STUDIORUM PROGRESSUS

Problems of Insect Tissue Culture[1]

By M. E. MARTIGNONI[2]

The excellent surveys of the literature on insect tissue culture recently completed by LOEB[3], HARTZELL[4], and DAY and GRACE[5], show that repeated subculturing of rapidly proliferating cell colonies has so far eluded all workers. At present no insect cell strains are available, and even a standard method for the routine production of primary cultures, comparable to the preparation of chick fibroblast cultures, is unavailable.

Due mainly to the work of TRAGER[6], WYATT[7], WYATT, LOUGHEED, and WYATT[8], and GRACE[9], media have been developed in which good survival of some cells of the silkworm, *Bombyx mori* (L.), is attained, and, in some instances, even moderate growth has been claimed. However, it has not been possible so far to prepare large numbers of uniform cultures for the study of growth requirements. This, in turn, has resulted in the poor knowledge of the requirements of insect cells *in vitro*. The present situation in insect tissue culture is a vicious circle, because the knowledge of cell nutrition and the production of cells *in vitro* are two interchangeably dependent factors.

Much of the success in the culture of mammalian cells derives largely from the extensive knowledge we have of the biochemistry and physiology of the higher animals. There is no such deep and extensive knowledge in insect physiology: this is a relatively new field, and what we know is based almost exclusively on studies of two Coleoptera, three Lepidoptera, three Diptera, three Orthoptera, and one species each of Hymenoptera and Hemiptera. A dozen species are very few compared to the more than 800000 insect species named up-to-date. Furthermore, function differs greatly, not only between insect species, but also between stages and instars of one single species, as in the case of the holometabola. For example, according to data compiled by BUCK[10], the hydrogenion concentration of insect blood varies interspecifically between pH 6 and pH 7·5. Intraspecifically it varies up to 0·7 pH units, while in man the pH of plasma varies only between 7·35 and 7·45. The osmotic pressure, expressed as g NaCl/100 ml, varies interspecifically between 0·60 and 2·01 in insects. In our laboratory, we found this value to vary intraspecifically, and for one single instar, between 0·80 and 1·03 for *Peridroma margaritosa* (Haworth). In man the variation is small, between 0·83 and 0·85. As BUCK puts it, «The concept of an 'average' insect blood has no validity.»

Evidently, the approach to insect tissue culture cannot be successful if one ignores these considerations. Insect tissue culture problems must be solved for every single species, or possibly for groups of species having a certain amount of physiological unity.

Since very limited success has been derived from the empirical application of vertebrate culture techniques to insect tissue culture, it becomes necessary to examine the basic differences between the two types of organisms. Such differences may be found particularly in (1) blood, (2) respiratory mechanism and metabolism, (3) metamorphosis, (4) tissues, and (5) bacterial contamination.

What differences do we find between the hemolymph of insects and blood of vertebrates? Hemolymph is the only body fluid present in insects, resembling both the blood of vertebrates and the lymph. It circulates mainly in direct contact with tissues, rather than through vessels. Of course, it will be impossible to consider here, even summarily, all the differences which exist between vertebrate and insect blood. Only some of these will be discussed, to give an idea of the diversity between the classes of organisms, and within the one class with which we are particularly concerned.

Until some years ago, insect hemolymph was considered to differ from the blood of other animals in having a high potassium and a low sodium content. This is not true: recent work (see BUCK[10]) has definitely shown that, here too, *natura non facit saltum*. There is a continuity between insects and other animals in the mole ratio Na/K (from 0·1 to 25). Usually, and with only a few exceptions, the phytophagous insects have ratios of less than 1, the carnivores have ratios greater than 1, and the omnivorous species have ratios which fall between these two. Man has a Na/K ratio of 29, close (and not too surprisingly) to the one of *Cimex lectularius* L., the bedbug. The Na/K ratio in insect hemolymph cannot be changed appreciably by changing their diets: it is genetically fixed, and insects maintain it through effective regulation of the ion content of their blood. The intracellular Na/K ratio, on the other hand, is the same for man and the few insect species investigated so far: that is, between 0·3 and 0·4. The fact that tissue with a high potassium rate is present in environments both with high and low potassium rates has led some to think that insect tissue may be more tolerant to ion concentration than vertebrate tissue. This however, remains to be proved.

The free amino acid content of insect blood plasma is very high (see Table). While in human plasma the amino nitrogen content amounts to about 5 mg%, in insects it may reach up to 70 times this value, as in pupae of some species of *Saturnia*. Again, the amino acid content of insect blood varies considerably interspecifically (see Table) as well as intraspecifically (between stages and instars of the same species). Amino acids contribute 40% or more of the total blood osmotic pressure in insects, while in man 92% of the osmotic pressure is due to inorganic ions, and only 1% to amino acids. WYATT, LOUGHEED, and WYATT[8] were able to establish only one constant factor in amino acid composition of the plasma of three species of insects: in two Lepidoptera and one species of Hymenoptera glutamine was a major component. In the silkworm there seems to be a constant preponderance of glutamine, histidine, and lysine during

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[3] MARCIA JOAN LOEB, Thesis Cornell University, Ithaca (N. Y.) (1957).

[4] A. HARTZELL, Proc. intern. Congr. Entomol., 10th Congr., Montreal *2*, 319 (1958).

[5] M. F. DAY and T. D. C. GRACE, Ann. Rev. Entomol. *4*, 17 (1959).

[6] W. TRAGER, J. exp. Med. *61*, 501 (1935).

[7] S. S. WYATT, J. gen. Physiol. *39*, 841 (1956).

[8] G. R. WYATT, T. C. LOUGHHEED, and S. S. WYATT, J. gen. Physiol. *39*, 853 (1956).

[9] T. D. C. GRACE, Austr. J. biol. Sci. *11*, 407 (1958).

[10] J. B. BUCK, in K. D. ROEDER, ed. *Insect physiology* (John Wiley and Sons, Inc., New York 1953), p. 147.

Amino acids	Blood plasma			Synthetic mixtures			
	Man	*Bombyx mori* (L.) mature larva	*Galleria mellonella* (L.) mature larva	Configuration	199	Configuration	Wyatt
Arginine	1·2 – 3·0	28	39	L	7	—	—
Arginine HCl	—	—	—	—	—	L	70
Histidine	1·0 – 3·8	273	136	L	2	L	250
Lysine	2·3 – 5·8	164	68	L	7	—	—
Lysine HCl	—	—	—	—	—	DL	125
Tyrosine	0·9 – 2·4	31	76	L	4	L	5
Tryptophane	0·9 – 3·0	—	—	DL	2	L	10
Phenylalanine	1·1 – 4·0	11	11	DL	5	L	15
Cystine	1·8 – 5·0	0	0	L	2	L	2·5
Methionine	0·25– 1·0	14	27	DL	3	DL	10
Serine	0·3 – 2·0	111	47	DL	5	DL	110
Threonine	0·9 – 3·6	36	62	DL	6	DL	35
Leucine	1·0 – 5·2	29	42	DL	12	DL	15
Isoleucine	1·2 – 4·2			DL	4	DL	10
Valine	2·5 – 4·2	23	29	DL	5	DL	20
Glutamine	4·6 –10·6	143	369	L	10	L	60
Glutamic acid	0 – 1·3	10	22	DL	15	L	60
Asparagine	—	59	13	—	—	L	35
Aspartic acid	0 – 1·2	10	38	DL	6	L	35
Alanine	2·4 – 7·6	50	225	DL	5	DL	45
Beta alanine	—	39	51	—	—	—	20
Proline	1·5 – 5·7	36	520	L	4	L	35
Hydroxyproline	—	—	—	L	1	—	—
Glycine	0·8 – 5·4	73	51	—	5	—	65
Cysteine	—	0	0	—	0·01	—	—
Cysteine HCl	—	—	—	—	—	—	8

The free amino acids of human plasma, the plasma of two insect species (Lepidoptera), and of two synthetic mixtures; mg/100 ml. (Data from SPECTOR[20]; WYATT, LOUGHHEED, and WYATT[8]; MORGAN, MORTON, and PARKER[21]; and WYATT[7])

the five larval instars; but otherwise, great variations were found. Cystine, which is present in considerable amounts in human plasma, has not been detected in the three species studied by WYATT, LOUGHHEED, and WYATT.

A third, and most striking finding recently reported (WYATT, LOUGHHEED, and WYATT[8]; WYATT and KALF[11]) is the presence of a disaccharide in large quantities in insect hemolymph. Glucose, which accounts for the largest part of the reducing substance in human plasma, is present (with fructose) only in traces in the hemolymph of many insects, and particularly in the larvae of Lepidoptera. The reducing substances in the blood of these insects are, for the most part, non-fermentable.

It has always puzzled physiologists why insect blood contains apparently only a trace of sugar. However, sugar has been found recently in large proportion by WYATT and KALF[11]. It is a non-reducing disaccharide, α,α-trehalose, and for this reason it has probably eluded previous workers. Trehalose makes up over 90% of the blood sugar in four species of Lepidoptera. Furthermore, it has also been found to be a good substrate for respiration of insect muscle homogenates. This has aroused the interest of some physiologists. The study of the metabolism of trehalose by insect tissue will definitely produce much valuable information for the comparative and the cellular physiologist. It is significant, in this connection, to note that TRAGER[6], in 1935, long before the discovery of trehalose in hemolymph, found maltose a very effective source of energy for cultured silkworm tissue. Maltose is a glucose α-glucoside, with a 1,4 linkage; trehalose has a 1,1 linkage. While the hexose composition of the two disaccharides is the same (two molecules of glucose), maltose is a reducing sugar and trehalose is not.

What are the striking characteristics of the respiratory mechanism and metabolism of insects? Because of their open type of circulation, insects cannot utilize blood effectively for the exchange of respiratory gases. In most species, the gas exchange is carried on through a complicated tracheal system. The tracheae end in fine tracheoles, which are in close contact with the various tissues. Whether or not the cytoplasm is penetrated by the tracheoles is still unknown. There are indications that tracheoles end abruptly on the surface of the tissues. We may say that each tissue has its own separate gas exchange pipelines. The final control of the exchange of respiratory gases between tracheoles and tissue has been studied only in a few species and various systems have been suggested and partly supported by experimental evidence. However, the end effect of the proposed regulatory mechanisms seems to be only one: to allow the selective exchange of respiratory gases just where this is needed, for example in muscles during flight, without generally increasing the oxygen concentration of blood. In fact, both the oxygen tension and the oxidation-reduction potential in insect blood are very low. Insect blood plasma cannot, without

[11] WYATT, G. R. and G. F. KALF, J. gen. Physiol. *40*, 833 (1957).

special precautions, be used or transferred as is done routinely with vertebrate serum: this would immediately unbalance the phenoloxydase-dehydrogenase system, which is of great importance *in vivo* and may be of great importance for the cultured tissue. There have been many attempts to inhibit the action of blood tyrosinase *in vitro*. Phenylthiourea has been used (SCHMIDT and WILLIAMS[12]) as a tyrosinase-inhibitor in insect tissue culture and has always been considered harmless. KURODA and TAMURA[13], however, have reported that this carbamide completely inhibits the growth *in vitro* of the intestinal melanotic tumors in organ cultures of two strains of *Drosophila melanogaster* Meig. It is therefore not surprising that WYATT[7] reported toxic effects of phenylthiourea 'or an impurity in it' for cultured insect tissue. WYATT prefers to inhibit blood and cell melanosis by precipitating the enzyme protein (heat treatment, 60°C for 5 min). We now have some evidence that a change in oxidation-reduction potential may be very critical when insect cells are removed from the intact animal (MARTIGNONI, unpublished material). It is known, in fact, that such changes occur *in vivo* at critical phases of the insect metamorphosis; furthermore, the activity of many enzymes playing an important role during the formation of adult tissues, is dependent on the potential of the hemolymph.

An interesting fact which emerged from the work of HARVEY and WILLIAMS[14] is the adaptation capacity of pupal heart tissue to low oxygen tensions. After exhaustion of the anaerobic reserve, the pupal heart is microaerophilic, and normal heartbeat is sustained by oxygen partial pressures lower than 0·5% atmospheres. According to HARVEY and WILLIAMS[14], this particular character of an insect tissue can be explained by the presence of a substantial titer of cytochrome oxidase in conjunction with only a trace of cytochrome *c*, with a majority of cytochrome oxidase existing in the oxidized state.

The failure of certain cells to propagate continuously *in vitro* has been considered to be related to the persistence of oxidative metabolism. The shift to glycolysis seems to be a characteristic of malignant cells of vertebrates, for example: these can be readily propagated in culture. The main question, for those interested in insect tissue culture, is: what is the best metabolic pattern for the insect cell *in vitro*? How prone are these cells to shift to a metabolic pattern which would permit continuous propagation?

Another point to consider when culturing tissues of insects is the existence of metamorphosis. Weight does not increase continuously throughout the insect's immature life but only at given periods, in relation to molting. In many insect species, there is a sequence of cellular breakdown and reconstruction at each molt, a system more complex than simple dichotomous division. During the phases between molting, there is very little or no increase in cell number. Growth in insects having complete metamorphosis (such as the silkworm, which has been largely used in tissue culture experiments) is regulated by at least two hormone-like substances: the *corpus-allatum* hormone or juvenile hormone (neotenin), and the growth and molting hormone (ecdyson) from the thoracic glands, with the addition of the so-called 'brain factor' (WIGGLESWORTH[15]). A primary culture must be established at periods when the hormonal balance of the insect is such as to allow growth; the media should contain insect blood plasma with high titer of ecdyson (SCHMIDT and WILLIAMS[10]). Insect extracts rich in growth and molting hormone may be a parallel to the embryonic extracts routinely used in vertebrate tissue culture.

The small size of insects (not to speak of insect embryos) is a further factor making tissue culture a difficult task. Dissections must be performed mainly under dissecting microscopes, and are very time consuming. The tissue mass is usually small; since insects do not have stratified epithelia, the number of cells one can obtain for a primary culture is limited. MARTIGNONI, ZITCER, and WAGNER[16] have obtained only 160000 cells by enzymatic dispersion of the three thoracic segments of one full grown larva of a cutworm, *Peridroma margaritosa*. The lack of tissue bulk may be a cause of failure in establishing primary cultures, unless one works with a very small volume of medium, in capillary tubes, for example. This may explain why the best success in the culture of insect tissues has been obtained almost in every case in hanging drops, where, as compared to other methods, the density of the cell population is high. Of all the insect tissues used so far, the inner envelope of the ovarian follicle of larvae of Lepidoptera has been maintained *in vitro* with the highest degree of success by many workers, beginning with TRAGER[6]. However, these cells may be identical with some type of hemocyte which has become sedentary, as suggested by WIGGLESWORTH[17], a phenomenon not at all uncommon in insects. In view of this, it seems that the use of hemocytes in tissue culture should be given further attention: insect blood would provide a great concentration of cells (up to 100000 per mm^3) for the establishment of cultures.

Last, but positively not least, is the ever present microbial contamination which is difficult to eliminate. Microbial contaminants appear not only outside the insect body, where sterilization is no problem, or in the intestinal tract, but also in the tracheospiracular apparatus and even, sometimes, in the hemolymph of the normal insect. Therefore, even if the operator succeeds in extracting tissue fragments without rupturing the delicate intestinal epithelium or one of the Malpighian tubules, it is still possible that the explant is naturally contaminated. One way of overcoming the pitfalls of contamination is to rear the insects under axenic conditions. This has been successful with only a limited number of species (DOUGHERTY[18]) such as the European corn borer, and the Asiatic rice borer. Unfortunately, phytophagous species of interest to the virologist, such as the cutworm *Peridroma margaritosa*, cannot be reared axenically, so far.

In conclusion, one could say that it is up to the biochemist to solve most of the problems just outlined. Two are of particular urgency: (a) more exhausting studies on hemolymph composition, and (b) studies on the nutrition and metabolism of insect tissues and cells maintained (even though not growing) *in vitro*.

These are only a few aspects of an area of 'biological ignorance' which needs the attention of more biologists. When we examine the massive bibliography of tissue culture (MURRAY and KOPECH[19]), knowing that the

[12] E. L. SCHMIDT and C. M. WILLIAMS, Biol. Bull. *105*, 174 (1953).

[13] Y. KURODA and S. TAMURA, Dobytsugaku Zasshi *65*, 219 (1956).

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[15] V. B. WIGGLESWORTH, Symposia Soc. exp. Biol. *11*, 204 (1957).

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[19] MARGARET R. MURRAY and GERTRUDE KOPECH, *A Bibliography of the Research in Tissue Culture 1884 to 1950* (Academic Press, Inc., New York 1953), 2 vols.

number of papers concerning insects amounts to little more than a hundred (DAY and GRACE[5]), we can not but feel discouraged. This is an area that badly needs more concentrated efforts.

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Riassunto

Non si è riusciti finora ad ottenere da un insetto un clone di cellule che si moltiplichi indefinitamente *in vitro*. D'altra parte, esistono numerosi cloni di cellule provenienti da vertebrati. Perchè questa discrepanza? Fra l'organismo degli insetti e quello dei vertebrati esistono differenze fondamentali. È chiaro, dagli studi finora pubblicati, che nel caso degli insetti non è possibile adottare direttamente i metodi di coltura da lungo tempo usati per cellule e tessuti di vertebrati. Per poter definire i fattori necessari alla nutrizione ed alla moltiplicazione cellulare è necessario, fra l'altro, che si approfondisca lo studio dell'emolinfa e che si analizzi in maggior dettaglio il metabolismo di cellule d'insetti *in vitro*.

[20] W. S. SPECTOR, *Handbook of Biological Data* (W. B. Saunders Co., Philadelphia 1956).

[21] J. F. MORGAN, HELEN J. MORTON, and R. C. PARKER, Proc. Soc. exp. Biol. Med. *73*, 1 (1950).

PRAEMIA

EIDGENÖSSISCHE TECHNISCHE HOCHSCHULE

Fonds für den Ruzicka-Preis

Ausschreibung des Preises für 1960

Aus dem Fonds für den Ruzicka-Preis wird alljährlich einem jungen Forscher für eine hervorragende veröffentlichte Arbeit auf dem Gebiete der allgemeinen Chemie ein Preis erteilt. Die chemischen Arbeiten, welche mit einem Preis ausgezeichnet werden sollen, müssen entweder in der Schweiz oder von Schweizern im Ausland ausgeführt worden sein.

Kandidaten dürfen in dem Jahre, in welchem sie den Preis erhalten, das 45. Lebensjahr nicht überschritten haben. Sie können dem Kuratorium von dritter Seite vorgeschlagen werden oder sich auch selbst um den Preis bewerben.

Die Preiserteilung erfolgt auf Antrag eines Kuratoriums durch den Schweiz. Schulrat. Die Höhe des Preises wird auf Antrag des Kuratoriums in jedem einzelnen Fall durch den Schweiz. Schulrat festgesetzt. Die Überreichung des Preises erfolgt im September 1960.

Bewerbungen und Anträge sind unter Angabe der chemischen Arbeiten, für welche der Preis erteilt werden soll, bis *spätestens am Samstag, den 30. April 1960* dem Sekretariat des Schweiz. Schulrates, Eidg. Technische Hochschule, Leonhardstr. 33, Zürich 6, einzureichen.

Zürich, 9. Februar 1960.

Der Präsident des Schweiz. Schulrates:
Prof. Dr. H. PALLMANN

CONGRESSUS

SCHWEIZ

Internationales Symposium über Polarisationsphänomene mit Nukleonen

Basel, den 4.–8. Juli 1960

In Basel findet vom 4. Juli bis 8. Juli 1960 ein Symposium über Polarisationsphänomene mit Nukleonen statt. An diesem Symposium werden zur Behandlung gelangen:

1. Herstellung von Quellen für polarisierte Kerne.
2. Erzeugung von polarisierten Nukleonen und Deuteronen durch Kernreaktion.
3. Reaktionen und Streuexperimente mit polarisierten Teilchen.
4. Theorien über Polarisationseffekte mit Nukleonen.

Anfragen sind zu richten an Prof. Dr. P. HUBER, Physikalisches Institut der Universität Basel, Klingelbergstrasse 82.