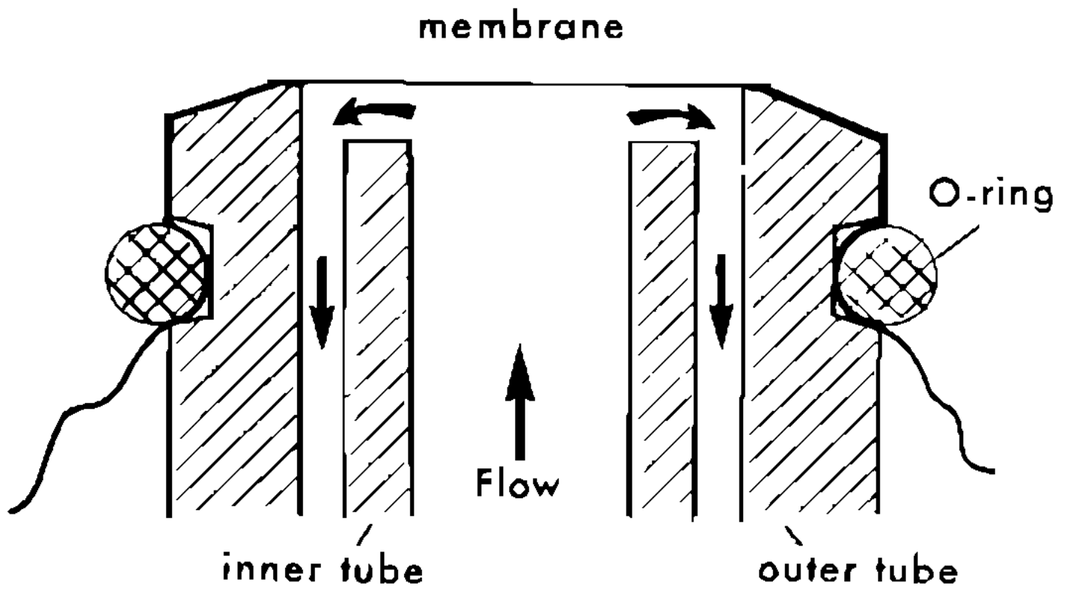

Fig. 5: Detail of the exchange area shown in Fig. 4, showing path of diet flow (Friend & Smith, 1977a).

tube straight, since even a gentle bend can cause the flow to be diverted to the outside of the bend, resulting in a "dead" region of old diet. Possible turbulence at the membrane due to rapid changes of diet velocity is minimized by keeping the cross-sectional area of the fluid pathway fairly constant. This is accomplished by making the inside diameter of the outer tube such that the annular cross-section of this part of the pathway equals that of the lumen of the inner tube. The end of the inner tube is also set away from the membrane by a distance of approximately one-quarter of its internal diameter. Thus the cylindrical area through which the diet passes between the end of the inner tube and the membrane is approximately the same as the cross-sectional area of the lumen of the inner tube.

Mechanical disturbance of the membrane during diet exchange was minimized by using a short length of wide diameter tubing as the outlet, and positioning the liquid level of the

outlet tube at about the same level as the membrane, thus keeping the pressure at the inner surface of the membrane approximately atmospheric. The level of the liquids in the diet reservoirs attached to the supply tubes were also kept just above the level of the membrane, to minimize presure differences that could cause the membrane to move excessively during exchange (Friend & Smith, 1977a).

PHYSICAL AND ENDOGENOUS FACTORS

Physical factors such as light levels, the presence or absence of a heat gradient, and the presence or absence of a membrane through which feeding takes place can affect the endogenous condition of a blood-feeding insect, changing its "central excitatory state" (Dethier, 1976). Changes in this state can affect the thresholds of response to stimuli, including phagostimulants, and can determine the destination of the ingested diet in the alimentary system (Friend & Smith, 1972, 1977b; Friend & Stoffolano, 1984; Friend, 1985a). Every effort should be made to test the insects in as uniform a manner as possible. Differences in the treatment of test insects prior to testing can have significant effects.

Nutritional condition

The sensitivity of *R. prolixus* to ATP increases during food deprivation. The relationship between sensitivity to ATP and the time elapsed since the previous blood meal is linear: sensitivity increases by about 20% of its value at day 15, for every 10 days the insect is further deprived of food (Friend & Smith, 1975).

The nutritional condition of the mosquito *Cu. inornata* prior to testing also affects its feeding responses (Friend, 1985a). After being removed from the colony, some insects were fed 1M sucrose and then held for 6 or 24h prior to further testing. These were compared with other insects held for the same periods but not fed when removed from the colony. When allowed to feed on water after the holding periods, the 24h unfed mosquitoes lost control of both diet destination and the amount they ingested; insects in the other three categories did not. The patterns of response to 1M sucrose presented after the holding periods were similar for the two unfed categories and for the insects held 24h after the initial meal. Insects fed 6h prior to testing still had their crops distended with the previous sugar meal, and although 64% still fed, they did not ingest as much as the other insects (Friend, 1985a). Obviously the state of nutrition of the insects prior to testing should be carefully considered, since it can affect the results to such a significant extent.

Membranes and mouthpart deployment

Most haematophagous insects will not feed normally on blood or on artificial diets containing phagostimulants if the diet is presented as a free liquid. Normal feeding occurs when the diet is covered with a suitable membrane which the insect can pierce, and consequently deploy its mouthparts as it would when feeding on a normal host (Friend & Smith, 1977b; Friend, 1978, 1985b; Friend & Stoffolano, 1984). The mouthpart deployment appears to lower the threshold of response to appropriate phagostimulants found in blood such as ATP. Unless the fascicle is extended from the labellar groove, *Cu. inornata* does not respond to ATP (Friend, 1985b). Spielman (1964) found that mouthpart deployment in which the labial sheath was retracted from the piercing mouthparts was essential for feeding fresh heparinized chicken blood from capillary tubes to the mosquito *Culex pipiens*. Similar mouthpart deployment was found to be necessary for Phlebotamine sandflies (Hertig & Hertig, 1927).

The selection of the most appropriate membrane is important. With the present state of knowledge, however, trial and error seems to be the only approach. Baudruche membrane (the outer layer of ox caecum) (Joseph Long Inc., Belleville, NJ, USA, 07109) works well with mosquitoes (Friend, 1978; Galun et al., 1985). Membranes of reconstituted collagen (sausage casings). are less effective but much cheaper (Wirtz & Rutledge, 1980). Condom latex works well as a feeding membrane for Triatomines and black flies (Friend & Smith, 1972; Smith & Friend, 1982). We have found slightly stretched Parafilm to be the best membrane for tabanids (Friend & Stoffolano, 1983). The tsetse fly researchers have found that thick membranes made of silicon and a fabric mesh are necessary for these flies to feed well (Bauer & Wetzel, 1976). In 1971, Galun reviewed the subject of membranes used for mass rearing blood-feeding arthropods.

Light levels

The level of light intensity at which the feeding trials are conducted can have significant effects on feeding behaviour. *T. nigrovittatus* feeds under laboratory conditions only if the

light intensity is high and the cage large enough for the insects to fly freely. Even the reflectivity of the cage is critical. Feeding takes place when the light level in a highly reflective aluminum cage is adjusted to 1200-1500 lux at the feeding membrane. This is approximately equal to the light intensity in the field at noon in temperate latitudes. At light levels below this, or if non-reflective cages are used, the insects will not feed (Friend & Stoffolano, 1983).

Immobilization and restraint techniques

We have found that the best way to immobilize *Cu. inornata* prior to operating on them is to chill them at -4°C for 12-15 min. This technique avoids the possibly harmful effects of anesthetics such as CO_2 or ether (Friend, 1978).

Bayberry wax, which has a low melting point, is useful for restraining insects. We attach *Cu. inornata* to applicator sticks by sticking their wings in a small ball of wax on the end of the stick. When we want to hold the mouthparts in a separated condition, a small drop of wax is first stuck to the side of the thorax. The fascicle is then carefully extricated from the labial groove and the labium is affixed gently to the drop of wax, leaving about 1 mm of the distal end of the labium free (Feir et al., 1961). A minimum amount of heat is used whenever the wax is melted. Insects treated in this way respond to water or sucrose diets in the same way as unoperated insects, and to ATP as if they were unrestrained mosquitoes feeding through a membrane (Friend, 1985a,b).

The heat is applied with a melting apparatus consisting of a small platinum wire loop mounted in pen-sized plastic tube and connected to a variable power source. If too much heat is used, the insects are traumatized and will not respond normally.

Sometimes, insects will not respond to artificial feeding situations unless a particular endogenous condition is invoked. The most difficult insect we have tested is the black fly *S. venustum*. Only flies caught *in vivo* while showing a characteristic probing behaviour would subsequently feed *in vitro*. This "blood-feeding mode" rapidly disappeared (ca. 2 min.) in the absence of stimuli eliciting actual probing, but for flies in this state, a small temperature rise was sufficient stimulus for probing (Smith & Friend, 1982).

DOSE-EFFECT ANALYSIS

The most appropriate technique for establishing the potency of a phagostimulant, a poison, or any other pharmacologically active substance is to determine the dose that gives a 50% response (the ED_{50}, or "effective dose for 50%"). Several concentrations of the test substance are chosen to obtain results both above and below the 50% response level.

The slope of the response plot should be as steep as possible, so that a minimum change in concentration of the test substance causes a maximum response in the test insects. Galun et al. (1984, in prep.) discovered that bicarbonate enhances the response to nucleotide phagostimulants in certain mosquitoes from 11 to 53-fold, and changes the slope of the response plot in *Ae. aegypti* from 0.48 to 1.26. This is an excellent example of how test conditions can be modified to produce the best results.

For dose-effect studies to be conducted, an "all-or-none" response must be established. For *R. prolixus* this is easy: either the insect gorges or it does not. For mosquitoes and tabanids, categories of engorgement had to be established. As long as these categories (e.g. "taste", "small", and "large" meals) are clearly defined, they will provide suitable data for analysis.

The data is best analyzed by probit techniques. A rapid graphic method which uses nomographs for the difficult conversions has been developed by Litchfield & Wilcoxon (1949). This method allows one to do probit analysis without even using a hand calculator. There are also rapid computer programs for probit analysis, such as one contained in the statistical software package from the Statistical Analysis System Institute (SAS) (Box 8000, Cary, NC, USA, 27511), available for various computers including personal computers.

Such pharmacological techniques have been used to determine the potency of phagostimulants such as nucleotide phosphates for blood-feeding insects. Relative potencies allow comparisons of how well a test molecule fits a receptor site. These techniques form the basis for Galun's discussion in this volume of the evolution of purinergic receptors.

DIET DESTINATION

Blood-feeding Diptera have complex feeding behaviours. Adult females display three modes of feeding: drinking, in which a small volume of water is taken into the midgut; nectar or sugar-feeding, in which a large amount of liquid fills the crop; and blood-feeding, in which a large amount of blood fills the midgut. Feeding

behaviour in each mode is the result of different patterns of stimulus-response events with feed-forward effects.

Appropriate stimuli early in the sequence can lower the threshold of response to appropriate stimuli that come later in the sequence. In the past, many authors studying feeding responses have not reported diet destination. This is regrettable, since the control of diet destination is such an important part of the sequence.

We have developed a simple technique to determine the amount ingested and the destination of the meal. Initially, we determined the amount of ingestion by adding C^{14} inulin to the diets and determining the amount of radioactivity in individual insects after they had fed. The radioactivity was measured by a scintillation counter (Friend, 1978). In addition to the radioactive inulin, we added 1% blue food coloring (Club House Brand) to the diet. Immediately after feeding, the insects were immersed in 70% alcohol and examined. The amount and location (crop or midgut) of the colored diet was easily observed through the abdominal cuticle. After using this technique for some time, we found that we could estimate the amount of ingestion by eye, and that these estimates were sufficiently accurate for us to rank the insects into four feeding categories; full, partially full, small meal or taste. Whenever there was any doubt about the amount of ingestion, the insects were scored in the lower category. Random checks in which our estimates were compared with quantitative data based on the radioactive counting convinced us that our estimates could be trusted within the limits of experimental error. Visual estimation techniques permit us to test many more insects than could possibly be handled using radioactive counting techniques.

In later studies the insects were immobilized in a freezer prior to dissection and examination. Under these conditions insects can be stored for several days before the determinations of diet ingestion and destination need be made.

These techniques have been successfully applied to studies on the mosquito *Cu. inornata* (Friend, 1978, 1981, 1985a, b) and the horsefly *T. nigrovittatus* (Friend & Stoffolano, 1983, 1984). They were also employed in recent investigations of the factors that control diet destination in these two Diptera. Interesting differences were found.

In the tabanid, diet destination is controlled by the osmotic pressure of the diet irrespective of the type of phagostimulant. Diets iso-osmotic to vertebrate blood are directed to the midgut whether they are fed as free liquids with sugar as the phagostimulant of through a membrane with ATP as the phagostimulant. Free liquid, sucrose diets, hyperosmotic to blood, are directed exclusively to the crop. Diets hyperosmotic to blood and fed through a membrane, with ATP as the phagostimulant, result in half the insects directing this diet to both the crop and the midgut, instead of the midgut alone as one would expect, since ATP phagostimulation is appropriate for the blood-feeding mode (Friend & Stoffolano, in prep.).

In the mosquito *Cu. inornata*, diet destination is not controlled by osmotic pressure but by specific stimulation of receptors that control the opening and closing of the valves that lead to the crop and midgut. Normally, when feeding commences, the valve to the midgut appears to be open and the valve to the crop closed. Sucrose causes the crop valve to open, and a strong sucrose signal causes the midgut valve to close. Under these conditions the sucrose diet would go to the crop as is the case in the nectar-feeding mode. Cellobiose is slightly phagostimulatory but cellobiose solutions usually go to the midgut. At high concentrations (0.5M) some opening of the crop valve occurs but there is no closing of the midgut valve. Combination diets of sucrose and cellobiose induce gorging, but diet destination is controlled by the concentration of sucrose (Friend et al., in prep.).

REFERENCES

BAUER, B. & WETZEL, H., 1976. A new membrane for feeding *Glossina morsitans* Westw. (Diptera: Glossinidae). *Bull. ent. Res.*, *65* :563-565.

DETHIER, V.G., 1976. *The Hungry Fly*. Harvard University Press: Cambridge, MA.

FEIR, D.; LENGY, J.I. & OWEN, W.B., 1961. Contact chemoreception in the mosquito, *Culiseta inornata* (Williston); sensitivity of the tarsi and labella to sucrose and glucose. *J. Insect Physiol.*, *6* :13-20.

FRIEND, W.G., 1978. Physical factors affecting the feeding responses of *Culiseta inornata* to ATP, sucrose, and blood. *Ann. ent. Soc. Am.*, *71* :935-940.

FRIEND, W.G., 1985a. Effects of mating, nutritional condition, and mouthpart separation on ingestion and destination of sugar and water by the mosquito *Culiseta inornata*. *Physiol. Ent.*, *10* :137-144.

FRIEND, W.G., 1985b. Diet ingestion and destination in *Culiseta inornata* (Diptera: Culicidae): Effects of mouthpart deployment and contact of the fascicle and labellum with sucrose, water, saline, or ATP. *Ann. ent. Soc. Am.*, *78* :495-500.

FRIEND, W.G. & HEWSON, R.J., 1978. A small volume, thermostatically controlled apparatus for

feeding radioactive diets to mosquitoes and other sucking arthropods. *Mosquito News, 38* :536-541.

FRIEND, W.G. & SMITH, J.J.B., 1972. Feeding stimuli and techniques for studying the feeding of haematophagous arthropods under artificial conditions, with special reference to *Rhodnius prolixus.* P. 241-255; In: J.G. Rodrigues (ed.) *Insect and Mite Nutrition.* North-Holland: Amsterdam.

FRIEND, W.G. & SMITH, J.J.B., 1975. Feeding in *Rhodnius prolixus:* increased sensitivity to ATP during prolonged food deprivation. *J. Insect Physiol., 21* :1081-1084.

FRIEND, W.G. & SMITH, J.J.B., 1977a. An apparatus for exchanging diets rapidly during artificial feeding of sucking insects. *Can. Ent. 109* :15-20.

FRIEND, W.G. & SMITH, J.J.B., 1977b. Feeding behaviour of blood-sucking insects in relation to transmission of parasites. P. 1-24. In: A.M. Fallis (ed.), *Parasites: Their World and Ours.* Proc. 18th Symp. Roy. Soc. Can.

FRIEND, W.G. & SMITH, J.J.B., 1982. ATP analogues and other phosphate compounds as gorging stimulants for *Rhodnius prolixus. J. Insect Physiol., 28* :371-376.

FRIEND, W.G. & STOFFOLANO, J.G. Jr., 1983. Feeding responses of the horsefly, *Tabanus nigrovittatus,* to phagostimulants. *Physiol. Ent., 8* :377-383.

FRIEND, W.G. & STOFFOLANO, J.G. Jr., 1984. Feeding responses of the horsefly *Tabanus nigrovittatus* to physical factors, ATP analogues and blood fractions. *Physiol. Ent., 9* :395-402.

GALUN, R., 1971. Recent developments in the biochemistry and feeding behaviour of haematophagous arthropods as applied to their mass rearing. P. 273-282. In: *Sterility Principle for Insect Control or Eradication.* Vienna: IAEA.

GALUN, R.; KOONTZ, L.C.; GWADZ, R.W. & RIBEIRO, J.M.C., 1985. Effect of ATP analogues on the gorging response of *Aedes aegypti. Physiol. Ent., 10* :275-281.

GALUN, R.; OREN, N. & ZECHARIA, M., 1984. Effect of plasma components on the feeding response of the mosquito *Aedes aegypti* to adenine nucleotides. *Physiol. Ent., 9* :403-408.

HERTIG, A.T. & HERTIG, M., 1927. A technique for the artificial feeding of sandflies (*Phlebotomus*) and mosquitoes. *Science, 65* :328-329.

LITCHFIELD, J.T. & WILCOXON, F., 1949. A simplified method of evaluating dose-effect experiments. *J. Pharmac. exp. Ther., 95* :99-113.

SMITH, J.J.B., 1979. The feeding response of *Rhodnius prolixus* to blood fractions, and the role of ATP. *J. exp. Biol., 78* :225-232.

SMITH, J.J.B. & FRIEND, W.G., 1970. Feeding in *Rhodnius prolixus:* responses to artificial diets as revealed by changes in electrical resistance. *J. Insect Physiol., 16* :1709-1720.

SMITH, J.J.B. & FRIEND, W.G., 1971. The application of split-screen television recording and electrical resistance measurement to the study of feeding in a blood-sucking insect (*Rhodnius prolixus*). *Can. Ent., 103* :167-172.

SMITH, J.J.B. & FRIEND, W.G., 1982. Feeding behaviour in response to blood fractions and chemical phagostimulants in the black-fly, *Simulium venustum. Physiol. Ent., 7* :219-226.

SPIELMAN, A., 1964. Studies on autogeny in *Culex pipiens* populations in nature. I. Reproductive isolation between autogenous and anautogenous populations. *Am. J. Hyg., 80* :175-183.

WIRTZ, R.A. & RUTLEDGE, L.C., 1980. Reconstituted collagen sausage casing for the blood feeding of mosquitoes. *Mosquito News, 40* :287-288.