Insect cell physiology

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Introduction

Viruses from the genus Baculovirus are attractive as pesticides and as vectors for producing foreign proteins in insect cells. Because insect cells are relatively easy to cultivate, faithfully perform some of the post-translational modifications, allow for complex formation to take place between the two proteins co-expressed in cultures infected with two hybrid viruses, and often can produce large quantities of proteins, baculovirus technology has found its way to a number of industrial applications for expression of a variety of proteins (see review Murhammer, 1991; O'Reilly *et al.*, 1992). In addition to proteins, production of baculovirus itself as insecticide represents a major potential for use of insect cell cultures (Wood, 1995). The final product yield, whether a protein or the virus, is influenced by several factors such as the cell line (eg. Lynn and Hink, 1980; McIntosh and Grasela, 1984; Hink *et al.*, 1991; Wickham *et al.*, 1992) and virus type (Fraser, 1989), passage number of virus (Wickham *et al.*, 1991), medium composition (eg. Cho *et al.*, 1989; Hink *et al.*, 1991), dissolved oxygen concentration (eg. Scott *et al.*, 1992; Wang *et al.*, 1993), time and the multiplicity of infection (MOI) (Licari and Bailey, 1991; Bedard *et al.*, 1994) nature of protein (Hink *et al.*, 1991), cell density (Wickham *et al.*, 1992) and stage of growth and metabolism (eg. Caron *et al.*, 1990; Lindsay *et al.*, 1992; Reuveny *et al.*, 1993).

Despite significant advances in the genetics of Baculovirus/insect cell expression system, our understanding of cellular physiology during pre- and post infection is relatively limited. An expanded understanding of metabolic features of insect cells will prove extreme-

ly useful to the bioprocess and biochemical engineers who work towards improving insect cell culture productivity. Several recent articles have addressed consumption of carbohydrate and amino acid and formation of the metabolic by-products in insect cell cultures. Those studies, reviewed first in this article, have significantly enhanced the metabolic knowledge of insect cells. Substantially more insights, however, may be obtained by a rigorous analysis of primary metabolic pathways. Our recent work (Ferrance *et al.*, 1993), has demonstrated the feasibility of metabolic flow analysis for insect cell growth in a serum-free medium. This manuscript also provides a summary of metabolic pathway analysis and highlights the assumptions, describes the lessons learned, and outlines the future work in a more critical fashion than is available in Ferrance *et al.* (1993). Finally, future research directions critical for attaining detailed metabolic insights are outlined in the last section of this manuscript.

Carbohydrate and amino acid metabolism

Carbohydrate metabolism

The range and efficiency of carbohydrate utilization differ for different insect cell lines. Stockdale and Gardiner (1976) studied the utilization of 21 sugars for the growth of *Trichoplusia ni* (TN 368) and found that only five supported cell growth: glucose, fructose, mannose, maltose and trehalose. Glucose and trehalose were consumed at a somewhat higher rate than fructose. Reuveny *et al.* (1992) also reported that a higher cell density of SF9 cells and recombinant protein (β -galactosidase)

production was achieved in glucose containing media than in media which contained either fructose or maltose. In the presence of both glucose and fructose in the media, fructose utilization begins upon depletion of glucose at a rate comparable to glucose (Bedard *et al.*, 1993). This behavior may indicate the presence of a phenomenon similar to the catabolite repression commanded by glucose as observed for some other cells (Mathews and Holde, 1990). Maltose consumption, in media which contain both glucose and maltose, usually occurs during early growth phase as is evident from the initial rise in glucose level and the decline in maltose concentration (Ferrance *et al.*, 1993; Bedard *et al.*, 1993). This observation could indicate the presence of an enzyme, on the cell surface or secreted in the media, capable of breaking the linkage between the two glucose of the dimer.

The high consumption of glucose and other glycolytic intermediates in various cultures, demonstrates the presence of an active glycolytic pathway in cultured insect cells. However, since lactate and pyruvate are unable to support SF9 cell growth (Reuveny *et al.*, 1992) the glucogenesis pathway may not be operating efficiently to facilitate cell growth in the absence of a glycolytic substrate.

Sucrose utilization, upon glucose depletion, has been reported for the insect cell line *Antheraea euclaypti* (Grace, 1966; Clements and Grace, 1967; Stockdale and Gardiner, 1975). Other reports (eg., Ferrance *et al.*, 1993; Bedard *et al.*, 1993) have noted that level of sucrose does not vary significantly in SF9 cell cultures. Because of high concentration of sucrose in the media, low rates of sucrose utilization can not be ruled out. Consumption of sucrose by SF9 cells during the postinfection period has been reported by Wang *et al.* (1993) which is in contrast with the finding of Wong *et al.* (1994).

Finally, it should be noted that different insect cell lines exhibit different patterns of carbohydrate utilization. For example, in sharp contrast to SF9 cells, the consumption of sucrose by BM5 cells, in a medium containing glucose, maltose, and sucrose is higher than other carbohydrates (Stravroulakis *et al.*, 1991a). Moreover, maltose is not substantially consumed by the BM5 cells (Stravroulakis *et al.*, 1991a). Furthermore, while SF9 cells do not accumulate significant amounts of lactate (Ferrance *et al.*, 1993; Bedard *et al.*, 1993; Wang *et al.*, 1993), BM5 cultures accumulate lactate to inhibitory levels (Stravroulakis *et al.*, 1991 and b).

Amino acid metabolism

Ferrance *et al.* (1993) have reported that amino acids aspartate, asparagine, glutamate, glutamine, and serine are utilized at a much higher rate than required for incorporation to cellular proteins and other structures. Arginine, asparagine, aspartate, glutamate, and glutamine can easily be oxidized to CO₂ via malic and the TCA cycle enzymes. One intriguing issue is whether the energy provided by the metabolism of these amino acids is required for biosynthetic functions or their presence at higher levels stimulate their uptake within a regulatory context somewhat different than a global energy optimization.

A useful index of coupling of the energetic and biosynthetic reactions is the ATP yield coefficient $Y_{x/ATP}$ (g. cells generated/mole ATP produced). The total ATP production and hence the value of the $Y_{x/ATP}$, can be obtained by either monitoring the oxygen uptake rate or the information on the metabolic flow of reactions which produce the reducing power, NADH and FADH, and the substrate level ATP (see section: Evaluation of the ATP yield coefficient). The theoretical maximum value for this coefficient varies slightly with the nature of the medium but it is close to a value of 30 (Stouthamer, 1979; Stouthamer *et al.*, 1990). Ferrance *et al.* (1993) found that for insect cells cultivated in media with high (IPL-41) and low (or more balanced) amino acid content (EMICG), the values of $Y_{x/ATP}$ were 5 and 22 (See Table 1), respectively. This finding signifies a substantially higher degree of coupling of energetic and biosynthetic reactions in the medium with a more balanced amino acid content.

There is a significant literature, primarily in bacterial systems, in which the discrepancy between the ATP production and consumption, termed maintenance requirements, has been attributed to membrane energization, macromolecule turnover, futile cycles, and possibly other unknown essential cellular functions. Our group has recently questioned such a notion and has demonstrated that a better coordination of primary pathways via media design, compatible with metabolic regulations, leads to an almost complete synergy between ATP production and consumption. This assessment is based on attaining, in a metabolically designed media, $Y_{x/ATP}$ values close to the theoretical maximum value of 30 which ignores maintenance functions (Goel *et al.*, 1995 a and b). The significantly higher ATP yield coefficient, in insect cells cultured in a medium with a more metabolically balanced amino acid composition suggests that, similar to bac-

Table 1. Summary of overall culture data¹ for experiments with IPL-41 and EMICG

Parameter	IPL-41	EMICG
Final cell density (x 10,000 cells/ml)	474	798
Initial cell density (x 10,000 cells/ml)	32	58
Cell mass formed	2728	4568
Initial Amino Acid ²	12700	5484
Final Amino Acid ²	9450	2742
Amino Acid used	3250	2742
Percent amino-acid used	26	50
Initial Glucose	2580	4000
Final Glucose	240	1000
Initial Maltose	1000	0
Final Maltose	0	0
Cell Yield ³ (cell carbon/carbon supplied)	0.20	0.54
Cell yield ³ (cell carbon/carbon used)	0.50	0.84
ATP yield coefficient ⁴ (g cell/mol ATP)	5	22

¹ Initial and final refer to concentration in, $\mu\text{g/ml}$, at the time of inoculation and at the end of exponential growth, respectively.

² Concentration of individual amino acids were also obtained and used in the flow calculations given in Table 2.

³ Cell yield were calculated based on a carbon content of 0.45 g/cell.

⁴ The ATP yield coefficient was obtained as discussed in section: Evaluation of the ATP yield coefficient.

terial systems, a significantly higher synergy between ATP production and consumption could be attained in insect cell cultures by appropriate media formation which closely reflects the biosynthetic and energetic demands. Moreover, it highlights the potential of $Y_{x/ATP}$ along with carbon yield (carbon in cells/total carbon consumed) as markers for assessing how far the culture performance is from an optimal value by comparing the $Y_{x/ATP}$ and carbon yield to the theoretical maximum values.

Formation of metabolic by-products

Experiments with different concentrations of glucose in the TNM-FH media unveiled that for the range of glucose concentrations examined (4–24 mM), the final cell density does not increase in proportion with the initial glucose concentration (Bedard *et al.*, 1993). How-

ever, glucose is fully consumed in all cases and alanine production is high in cultures cultivated with a high initial glucose concentration. Upon the exhaustion of glucose, ammonia begins to accumulate and alanine concentration drops. The drop in alanine concentration may indicate consumption of alanine at later stages of growth (Bedard *et al.*, 1993). Moreover, while glutamate consumption was higher in cultures with higher initial glucose concentration, glutamine consumption was higher for the cultures with lower initial glucose concentrations. Furthermore, the ammonia production was lowest in cultures with the highest initial glucose concentration.

Due to the complexity of metabolic reaction networks, it is difficult to obtain a coherent picture of these important observations. However, speculation can be made to the possible routing of the key amino acids and glucose. We suspect that a low level of alanine production in culture with the low initial glucose concentration (4 mM) indicates almost full utilization of glucose to biosynthetic precursors derived from glucose through HMP pathway and those obtained from glycolytic intermediates. This hypothesis is also consistent with the highest level of ammonia production and glutamine consumption as a lower glucose flux could lead to a significantly lower incorporation of glucose carbon to TCA cycle intermediates and thus a higher utilization of amino acids. Relatively higher consumption of amino acids, for fulfilling the energetic requirements, will in turn increase ammonia production. Finally, higher glutamate consumption in cultures with higher glucose concentration may reflect the glutamate conversion to alanine via the alanine transaminase catalyzed reaction. Metabolic flow analysis, discussed next, provides a powerful tool for a more rigorous analysis of metabolic consequences of various feeding strategies.

Metabolic flow analysis

Formulation of pathways and the assumptions

The details of the approach used to evaluate the metabolic flows of the key pathways which provide energy and metabolic precursors is given elsewhere (Ferrance *et al.*, 1993). In this manuscript, a summary of the approach is first presented followed by a more critical discussion of the results than is available in the report by Ferrance *et al.* (1993).

Figure 1 depicts reactions/pseudo-reactions com-

Figure 1. Scheme of the reaction networks used in the analysis of the molar flow of metabolites.

Thirdly, it was assumed that although the medium itself contains fatty acids, the lipid requirements are satisfied by *in vivo* synthesis. This assumption was based on reports indicating that *Spodoptera* cells produce, and even excrete fatty acids during growth rather than using the lipids available in the medium (Louloudes *et al.*, 1973) (Some components of the lipid emulsion, however, are needed for cell growth). In the model, the lipid requirements are satisfied by using acetyl CoA as a precursor. The $r_{\text{ACoA-Lip}}$ was obtained using the information on the lipid contents of various insect cell lines (Mitsuhashi *et al.*, 1982). Finally, the variation in cell composition was considered to be small. It should be noted that the key fluxes are not sensitive to variations in cell composition. For example 10% variations in the cell's macromolecular composition only marginally affects the evaluated metabolic fluxes of glycolysis and TCA cycle.

Evaluating the metabolic flows

The flows labeled with i's indicate the amount (mmol/ml) of a component which is taken up by the cells from the medium. For all the amino acids and other components except glutamine, this amount was determined by the change in the medium concentration at the beginning of cultivation and a time at the end of exponential phase of growth. For glutamine, however, the amount uptaken by the cells is obtained by subtracting the amount of glutamine degraded from the value of change in the medium. The flows labeled with p's represent the incorporation of various amino acids to proteins. These values were obtained from the amino acid analysis of cell extract.

In total, there are 45 flow values that must be defined. The values for 27 of them are obtained from experimental measurements and stoichiometry, and the remaining 18 are evaluated from balances conducted around the pools of arginine, glutamine, glutamate, citrate, α -ketoglutarate, asparagine, aspartate, fumarate, malate, oxaloacetate, threonine, glycine, serine, glucose 6-P, ribose, pyruvate, alanine, and acetyl CoA. For example, the balance equations for glucose 6-P and pyruvate are:

$$r_{\text{G6P-Pyr}} = 2 \times (r_{\text{G6P}} - r_{\text{G6P-NA}}), \text{ and}$$

$$r_{\text{Pyr-ACoA}} = r_{\text{Ser-Pyr}} + r_{\text{G6P-Pyr}} + r_{\text{Mal-Pyr}} -$$

$$r_{\text{Pyr-Ala}} - r_{\text{Pyr-Lac}}.$$

The pseudo-stead-state balances for these components are valid because their intracellular concentrations are

insignificant relative to the size of inputs to and outputs from the pools.

Discussion of the molar flow data

A summary of the experimental measurements for cells grown in EMICG and IPL-41 is given in Table 1. The cell yield (g cell carbon/g carbon in carbohydrate and amino acids consumed) was higher for the cells grown in EMICG (0.84) than in IPL-41 (0.50). The amino acids were also used to a larger extent in the EMICG experiments (50%) than the IPL-41 (26%); thus the yield based on total carbon supplied in the medium is significantly higher in the EMICG (0.54) than the IPL-41 (0.20) medium. Table 2 lists the key molar flows for cells grown in both IPL-41 and the EMICG media. The molar flows are presented as the total flow followed by the normalized value, in parenthesis, obtained by dividing the total flow by the cell concentration toward the end of the growth. As the total amount of glucose consumed is similar for the EMICG and IPL-41 grown cells (see the flow of Gluc-G6P), the total flows may also be thought of as normalized per glucose used.

Note that the flow for aspartate to oxaloacetate for the cells grown in EMICG is negative. This negative flow implies a lower uptake of aspartate than needed for the biosynthesis of amino acids and nucleotides. Since it is highly likely that yeastolate contains some nucleotide derivatives and bases, this flow may become a small positive value if the possible contribution of yeastolate to the precursor pools of nucleotide pathways was available and accounted for in the model. Of course the negative flow for aspartate to oxaloacetate can not be observed in the IPL-41 medium in which most of the amino acids are far in excess of biosynthetic requirements. The negative flow for alanine, fumarate, and α -ketoglutarate signifies their secretion to the culture. Overall, the flows for the cells grown in EMICG indicates that the efficient incorporation of carbon to biomass in this medium results from a tight coupling of the biosynthetic and energetic pathways as assessed by lower values of alanine production (See the normalized values in the parentheses in Table 2), glucose and amino acid uptakes, and the higher values of the cell and ATP yield coefficients.

The molar flow data may also be used to answer the following questions.

Is there a fully operational TCA Cycle? The $r_{\text{ACoA-Cit}}$ (Table 2) for the cells grown in IPL-41 (a medium with high amino acid content) and EMICG (a medium with

Table 2. Flows in millimolar and (mmole/g-cell) for reactions in Figure 1

Flow	IPL-41	EMICG
i Arg	2.08(0.71)	3.33(0.73)
i Gln	1.21(0.41)	2.68(0.59)
i Glu	7.21(2.46)	4.63(1.02)
i Asn	4.72(1.61)	1.78(0.39)
i Asp	4.39(1.50)	2.19(0.48)
i Thr	1.22(0.42)	0.99(0.22)
i Gly	0.87(0.30)	2.84(0.62)
i Fum	-0.71(0.24)	0(0)
i KetoG	-0.14(-0.047)	0(0)
i Ser	2.18(0.75)	3.42(0.75)
i Ala	-7.76(-2.65)	-4.56(-1.00)
p Arg	0.51(0.17)	0.77(0.17)
p Gln	0.54(0.18)	0.82(0.18)
p Glu	0.53(0.18)	0.82(0.18)
p Asn	0.50(0.17)	0.77(0.17)
p Asp	0.50(0.17)	0.77(0.17)
p Thr	0.45(0.15)	0.68(0.15)
p Gly	0.81(0.27)	1.22(0.27)
p Ser	0.49(0.17)	0.77(0.17)
p Ala	0.88(0.30)	1.36(0.30)
Asp-NucAcids	1.70(0.58)	2.51(0.58)
Gly-NucAcids	0.85(0.29)	1.32(0.29)
G6P-Rib	1.70(0.58)	2.63(0.58)
Rib-NucAcids	1.70(0.58)	2.63(0.58)
ACoA-Lip	10.98(3.72)	16.89(3.72)
Pyr-Lac	0(0)	0(0)
Gln-Glu	0.67(0.23)	1.82(0.40)
Glu-KetoG	8.92(3.05)	8.14(1.79)
Arg-Glu	1.57(0.54)	2.56(0.56)
Asn-Asp	4.22(1.44)	0.97(0.21)
Asp-Oxalo	6.41(2.19)	-0.48(-0.11)
Mal-Pyr	14.48(4.95)	7.66(1.68)
Thr-Gly	0.77(0.26)	0.26(0.06)
Gly-Ser	-0.02(-0.01)	0.37(0.08)
Ser-Pyr	1.67(0.57)	2.99(0.66)
Gluc-G6P	18.84(6.44)	16.61(3.64)
G6P-Pyr	34.28(11.72)	27.54(6.04)
Pyr-Ala	8.64(2.95)	5.99(1.31)
Pyr-ACoA	41.79(14.28)	32.21(7.06)
ACoA-Cit	30.81(10.53)	13.80(3.03)
Oxalo-Cit	30.81(10.53)	13.80(3.03)
Cit-KetoG	30.81(10.53)	13.80(3.03)
KetoG-Fum	39.59(13.53)	21.94(4.81)
Fum-Mal	38.88(13.29)	21.94(4.81)
Mal-Oxalo	24.40(8.34)	14.28(3.13)

The numbers enclosed in parentheses are normalized flow (mmole/ g-cell).

Amino acids listed with common three letter abbreviations. 2-Ketoglutarate, fumarate, oxaloacetate, acetyl CoA, glucose 6-phosphate, and nucleic acids abbreviated as KetoG, Fum, Oxalo, ACoA, G6P and NucAcids.

lower amino acid content) is 10.5 and 3.03, respectively. The molar flows were obtained making the assumption that fatty acid requirements are satisfied entirely from acetyl CoA. If the fatty acid requirements are partially satisfied from the uptake of the medium components, then the $r_{\text{ACoA}-\text{Cit}}$ will be larger than the value given in Table 2. The high value of $r_{\text{ACoA}-\text{Cit}}$ for cells grown in IPL-41 and the lower value of the sum of $r_{\text{Mal}-\text{Pyr}} + r_{\text{Ser}-\text{Pyr}}$ than the $r_{\text{ACoA}-\text{Cit}}$ indicate that the SF9 cells possess a fully operational TCA cycle and glucose carbon enters the TCA cycle.

How to reduce the overproduction of alanine? The combination of lower specific glucose and amino acid uptakes in the EMICG as compared to IPL-41 media (see the number in parenthesis in Table 2) leads to a significantly lower specific alanine secretion for the cells grown in EMICG. The formation of large amounts of ammonia, produced as part of the amino acid catabolism, points to the production of alanine as a possible sink for ammonia. That is the presence of high levels of amino acids in the media such as IPL-41 could lead to higher than “needed” uptake rates which in turn results in excessive alanine formation. Thus, formulating a balanced medium with an amino acid composition closely reflecting the energetic and biosynthetic demands may lead to a drastic decrease in alanine production. Such formulation will be greatly aided by a better understanding of the interrelations between glucose and amino acid catabolism.

Oxygen consumption and carbon dioxide production. Several reports have indicated that cultures in which dissolved oxygen concentrations were maintained at relatively high levels attained significantly higher culture productivity (greater than 5% of saturation). A nice review of the effect of dissolved oxygen and oxygen uptake rate on culture productivity is available in a report by Taticek *et al.* (1995). A better understanding of the nature of shear sensitivity of insect cells and design of reactor configurations that allows for a higher oxygen transport rate would lead to more productive insect cell cultures. The issue of oxygen requirements, however, has not been viewed from a physiological stand point. An improved knowledge of the physiological and nutritional requirement will facilitate the design of productive culture media or feeding strategies for reducing the oxygen requirements. The oxygen uptake can be regulated by controlling the production of the reducing power NADH. The tight coupling of energetic and biosynthetic reactions can significantly

reduce the NADH generation. An instructive example entailing a comparison of the NADH generation in a medium with a low amino acid content (EMICG) and a medium with a high amino acid content (IPL-41) is discussed below.

The relation between oxygen consumption and the NADH production is $Q_{O_2} = 1/2 r_{NADH}$. Based on the formulation shown in Figure 1, NADH production is r_{NADH} (mmole/g-cell) = $r_{G6P-Pyr} + r_{Pyr-ACoA} + r_{Cit-KG} + 2r_{KG-Fum} + r_{Mal-Oxalo} - (18 - r_{Mal-Pyr} - 2r_{G6P-Rib})$. It is assumed that FAD is equivalent to NADH with respect to ATP generation. The first five terms reflect the NADH production and the last term accounts for the NADH conversion to NADPH by the action of transhydrogenases. The NADPH/g-cell required for biosynthetic reactions was assumed to be 18 mmole/g-cells which is the value reported for bacterial systems. The $r_{Mal-Pyr}$ and $r_{G6P-Rib}$ are accounting for the NADPH synthesized in those reactions. Thus, $(18 - r_{Mal-Pyr} - 2r_{G6P-Rib})$ represents the net requirement of NADPH provided for by the action of transhydrogenases. In the above calculations, we assumed that the NADPH requirements will be satisfied by the reactions catalyzed by transhydrogenases. It should be noted that we have shown that if the HMP pathway, instead of the transhydrogenases reactions was used for production of NADPH, r_{NADH} will not be significantly different.

Using the above equation, the oxygen consumption for the EMICG medium and the IPL-41 medium are 13 and 60 mmole/g-cell respectively. Thus, a drastic reduction in oxygen requirements (on per gram cell basis) is attained in a medium with a composition more closely matched to the biosynthetic and energetic requirement.

There is very little information on the effect of dissolved CO_2 ; we have just begun to examine how the dissolved CO_2 affects the culture productivity. The amount of evolved CO_2 can be calculated in insect cell by measuring the flux of the reactions producing CO_2 . Ferrance *et al.* (1993) calculated the evolved CO_2 by evaluating the flux of reactions coupled to CO_2 formation ($r_{Pyr-ACoA} + r_{Cit-KG} + r_{KG-Fum} + r_{Mal-Pyr} + r_{G6P-Rib}$) and found it to be very close to the measured value.

Evaluation of the ATP yield coefficient. The flows given in Table 2 can also be used to calculate the ATP yield coefficient ($Y_{x/ATP}$, g-cell mole-ATP). The ATP production r_{ATP} (mmole/g-cell) = $3 [(r_{G6P-Pyr} + r_{Pyr-ACoA} + r_{Cit-KG} + 2r_{KG-Fum} + r_{Mal-Oxalo}) - (18 - r_{Mal-Pyr} - 2r_{G6P-Rib})] + 2(r_{G6P-Pyr} - r_{Gluc-G6P} - r_{G6P-Rib})$. The

terms in brackets represent the NADH production (coupled to ATP formation) as discussed earlier. The value of 3 is the P/O ratio (the number of ADPs phosphorylated per electron pair transferred to oxygen). The last three terms represent ATP produced/consumed via substrate level phosphorylation. Using the above equation, the value of $Y_{x/ATP}$ can be obtained as: $Y_{x/ATP} = 1000/r_{ATP}$. The values of $Y_{x/ATP}$ for cells grown in IPL-41 and EMICG medium were given in Table 1, as 5 and 22, respectively.

Post infection metabolism

Several groups have examined the consumption of glucose, glutamine and other amino acids, alanine production, and oxygen uptake during the post-infection period (Weiss *et al.*, 1990; Kamen *et al.*, 1991; Wang *et al.*, 1993; Henseler and Agathos, 1994; Wong *et al.*, 1994). Despite multitudes of factors affecting the post-infection metabolism such as variations in medium composition, the time of infection, multiplicity of infection, etc., some common features may have emerged from the recent metabolic studies. First, as noted, maintaining a relatively high dissolved oxygen concentration leads to a higher recombinant protein productivity (Scott *et al.*, 1992; Wang *et al.*, 1993). Secondly, increased culture productivity is attained by replacement or dilution of spent medium, at the time of infection, with the fresh medium (Caron *et al.*, 1990; Lazarte *et al.*, 1992; Lindsay and Betenbaugh, 1992; Wang *et al.*, 1993). Thirdly, supplementing the culture with glucose and glutamine at the time of infection may somewhat enhance the concentration of the recombinant protein (Wang *et al.*, 1993). Finally, supplementation of yeastolate along with glucose and glutamine coupled with media replacement substantially elevates the rate of protein production (Reuveny *et al.*, 1993). A recent report has revealed that SF9 cells infected at a high cell number density of 7×10^6 ml⁻¹ attains high recombinant productivity when supplemented, without media change or a significant dilution at the time of infection, with yeastolate and a mixture of all other amino acids (Bedard *et al.*, 1994). This finding may suggest that low productivity for SF9 cultures infected at the late exponential phase (e.g. Caron *et al.*, 1990; Lazarte *et al.*, 1992; Reuveny *et al.*, 1993) reflects a nutrient limitation rather than the build-up of toxic metabolic by-products. This report also supports the observation that cell-cell contact inhibition at higher cell densities appears to be insignificant for SF9 and

SF21 cells (Wickham *et al.*, 1992).

The effect of yeastolate on protein productivity may reflect the supply of a limited nutrient by the mixture. Since addition of vitamins and lipid components does not produce productivity enhancement achieved by yeastolate (Reuveny *et al.*, 1993), we suspect that some other essential compound(s), perhaps a component of the nucleotide pathways is supplied by yeastolate. Nucleotides serve as building blocks for the synthesis of nucleic acids, structural moiety of coenzymes, metabolic regulators and signal molecules, and are critical elements of energy metabolism. Because of the significant role of nucleotides in cellular regulation and DNA replication, efforts aimed at a better understanding of nucleotide metabolism of insect cells will be highly desirable.

Future research

Media without yeastolate

Despite limited knowledge on insect cell metabolism during pre- and post infection, significant progress has been made. The original media for cultivating insect cells contained serum or hemolymph. The presence of serum makes purification of the target protein more difficult and leads to variation in product yields due to the variation in the composition from lot to lot. It also significantly complicates detailed metabolic studies. Work on replacing serum with other components has yielded serum-free media. The serum-free media contain sugars, salts, amino acids, organic acids, vitamins, trace elements, and ultrafiltered yeastolate. Although it is feasible to gain some metabolic insights using insect cells cultivated in yeastolate containing media, its substitution with chemically defined components will vastly enhance the range and the scope of such studies. The chemically defined medium will also ensure reproducibility of optimized process conditions.

Wilkie *et al.* (1980) have claimed a chemically defined medium (CDM) lacking yeastolate. We prepared this medium from the individual components. Several attempts to subculture the cells in CDM proved unsuccessful. It was found that although the cells will grow in CDM for the first subculture, they do not survive past one subculturing in shake flasks. Successful subculturing of the cells became possible only when the CDM was supplemented with yeastolate. However, recently we have made progress in substituting

yeastolate with chemically defined components. These findings will be communicated at a later time.

Performance of metabolite routing studies

We have shown that significant insight may be gained by the stoichiometry-based analysis of the primary metabolic pathways. More metabolic flow studies under different nutritional conditions will expand the current metabolic knowledge of insect cells. However, for elucidating regulatory and flux routing details, these studies must ultimately be coupled to measurements on concentrations of intracellular metabolites and enzymes, and tracer compound studies. Such experiments, currently underway in our laboratory would supplant the fluxes identified from using overall balances and observables by filling in gaps and eliminating uncertainties regarding the glucose-6-phosphate, pyruvate, and malate branch points. The metabolic routing studies with radiolabelled substrates is also critical for an improved understanding of post-infection metabolism.

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