INTRODUCTION

Biological products are derived from living sources, which presents special challenges for a heating, ventilation, and air-conditioning (HVAC) system serving a facility that processes and handles these products. The most important challenge is to prevent microbial contamination, which to a large measure is controlled by the HVAC system by utilizing classified spaces, providing containment, and main-
taining pressurization. Biological facilities are subject to federal regulation. The Food and Drug Administration's (FDA's) Center for Biologic Evaluation and Research (CEBR) is the primary regulating agency in the United States and its principal concern is the prevention of cross-contamination between different products produced in a multiproduct facility (1). Bioprocess procedures are generally conducted in environments that are cleaner than standard buildings. The HVAC system assumes a large part of the responsibility in maintaining these clean environments. Air used to condition a space must be highly filtered, properly directed, and able to maintain pressure relationships within and between adjacent spaces.

HVAC DESIGN PROCESS

Initial Effort
The HVAC design process begins with meetings with process engineers, architects, and representatives from the owner or facility user. The process and instrument diagrams (P&IDs) are reviewed, and a general understanding of the process is conveyed to all interested parties. Operations of the facility are reviewed, and any plans for future additions or modifications are discussed.

Basis of Design
After the initial meeting, a written basis of design is produced that describes the regulations and codes that will govern the design. Spaces are defined by function, and temperature and humidity requirements are determined. Room classifications are listed and adjacency of spaces and pressure relationships are documented. Any unusual or unique facility requirements must also be designed into the HVAC system at this time, such as emergency backup or redundancy for HVAC systems. This is also the stage of the design process during which alternate studies are conducted to compare options for the HVAC system. The cost of a backup or redundant HVAC supply system may be compared with the cost of product loss or experiment interruption should temperatures or airflow go out of control or specification. Heat recovery from exhaust systems and thermal storage are examples of other potential areas for study (2).

Airflow diagrams are produced that show areas served by a particular air handling system including supply, return, exhaust, and transfer air between spaces. The basis of design also describes major equipment to be used and the level of quality of components and construction material.

Cost Estimate
From the basis of design and a plan of the area, a factored estimate can be developed for budgeting purposes. Preparation of the estimate usually includes a dollar per cubic feet per minute (CFM) quote or a budget quote from vendors, as well as previous history to determine air handling unit costs. Estimates are also made for ductwork, as derived from pounds per CFM history and historic cost data. Costs for fans, high-efficiency particulate air (HEPA) filters, terminal boxes, and building management systems are also included. The level of effort expended to produce the basis of design will affect the accuracy of the estimate. Estimates from a basis of design can range from 15 to 25% accuracy. The estimate at this stage of the project is usually developed for capital appropriation.

Document Preparation
After owner or user approval of the basis of design, final plans and specifications are prepared using manual or computer-aided design tools for preparation of plans and sections. The utilization of three-dimensional design systems for document preparation is becoming increasingly popular. In a three-dimensional system, an electronic model is built that includes all disciplines, including HVAC, architectural, structural, electrical, piping, equipment, and so on. From the model interference, checks can be made that indicate physical conflict. These conflicts are resolved in the design process, avoiding potentially expensive field modifications and possible project delays.

Bidding and Award
When plans and specifications are completed they are sent to HVAC contractors for bid and eventual award. Design and build is also becoming a very popular option, in which design and construction are performed by a single entity with potential cost and schedule savings (3). When awarding a contract the engineer usually meets with the contractor to review the bid for completeness and technical accuracy, and to review the contractor’s history on previous projects performing similar work. Since this is specialized HVAC work, it is necessary to have a contractor with sufficient resources and similar experience to avoid delays and potential rework that could increase costs.

REGULATION AND CODE CONSIDERATION

Code Compliance
Codes affect separation of air systems, gowning of people, airflow rates, exhaust requirements, construction materials, and many other aspects of an HVAC system for bioprocess facilities. If codes are not met, the result can be a danger to people working in the facility, products that do not meet specifications, and failure to license the facility.

Classified Spaces
Clean rooms provide clean air in aseptic spaces and are defined or classified in the United States by Federal Standard 209E of September 1992. The European Economic Community (EEC) published guidelines called "Guide to Good Manufacturing Practice for Medical Products in the European Community," which are more stringent than U.S. FDA regulations (4). Room classifications are established by measurement of the number of particles 0.5 μm and larger that are contained in 1 ft³ of sampled air. Generally class 100 to 100,000 rooms are used in the bioprocess industry. Rooms may be classified as clean at class 1 or 10 for other applications, particularly in the microchip indus-
try. Table 1 (derived from Federal Standard 209E) shows
the air cleanliness classes.

EEC guidelines classify spaces by alpha character, as
indicated in Table 2, and Table 3 shows the U.S. and EEC
comparison.

Laboratory Spaces

Laboratory spaces are associated with bioprocess facilities.
These classifications range from BL-1 through BL-4, with
BL-1 being the least hazardous. BL-1 compares to a school
science laboratory, while BL-4 labs must provide ultimate
containment and require glove boxes and double HEPA fil-
tration. These laboratory classifications were formulated
in the United States by the National Institutes of Health
(NIH) and are published as guidelines in the Federal
Register. The guidelines contain requirements for filtration of
air, recirculation of air, containment, and biosafety cabinet
requirements. There are various classifications of biosafety
cabinets, depending on the level of containment required.
The American Society of Heating, Refrigerating, and Air-
Conditioning Engineers, Inc. (ASHRAE) guide should be
consulted and owner requirements considered when se-
lecting the required class and type of cabinet (5).

Other Regulations

Other regulations and guidelines may include those pub-
lished by insurance agencies and corporate or plant guide-
lines formulated from specific industry experience and cur-
rent good manufacturing practices (cGMPs) as published
in the U.S. Code of Federal Regulations parts 210 and 211.
The cGMPs apply to manufacture, handling, or processing
of drugs to ensure safety, purity, and quality of the product.
C-GMPs regulate space temperature, and humidity and re-
quire a pressure differential between adjacent rooms of dif-
ferent cleanliness levels and a minimum air change rate
of 20 air changes per hour. The regulation also addresses
class 100 requirements at filling lines and microbiological
testing sites, in addition to laminar flow control equipment
parameters and testing at fill lines. Good practice usually
places class 100 areas in class 10,000 backgrounds, which
have between 35 and 60 air changes per hour in the space,
depending on the activity and particulate generation
within the space. Walls, floors, and ceilings for cGMP areas
are to be constructed of smooth, cleanable surfaces, imper-
vious to sanitizing solutions and resistant to chipping, flak-
ing, and oxidizing. Horizontal ducts, pipes, or crevices that
foster dust accumulation and cannot be easily cleaned are
not permitted.

Figure 1 is a sample graphic depiction of an air classi-
fication drawing that is prepared to communicate to the
design team and reviewing agencies the classification of
each area. This is usually a full-size drawing (scale: 1/8 ft
= 1 ft) and uses various shading symbols to identify each
classified area.

TEMPERATURE, HUMIDITY, AND AIRFLOW

Space Temperature

Generally areas are designed to provide room tempera-
tures from 67 and 77 °F (19 and 25 °C) with a control point
of 72 °F (22 °C). Control of humidity from 40 to 55% is
necessary for personal comfort, to prevent corrosion, to
control microbial growth, and to reduce the possibility of
static electricity. Calculations are made to determine in-
ternal heat gains from lights, people, and equipment in
addition to transmission gains from adjacent spaces to de-
termine if supply airflow quantities are adequate to pro-
vide design space temperature conditions. These calcula-
tions are compared with airflow quantities required by
classification to establish the minimum air required to sat-
fy both the space cooling load requirements and air clean-
liness classification. Fan heat from recirculating fans can
also be a large heat contributor in clean spaces.

Heat-loss calculations must also be made to determine
heat loss through walls, roof, and floor. It may be necessary
to add additional heat to a reheat coil to overcome these
losses. No credit should be taken for process heat gain in
this calculation, since the process could be dormant and
the space would still need to be maintained at proper
temperature. Lower space temperatures may be required
where people are very heavily gowned and would be un-
comfortable at "normal" room conditions. Humans are gen-
erally the largest contributors to airborne particulate in
clean spaces; personal discomfort may cause perspiration,
which significantly adds to the generation of airborne par-
ticulate. To achieve colder room conditions, the tempera-
ture of the supply air to the rooms may have to be colder
than the normal 50–55 °F (10–13 °C), resulting in a greater
dehumidification demand on the air-conditioning system.
The amount of dehumidification required can be deter-

<table>
<thead>
<tr>
<th>Class</th>
<th>SI</th>
<th>English</th>
<th>Measured particle size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sl</td>
<td>m³</td>
<td>ft³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>M 1.5</td>
<td>1</td>
<td>1,240</td>
<td>35</td>
</tr>
<tr>
<td>M 2.5</td>
<td>10</td>
<td>12,400</td>
<td>350</td>
</tr>
<tr>
<td>M 3.5</td>
<td>100</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>M 4.5</td>
<td>1,000</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>M 5.5</td>
<td>10,000</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>M 6.5</td>
<td>100,000</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Note: Limits listed are maximum concentrations of particle sizes equal to and larger than the measured particle sizes shown.
Table 2. Classification of Clean Areas Defined by the European Economic Community

<table>
<thead>
<tr>
<th>Grade</th>
<th>Maximum permitted number of particles per m³ equal to or above 0.5 µm</th>
<th>Maximum permitted number of viable microorganisms per m³</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3,500</td>
<td>&lt;1</td>
</tr>
<tr>
<td>B</td>
<td>35,000</td>
<td>5</td>
</tr>
<tr>
<td>C</td>
<td>350,000</td>
<td>100</td>
</tr>
<tr>
<td>D</td>
<td>3,500,000</td>
<td>500</td>
</tr>
</tbody>
</table>

- Laminar airflow speed: 0.3 m/s for vertical flow 0.45 m/s for horizontal flow.
- Air changes per hour for grades B, C, and D should be higher than 20.
- U.S. Federal Standard 209E corresponding particle counts are:
  - Class 100 (Grades A and B)
  - Class 10,000 (Grade C)
  - Class 100,000 (Grade D)
- It is accepted that sometimes conformity with particle counts may not be met at point of fill due to generation of particles or droplets from the product itself.

Table 3. Air Cleanliness Classification for Aseptically Produced Products

<table>
<thead>
<tr>
<th>Operation Parameters</th>
<th>United States</th>
<th>European Economic Community</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsterilized product or container (U.S.: controlled area)</td>
<td>Class 100,000</td>
<td>Grade C (Class 10,000)</td>
</tr>
<tr>
<td>Clean room type</td>
<td>100,000/ft³</td>
<td>350,000/m³</td>
</tr>
<tr>
<td>Maximum particle size 0.5 µm or above</td>
<td>(3,531,000/m³)</td>
<td>(9,900/ft³)</td>
</tr>
<tr>
<td>Maximum viable organisms</td>
<td>2.5/ft³</td>
<td>100/m³</td>
</tr>
<tr>
<td>Airflow rate</td>
<td>Minimum 20 air changes/h</td>
<td>Higher than 20 air changes/h</td>
</tr>
<tr>
<td>Space pressurization</td>
<td>0.05 in water</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Sterilized product or container (U.S.: critical area) | Class 100 environment in class 10,000 background | Grade A (class 100) environment in grade B (class 100) background |
| Clean room type      | Class 100 environment in class 10,000 background | Grade A (class 100) environment in grade B (class 100) background |
| Maximum particle size 0.5 µm or above | 100/ft³ | 3,500/m³ |
| Maximum viable organisms | 0.1/ft³ | (99.1/ft³) |
| Airflow rate         | 90 ft/min ± 20% | Grade A: laminar flow work station |
| Space pressurization | 0.05 in water | Vertical: 0.3 m/s |

Space Humidity

In some cases where products sensitive to moisture are handled, the room relative humidity requirement may be as low as 15 to 20% and may require the use of chemical dehumidifiers. It is necessary to establish this criteria early in the design process to allow selection of dehumid-
HEATING, VENTILATION, AND AIR-CONDITIONING

The lower the required temperatures and humidity, the more sophisticated the HVAC equipment becomes, resulting in higher start-up and operating costs. Range of temperature control also affects the cost of the control and HVAC delivery system. The closer the space temperature tolerances are, the more sensitive the controls must be to react. To achieve these temperature tolerances, the air distribution system must be more extensive and elaborate to prevent hot or cold spots. An HVAC room balance, shown in Table 4, is a sample of a preliminary design document that indicates room temperature and humidity design conditions, in addition to dimensions. Table 4 also lists room classification air changes per hour, supply, exhaust, return, and transfer air requirements. This information is useful in communicating design conditions to the design team and reviewing agencies. The table should be kept up to date, because it is useful to installing contractors and the construction supervision team as a check on ultimate system performance. When the project is near completion this information is usually transferred to the construction documents for ease of validation and agency approval.

Calculation of Air Changes

Table 4 indicates a typical range of air change rates generally used to achieve the desired room cleanliness classifications and to meet federal and local regulations. These air change rates vary widely in actual practice due to the level of activity, number and type of particulate generators in a room (such as people and equipment), and room size and quality of air distribution. It is generally best to use historic data to establish airflows, which is usually done with significant input from the owner based on past experience or preference. There is nothing sacred about an air change rate as long as minimum airflow rates required by code are maintained. The goal is to achieve desired particulate cleanliness levels and stay at or above a 20 air changes/h minimum.

To calculate airflow requirements from room air change rates, the following formula should be used:

\[
\text{air flow (CFM)} = \frac{\text{room volume (ft}^3) \cdot \text{Number of air changes/h}}{60}
\]

or (metrically)

\[
\text{air flow (m}^3/\text{h)} = \frac{\text{room volume (m}^3) \cdot \text{Number of air changes/h}}{60}
\]

Adjacency

In the early stages of design, the HVAC engineer must work with the architect, process engineer, user, and layout planner, if one is involved, to establish adjacency of spaces. It is suggested that a computerized drafting system with block layouts be used to allow the frequent manipulation that is required to arrive at an optimum arrangement. During this exercise the layout is primarily determined by process function and spaces are classified as clean, dirty, or contained. A contained space is used to contain a product or material that could be spilled into an occupied space or find its way into the building air system. From an HVAC standpoint it is desirable to keep similarly classified areas as physically close to each other as possible so they can be connected to the same air handling system, thereby minimizing duct runs, cost, and air system complexity. It is also imperative that spaces be arranged to allow people to move around without disrupting the cleanliness or containment of the spaces.

Ultimately, as the layout develops, the suites or groups of spaces will be established and groupings of rooms can be made for assignment to separate air handling systems. It is not desirable to mix dirty and clean systems or suites that may allow the possibility of cross-contamination from one suite to another. Leaks can develop in a filter, or some source of contamination could find its way through the air.

<table>
<thead>
<tr>
<th>Room name and number</th>
<th>Room temperature</th>
<th>Relative humidity (%)</th>
<th>Area (ft²)</th>
<th>Room height (ft)</th>
<th>Air changes per hour</th>
<th>Hood, general exhaust</th>
<th>Required return CFM</th>
<th>Total supply CFM</th>
<th>Transfer air CFM</th>
</tr>
</thead>
</table>
supply or return systems, providing a source for cross-contamination.

PRESSURIZATION

Requirement and Theory

Pressurization of spaces is required to keep products from being contaminated by particulate or to protect people from contact with harmful substances by physical means or inhalation. Where very severe potential exposure exists, air packs and breathing apparatus are employed.

Pressurization can best be explained by thinking of air leaking from a balloon. Pressure is built up inside the balloon and air escapes from a small leak in the balloon. If the hole stays the same size, a greater pressure differential between the inside and outside of the balloon will allow the air to escape at a higher velocity, which can be termed a higher level of pressurization. As the air in the balloon is depleted, the pressure differential drops, the rate of air escaping diminishes, and the velocity decreases. The same principle applies in bioprocess facilities where rooms are made as tight as possible to contain air, allowing the air handling system to build up or reduce pressure in rooms. These conditions are maintained by airflow, or leakage, between adjacent spaces caused by pressure differentials created by the air supply, return, and exhaust systems. Where major demarcations of pressure are required, air locks are used. These are small rooms with controlled airflow acting as barriers between spaces. Standard 209E recommends maintaining pressures of 0.05 in water (12 Pa) between adjacent spaces with doors closed. During facility operation as doors are opened the design differential is greatly reduced, but air must continue to flow from the higher to lower pressure space, even though at a reduced flow rate. To maintain a differential of 0.05 in water (12 Pa), a velocity of approximately 900 ft/min (4.7 m/s) should be maintained through all openings or leaks in the room, such as cracks around door openings. In theory the actual required velocity is less, but in actual practice it is prudent to use 900 ft/min. Table 5 gives the velocity pressures for airflow from 0.01 to 0.20 in water (2.4–48 Pa), which can be used in approximating airflow between rooms. Limits on pressure drops must be set during the design (usually 0.05 in water) and observed. The pressure differential exerts a force on the door that can be calculated. If the force is too great (0.15 in water/36 Pa), the door may not close fully or may be difficult to open. This is particularly important in large complex facilities where many levels of pressurization may be required. Many facilities now use sliding doors, and it is essential that the seals be carefully designed to allow minimum leakage and proper containment or pressurization.

Pressurization Diagram

Figure 2 indicates levels of higher to lower pressurization with arrows showing the direction of airflow, (leakage) between the spaces. In actual practice the calculated airflow quantities are approximate since in constructing a facility it is very difficult to build everything absolutely airtight. A very small unexpected leak or a seal that is not as tight as expected in design can affect the pressurization and airflow required to maintain the design value of differential. Some flexibility must be built into the air handling system capacity and controls to compensate for these variations.

One technique to establish pressurization in separate rooms is to have a common reference for the control sensors. This common reference is usually a mechanical room or an interstitial space where the pressure is not affected by changes in the controlled spaces. It may be necessary to build an elaborate zero pressurization chamber to house the common sensor to negate the effects of wind or cross-currents. Referencing spaces to each other can lead to problems if doors are frequently opened and upsets in pressure occur. The controls may never settle down and tend to constantly modulate, a problem termed hunting.

Room Seals and Doors

In most facilities the openings around the doors between rooms are where leaks occur due to pressure differentials between rooms. In making rooms tight any room openings

| Table 5. Velocity Pressure to Velocity Conversion: Standard Air |
|-------------------|-------------------|-------------------|-------------------|-------------------|
| VP   | V   | VP   | V   | VP   | V   | VP   | V   |
| 0.01 | 401 | 0.06 | 981 | 0.11 | 1328| 0.16 | 1602|
| 0.02 | 566 | 0.07 | 1060| 0.12 | 1387| 0.17 | 1651|
| 0.03 | 694 | 0.08 | 1133| 0.13 | 1444| 0.18 | 1699|
| 0.04 | 801 | 0.09 | 1201| 0.14 | 1499| 0.19 | 1746|
| 0.05 | 896 | 0.10 | 1266| 0.15 | 1551| 0.20 | 1791|

Note: VP, velocity pressure; V, velocity.

Figure 2. Pressurization Diagram.
must be sealed with a proper sealant that will not promote growth of organisms and can be easily cleaned. Areas to be sealed include ceiling tiles, lighting fixtures, pipe penetrations, telephone outlet penetrations, and any cracks or openings that appear in the structure. A typical door would have the following dimensions and crack area at the perimeter: door size, 3 ft (0.915 m) wide by 7 ft (2.41 m) high; cracks at top and sides, 1/8 in (3.2 mm) with an undercut of 1/4 in (6 mm). The calculated area around the door is equal to 0.24 ft² (0.024 m²). To achieve 0.05 in water (12 Pa) pressure differential across the door, approximately 215 CFM (375 m³/h) of airflow through the cracks is required. Door seals around the top and sides are usually made of closed cell neoprene and should generally be used to reduce the crack area. To reduce the undercut, a drop-type seal, which is commercially available, should be used. The drop type is preferred to a wipe type, since it will not mar or leave residue on the floor. Air used for pressurization must be accounted for in system calculations. Air through cracks or openings is accounted for as transfer air and shown in the HVAC room balance table.

Of particular importance is exhaust air from equipment and hoods that may be on or off at different times during occupied periods. These variations must be dynamically compensated for to maintain room pressurization. To maintain the required balance, numerous systems are employed using manual and automatic dampers, constant and variable volume air control boxes, and elaborate airflow sensing devices. These components are combined with control systems and sensing devices to ensure that the room pressurization is maintained.

AIR HANDLING SYSTEMS

Constant Volume Systems

After block layouts, adjacency layouts, room design criteria, and pressurization levels have been established, the number and type of air handling systems can be selected. Air handling systems most commonly used are constant and variable volume with and without reheat coils for control. For controlled spaces the most reliable system is a constant volume system with terminal reheat (CVRH). This system is not used in less critical or commercial applications. In a terminal reheat system the air leaving the cooling coil is set at a fixed temperature, and the terminal reheat responds to a space thermostat, turning on heat to satisfy the load. This can waste energy, since air is cooled and then reheated. Reheat systems require energy to both simultaneously heat and cool the air, as can easily be demonstrated on a psychrometric chart. Many energy codes prohibit this practice for comfort applications, however, where close control of temperature and humidity is required for process areas the energy conservation requirement is waived. The advantages of reheat systems are that humidity is always controlled (since dehumidification always takes place at the cooling coil) and each space or zone that needs temperature control can easily be accommodated by adding a reheat coil and thermostat. Another advantage of the CVRH system is that airflow is constant, which makes balancing and pressurization easier to maintain. A reheat system is probably the simplest and easiest of all systems to understand and maintain. Figure 3 is a typical depiction of an airflow diagram for a CVRH system.

Variable Air Volume Systems

A variable air volume (VAV) system is generally used in administrative areas and some storage spaces where pressure control is not critical, humidity control is not essential, and some variations in space temperature can be tolerated. The VAV system works by delivering a constant-temperature air supply to spaces with reductions in airflow as cooling loads diminish. This reduction in airflow results in a reduced cooling effect, allowing the airflow or cooling to match the actual room load, which provides the design space temperature condition. This eliminates the energy used for reheat and saves fan energy, because the total amount of air moved is reduced. Some form of perimeter heating must be supplied for spaces with exterior walls or large roof heat losses. The perimeter heating can be baseboard radiation or some form of air heating using heating coils. Finned radiation or convection heating devices should not be used in clean spaces, since they are not easily cleaned and allow places for unwanted particulate buildup. Combinations of systems can be used, especially if variable quantities of supply and exhaust air are required for fume hoods or intermittent exhausts.

Figure 3. Airflow diagram: 1, 35% filters; 2, 85% filters; 3, preheat coil; 4, cooling coil; 5, fan section; 6, humidifier; 7, air monitoring device; 8, constant volume reheat box; 9, constant volume box; 10, return fan; 11, relief air damper; 12, return air damper; 13, outdoor air damper.
Recirculation Systems

In bioprocess facilities large quantities of air may be required to promote unidirectional flow and air cleanliness. This is particularly true in a class 100 space. In many cases the large quantities of air exceed the requirements for cooling, so it is desirable and possible to recirculate air within the space and only pass enough air through the air handling unit to perform the heating or cooling. In many cases the recirculating systems are supplied as a separate package installation. Numerous manufacturers provide these packages, which include supports, fans, and filters. Some vendors provide fans that are self-contained with each 2-ft by 4-ft filter module.

Airflow Sheets

The airflow sheets should be developed on full-size drawings using computer drafting. They should list each space and show air quantities supplied, returned, and exhausted from each space. They also must show air transferred into and out of spaces, and, while quantities should be shown, they will probably require field modification to attain pressurization. The airflow sheet becomes a useful tool for transfer of information to the owner or user, for agency reviews, for transmission of information to HVAC designers, and for other engineering disciplines. These documents are also invaluable to construction contractors and for system checking by construction managers and balancing contractors. Airflow sheets provide a pictorial description of each air system and show how the elements comprising the system are related.

DEHUMIDIFIERS

Need for Dehumidification

In many cases low relative humidity is required, such as in drying or powder areas where hygroscopic materials are handled and would absorb unwanted moisture from the air. A chemical dehumidifier may be required to obtain the desired low humidity conditions. The need for a dehumidifier can be determined by utilizing a psychrometric chart, which will establish a leaving-coil dew point. Normalifier can be determined by utilizing a psychrometric chart, desired low humidity conditions. The need for a dehumidifier may be required to obtain the handled and would absorb unwanted moisture from the in drying or powder areas where hygroscopic materials are handled and would absorb unwanted moisture from the air. A chemical dehumidifier may be required to obtain the desired low humidity conditions. The need for a dehumidifier can be determined by utilizing a psychrometric chart, which will establish a leaving-coil dew point. Normalifier can be determined by utilizing a psychrometric chart, desired low humidity conditions. The need for a dehumidifier may be required to obtain the handled and would absorb unwanted moisture from the in drying or powder areas where hygroscopic materials are handled and would absorb unwanted moisture from the air. A chemical dehumidifier may be required to obtain the desired low humidity conditions. The need for a dehumidifier can be determined by utilizing a psychrometric chart, which will establish a leaving-coil dew point. Normalifier can be determined by utilizing a psychrometric chart, desired low humidity conditions. The need for a dehumidifier may be required to obtain the handled and would absorb unwanted moisture from the in drying or powder areas where hygroscopic materials are handled and would absorb unwanted moisture from the air. A chemical dehumidifier may be required to obtain the desired low humidity conditions. The need for a dehumidifier can be determined by utilizing a psychrometric chart, which will establish a leaving-coil dew point. Normalifier can be determined by utilizing a psychrometric chart, desired low humidity conditions. The need for a dehumidifier may be required to obtain the handled and would absorb unwanted moisture from the in drying or powder areas where hygroscopic materials are handled and would absorb unwanted moisture from the air. A chemical dehumidifier may be required to obtain the desired low humidity conditions. The need for a dehumidifier can be determined by utilizing a psychrometric chart, which will establish a leaving-coil dew point. Normalifier can be determined by utilizing a psychrometric chart, desired low humidity conditions. The need for a dehumidifier may be required to obtain the handled and would absorb unwanted moisture from the in drying or powder areas where hygroscopic materials are handled and would absorb unwanted moisture from the air. A chemical dehumidifier may be required to obtain the desired low humidity conditions. The need for a dehumidifier can be determined by utilizing a psychrometric chart, which will establish a leaving-coil dew point. Normalifier can be determined by utilizing a psychrometric chart, desired low humidity conditions. The need for a dehumidifier may be required to obtain the handled and would absorb unwanted moisture from the in drying or powder areas where hygroscopic materials are handled and would absorb unwanted moisture from the air. A chemical dehumidifier may be required to obtain the desired low humidity conditions. The need for a dehumidifier can be determined by utilizing a psychrometric chart, which will establish a leaving-coil dew point. Normalifier can be determined by utilizing a psychrometric chart, desired low humidity conditions.

Chemical Dehumidification

Chemical dehumidifiers are commercially available air handling units that contain a sorbent material (desiccant) that can be a solid or liquid. Wet dehumidifiers use adsorbents that change physically during the process. Lithium salt solutions are generally used to remove moisture from conditioned air and are then regenerated by heat, usually using a steam heat exchanger. Dry dehumidifiers use adsorbents that do not experience phase changes dur-
contain good access doors, view ports, electrical convenience outlets, and interior lighting for maintenance. The casings should be tightly sealed and designed for pressures that are higher than commercial applications due to the generally high system air pressures required for bioprocess applications. All sealants and lubricants exposed to the airstream should be food grade to minimize the chance of air contamination. Units designated as draw-through have the coils on the suction side of the fan. Blow-through units have the coils on the discharge side of the fan and have the advantage of a filter downstream of the coils, reducing potential contamination of the supply duct system. On blow-through units an air distribution plate must be installed to properly distribute air evenly over the filter and coils. It should be noted that if the unit contains a HEPA filter and the air is ducted directly to the clean space without a terminal HEPA filter, the duct should be constructed of a clean, smooth, nonflaking material, usually stainless steel.

Air Handling Unit Location

It is best to locate units indoors for ease of service and to extend the life of the units. They should also be located as close as possible to the main rooms they are serving to minimize larger and longer duct runs. Airflow measuring devices and humidifiers must also be installed, and unit location must allow enough straight run of duct for installation and proper operation of these devices. Location of outdoor air-inlet louvers must be carefully considered. Intakes are generally located on the building sidewall high off the ground to minimize dust intake. Intakes should also be away from truck docks or parking lots, where undesirable fumes and particulate are generated. In locating inlets the prevailing winds should also be considered, and any nearby exhausts or fume concentrations should be avoided to prevent recirculation of exhaust air back into the supply system.

RETURN AND EXHAUST FAN SELECTION AND LOCATIONS

Return Fans

Return and exhaust fans are integral parts of the air handling systems and also must be shown on airflow diagrams. Return fans are used on systems with long duct returns or that have return system pressure drops greater than 0.5 in water (120 Pa). This allows proper total system balance and minimizes suction pressure required from the supply fan. If a return fan is not used, the capacity of the supply fan can be overextended and it may be difficult to limit and properly control the amount of outside air being admitted to the unit. Outside air fluctuations are also more susceptible to exterior wind conditions. Return fans are also needed when required to provide a negative pressure in rooms that require containment. Return fans can be of standard centrifugal type or an in-line type, which works nicely for installation directly into return ducts in crowded equipment rooms. Return fans may also be required to handle varying quantities of air or provide a constant flow of air at varying pressure conditions. To achieve these conditions some form of damper control, inlet vane, or variable frequency drive motor control is generally used.

Exhaust Fans

Building exhausts are generally collected and ducted to exhaust fans in groups or clusters. Exhaust fans should be located as near to the building discharge as possible since this keeps the duct under a negative pressure and any leaks will be into the duct, and not contaminated air from the duct into an occupied space or mechanical room. For this reason roof locations of fans are preferred, even though this may make service difficult in severe weather conditions. When fans are located in mechanical rooms or interstitial spaces, it is essential to tightly seal the discharge duct before it exits the building in a roof vent or wall louver. Roof penetrations should be kept to a minimum to prevent leaks. Fumes and toxic exhausts should be extended through the roof and terminated well above the roof line in a suitable stackhead. Designs for stackheads are found in most duct manuals and are used to direct exhaust vertically at high velocities to enhance dispersion of fumes and prevent water from finding its way into the exhaust system (8). Extremely toxic or dangerous active biological agents may require HEPA filtration or other treatment, such as incineration, before exhaust to the atmosphere.

Redundancy

Another important consideration occurs when return or exhaust fans are used as part of the containment system. Should the fans be essential to maintaining containment, it may be desirable to have a backup fan or redundant system. This is essential if loss of containment can be harmful to humans or would result in an expensive loss of product. Airflow switches, which give a warning in case of fan system failures, are also desirable options for critical systems. The airflow sensing method to prove flow is preferred to an electrical motor indication since the motor could be running with a broken fan belt and the operator would not know that the fan is not moving air.

HEPA FILTERS

HEPA Filter Description

HEPA filters are used in the final cleaning stages of air to remove very fine particles. Rough and intermediate filters that are not as efficient as HEPAFs are used to remove larger particulate matter. This prolongs the life of the more expensive HEPAFs, significantly reducing the cost of filter element replacement. A HEPA filter, by definition, removes 99.97% of particles of 0.3-micron size. HEPA-type filters called ultraparticulate arrestences (ULPAs) can be even more efficient and are commercially available at a greater cost. Pressure drop across filters is rated for nominal flow at new clean conditions and is usually about 1 in (25.4 mm), and the systems are designed to change out filters at approximately 2 in (50.8 mm) or double the clean ratings, although filters can be operated at higher pressure drops, which some owners prefer. The higher operating pressures
result in a greater use of electrical energy to power the fan to overcome the increased resistance that it must operate against.

Location

HEPA filters are usually located downstream of heating and cooling coils in air handling systems since coils are potential sources of contamination. The most popular HEPA filter location is in the room ceiling using standard laminar flow outlets nominally 24 in by 48 in (0.6 m by 1.2 m). These outlets contain manual control dampers, testing sample ports, a diffusion panel, and a HEPA filter element. They are commercially available in permanent or throw-away units where the entire assembly is disconnected from the flexible duct supply terminal and replaced when the filter is dirty. Sealing of filters to frames is an installation problem and is best solved by using a filter frame with a gel-like seal into which the filter fits. This material does not support biological growth, and these filters are offered by several manufacturers.

Bag-In Bag-Out Housings

Where danger of exposure to filter changing maintenance personnel exists, filters are placed in special enclosures called bag-in bag-out housings. These units are quite expensive and employ elaborate sealing methods and a double bag arrangement that allows changing of the filter element(s) without ever exposing the changer to the dirty filter or its collected contaminant.

Laminar Flow Workstations

HEPA filters are also used in class 100 workstations in single or double filtration of air to provide sufficient cleaning before unidirectional (laminar) air distribution to the work space. These workstations are commercially available in horizontal or vertical flow patterns generally recirculating within the clean space. Filters are commercially available in many standard sizes and come with many frame arrangements with different methods used to form a seal between the filter element and the frame.

TERMINAL AIR CONTROL DEVICES

In most bioprocess applications the volume of airflow must be controlled to ensure proper air changes and provide air for pressurization. The choices to control airflow are (1) a variable flow responding to some pressure or temperature actuated signal or (2) constant volume to provide a uniform quantity of air under varying upstream pressure conditions. Many commercial devices, usually called boxes, or terminal control units, are available that utilize a variety of air dampening designs—from a simple blade type to pneumatic bladder type and conical type. Each type has its own application and associated cost implications. The published damper design and test data for flow and repeatability should be studied against the suspected operation and matched to the proper application. Dampers are controlled from pneumatically controlled operators or electric motors responding to electric or electronic signals. Pneumatic dampers generally react more quickly to changing airflow requirements. Dampers and controls should be selected for linearity of response, repeatability, and percentage of error when compared with desired flow. The damper is generally housed in a device called a volume control box, which contains deflecting baffles and flow-measuring or pressure-sensing devices that are used for flow control. Accuracy of flow control boxes, combined with the controlling system, must be aggregated to ensure that the actual flow will be within acceptable limits to control pressurization. The accuracy of control devices can vary between 5 and 10% of maximum flow. This built-in inaccuracy must be calculated when establishing flow differential rates. For commercial applications, control boxes are lined with insulating acoustical material, which should be avoided on boxes used for critical or controlled areas to avoid dirt catchers or host locations for unwanted growth.

Some vendors offer complete control box systems, including controls, supply and return boxes, and a system to allow tracking. Tracking is a term used to describe when the control system monitors the supply flow and automatically adjusts the return or exhaust flow to maintain a fixed CFM differential between supply and return or exhaust. This maintains the space pressure differential.

AIR TERMINAL OUTLETS

Supply Terminals

In clean spaces the desired distribution of air is unidirectional or acts as a piston of air that reduces turbulence and eddy currents in the airflow. This piston of air carries particulate from the ceiling to the floor return and helps to prevent airborne particulate matter from recirculating and contaminating the work space. In most cases it is desirable to recirculate air within a space through a filter since the return air has less particulate than typical outdoor air and does not require extensive heating and cooling. Air terminals should be selected of materials that are nonflaking, nonoxidizing, and are easily wiped clean.

Location

Placement of outlets should be above work areas since the cleanest air should be introduced at these spaces. If the product at the workstation is hazardous to humans the air should be supplied at the operator’s back and pulled into the work area to ensure that flow is away from the operator and any particulate will be captured in the airstream. Containment velocity at the inlet of a biosafe hood or enclosure should be about 100 ft/min (0.508 m/s). Airflow near hoods or enclosures should avoid any air disturbance that could cause eddy currents or air turbulence. These currents will upset capture patterns of the hood and make verification of hood face velocity difficult. Many of the cabinet manufacturers have extensively tested capture velocities and provide excellent data on the actual operating conditions. When locating terminals and enclosures it is important to consider movement of personnel within the space and identify frequently opened doors, which also tend to upset airflow patterns. Should the operator pose a threat to the
product. the airflow pattern should be reversed, with the air supplied behind the product and blowing past the product to avoid contamination from the operator.

Return Terminals

Return terminals are also an important consideration and are generally located low in the walls for clean rooms. In class 10,000 to 100,000 rooms low cleanable wall registers are generally used. In cleaner areas low return wall systems, termed air walls, are used. The air wall is an almost continuous opening at the base of the wall with the air ducted up in the wall system and collected for return to the air handling system. Air wall inlets are generally located not more than 15 ft (4.5 m) in plain view from a supply terminal to reduce the likelihood of turbulence. Figure 4 shows a typical return air wall detail.

DUCT MATERIALS, PRESSURE, AND CLEANABILITY

Materials of Construction

Unlined galvanized steel ductwork is used in rectangular, round, and elliptical (or flat oval) configurations for the majority of the systems. Because galvanized duct can flake off or rust, it should not be used downstream of the HEPA filters to avoid contamination from the duct system itself. When the HEPA filter is located upstream of the room terminal and a long run of duct is present, the material of choice for the duct is stainless steel, but this is expensive and its use should be minimized. Many systems may also be fumigated or cleaned in place, and the duct material chosen should not be affected by the cleaning agent.

Duct Pressures

Ductwork systems in bioprocess facilities tend to have higher system pressure requirements than commercial systems due to extensive use of filters, volume control devices, and physically complex arrangements. The duct system pressures must be calculated and clearly stated on the contract documents to allow the fabricator to provide the proper metal thickness and construction methods for the required system pressures. System pressures will also change as the system is operated with filters that get dirty or space pressure conditions that vary. Duct systems must allow for these pressure fluctuations, and the fans may require speed controls, inlet vanes, or variable pitch blades to match the varying flow and pressure conditions.

Cleanability

Cleanability of duct systems is important to ensure that if an installed system gets dirty or contaminated it can be cleaned. In the design stage care must be taken to locate access doors in the duct, where they can be easily reached without compromising a process or violating a controlled space. It is best to minimize entry of facility system maintenance personnel into the clean spaces. All sealed duct shipped to the site should have only end seals broken, and then quickly resealed, during final installation. In very critical applications the duct is factory cleaned and sealed before shipment to the site. This step removes the oil and other contaminants present during duct construction but is expensive. It may be difficult to find sheet-metal fabricators willing to do this work, since they are not always set up for such procedures and it makes shipping the completed product difficult.

SYSTEM OPERATING PROCEDURES

Operating Procedure

When the airflow sheets are completed it is appropriate to finalize the system operating procedure. This is usually a written document stating how systems are turned on and off, and it gives a description of their frequency of operation. For clean spaces general practice is to operate the air system 15–30 min for purging before beginning production or use of the space. In some cases it is necessary to operate a system continuously to ensure that pressurization or containment is always maintained. When analyzing system operation it is important to assess how an unexpected system shutdown or flow reduction will affect an adjacent sys-
The HVAC system is called by many names: the automatic control system that controls and monitors the HVAC system. At this stage of design, procedures should be developed for the air system to accommodate a product spill or accident in a contained space. The ramifications of a spill on the air system, controlled space, and adjacent operations must be evaluated. Cleanup procedures could include fumigation of the air system, which would require operation of a relief connection to the ductwork for venting the fumigant.

System Alarms and Emergency Measures

Alarms that sound to indicate loss of pressurization are valuable features and essential in the HVAC design of critical areas. At this stage of design, procedures should be developed for the air system to accommodate a product spill or accident in a contained space. The ramifications of a spill on the air system, controlled space, and adjacent operations must be evaluated. Cleanup procedures could include fumigation of the air system, which would require operation of a relief connection to the ductwork for venting the fumigant.

Operations Review

The entire step of establishing operating procedures should be included in the system, such as a night temperature setback or reduced ventilation and exhaust rates during unoccupied periods.

System Architecture

The control system of choice for major facilities, and even for some small systems, is a direct digital control (DDC) system. Most major control system vendors and many of the smaller vendors offer DDC systems that are similar but contain internal differences. The systems are computer-based and have the ability to communicate within and outside the system by coded digital signals. System architecture refers to the major components of the DDC system and their interrelationship. The architecture is developed by determining what components are initially required, what may be required in the future, and how the system may expand as additional requirements are added. Figure 5 shows a typical DDC system architecture.

Points List

After the sequence of operation is completed and the airflow diagrams are defined, the next step is to develop the alarm, control, and monitoring points list. This is an all-inclusive list of points that are to be connected to the DDC system. There are two major types of points: digital and analog. A digital point is simpler, generally less expensive, and works on a simple on–off or contact principle. Digital points are used to start and stop fans, indicate an on–off condition, or anything that requires only a single contact. An analog point is used to measure variables such as temperature, pressure, and flow rate. These points generally use 4- to 20-mA signals that provide varying signals in response to the parameter measured. The electronic signals used by the BAS may be transduced from variable pneumatic or pressure signals. The points list should include analog control points such as cooling coil valves and room temperatures. Monitoring points can be digital or an-

EMERGENCY ELECTRICAL POWER

An essential step in the HVAC design process is coordination with the electrical design team. Motor lists for HVAC equipment must be prepared and reviewed with the electrical design team. The need for motors designated for emergency power, variable speed, reduced voltage starting, or other special characteristics must be communicated to the electrical designers early in the design process. The sizing of the emergency generator can be greatly affected by motors required on emergency power from the HVAC system. Fans, equipment, or sensing devices that require interlocks must also be picked up by the electrical designers. The motor list must be kept up to date from project inception through commissioning. The motor list is useful for a reviewing agency, a valuable tool in training plant operators, and a great aid in understanding the HVAC system.

BUILDING CONTROL AND AUTOMATION SYSTEMS

Sequence of Operation

The automatic control system that controls and monitors the HVAC system is called by many names: the automatic temperature control system (ATC), the energy management and control system (EMCS), the building automation system (BAS), or the building management system (BMS). The first element in the design of the system is the development of a sequence of operation, which is a written description of the HVAC and related systems operation. A separate sequence is usually written for each air handling system, describing the complete operation of the system from control of coils and humidifiers to control of the room temperature and humidity. Starting and stopping of the air handling unit fans is outlined, along with interlocking of exhaust or return fans in relation to the main air system fan operation. Generally all fans operate at the same time, which is necessary to maintain pressurization. The sequence also addresses abnormal occurrences such as a smoke detection alarm or failure of an exhaust fan. The sequence describes what happens to system components during an abnormal occurrence. It may be necessary to shut down the system if a major exhaust fan should fail to prevent or minimize the loss of pressurization. The sequence also describes any energy management strategies to be included in the system, such as a night temperature setback or reduced ventilation and exhaust rates during unoccupied periods.
Estimate of System Cost

The automatic control and monitoring system is a major cost element in the overall HVAC system for a bioprocess facility. After the points list is developed a good estimate can be made for the system cost. Several estimating numbers can be used in providing an educated guess of the cost, with a general range from a low of $500/point to as high as $1,200/point. Larger systems have lower dollar-per-point costs because the cost of the computers, monitors, and printers are diluted over many points. Smaller systems have higher costs because this dilution effect does not come into play. Systems with a higher percentage of analog points over digital points also tend to raise dollar-per-point costs. Control wiring is also a significant component of the BAS cost, and long runs of wiring tend to raise the dollar-per-point cost. When a floor plan is completed, the system architecture is established, and the points list is completed, this information should be given to one or more DDC vendors for a budget estimate. Vendors will make estimates based on the information provided, take off wiring runs, and suggest panel locations in arriving at the budget cost. At the completion of this exercise the cost of this major element can be locked in. Should the cost be greater than desired some points can be eliminated or the scope of the system reduced to bring the cost within the budget. When a system is bid it is recommended that component unit pricing be obtained from the vendor to lock in future costs should additions or modifications be desired. It is essential that vendors give classes and instructions on their systems before final turnover to the owner's operators.

Future System Flexibility

The trend is that after a DDC system is installed the number of points grows as the user becomes familiar with the system. Additional points may be added for maintenance, such as filter change out pressure devices. Run time logs set up in the system software are useful for lubrication schedules, belt checks, and so on. This requires software to log in and integrate data and to print out notices at preselected intervals of run time. Another useful feature is the phone modem, which would allow an operator to call in from a remote location and check on the system operation. This feature can save a trip to the facility if an alarm situation develops. Someone at the plant, such as the security guard who received the alarm, could place a call to the operator at home. The operator could dial into the BMS by modem and possibly analyze the problem by reading some key parameters. He or she could then determine the severity of the problem and either ignore it until the next shift or instruct someone on-site to take corrective action. Even though the original budget may not allow all features that are ultimately desired, it is good to look toward the future in anticipation of system expansion and ensure that the purchased system is capable of desired expansion.

TESTING, BALANCING, AND CLEANING

Testing and balancing for bioprocess systems is more critical than for commercial systems due to the very complex systems and higher pressures encountered. Associations of testing and balancing agencies publish excellent standard forms and procedures that are used in balancing air and water systems. Air balancing consists of setting terminal devices and air-moving equipment to deliver the proper quantities of air. For bioprocess facilities the additional requirement of establishing pressure differentials between adjacent spaces must also be set. These differentials are obtained by adjusting airflows, smoke tests, taking pressure readings, and setting controls. This effort can take some time as each facility is different and each room has different leakage characteristics that affect pressurization. As part of the balancing it may be discovered that duct systems or rooms are not as tight as designed and may require additional sealing to obtain the required pressure differentials. Recall that airflows shown on drawings are design values and generally require minor adjustment to achieve the required pressure differentials. A simple solution to many pressurization problems is to keep increasing outdoor air to the system. This can lead to problems if
design values are exceeded, with heating or cooling coils not meeting this need, resulting in off-design room temperatures and humidity levels. The air handling unit coils will use available cooling capacity to condition excessive quantities of outdoor air, resulting in room supply temperature higher than as designed.

In these cases the rooms or systems should be tightened to get closer to design values. The optimum time to balance is when few construction workers or facility personnel are in the spaces. The balance should be done with all doors closed, since opening and closing result in system pressure upsets and make balancing difficult. Before the system is turned over to the owner, the rooms and ductwork system must be thoroughly cleaned to allow certification of room cleanliness conditions. During construction the work areas should be broom-cleaned after each day’s work and open duct systems kept closed. If followed during construction, these procedures will result in an easier final cleanup before turnover.

VALIDATION

When a bioprocess facility is to be validated, the validating agency will peruse the HVAC documentation and should communicate with the design engineers to establish the validation protocol as it relates to the HVAC system. If the design is proper, the contractor has properly installed the system, and the components perform as specified, the systems should be easily validatable. The validator will follow a master plan and protocols to verify the actual system installation and operation against design values and intent (10). The physical parameters reported by the BAS system will be verified by measurements using calibrated instruments to verify accuracy. Airflow measurements will be made at supply and exhaust outlets, as well as traverses across the face of hoods, to verify proper flows and capture patterns. Filters will be physically tested or challenged to uncover leaks in the filter media or at seals between the holding frame and elements. Any leaks will be field corrected. Samples of air will also be taken by the validator to ensure that the cleanliness requirement for the controlled space is actually maintained by the installed operating air system. If problems occur they are usually in the form of pressurization deficiencies or filter leaks. Even after the mechanical contractor has completed his or her work, a hole for a pipe could be cut in a room and not properly sealed, resulting in a change in room pressurization. Filter elements may not be properly sealed with the frame, resulting in leaks, or small holes may be discovered in the filter element as a result of handling or damage during installation. Should any of these problems occur, and are not easily resolvable by the validator, it may be necessary for the HVAC contractor and engineer to lend assistance in making the system valid. Another important criterion at this stage is the calibration of airflow monitors, which should be installed in supply and return systems to critical areas to ensure that airflow rates are as designed. Temperature and humidity sensors at critical areas should also be checked for accuracy at this time by actually reading space conditions and checking against values reported by the BMS.

SUMMARY

The HVAC system is an essential component in the successful operation of a bioprocess facility and must be given a proper amount of attention during the planning and design stage. The space requirements for air walls, control boxes, extensive duct runs, and large air handling units must be established early in the design process to prevent unsatisfactory compromises later on. The owner and operators must work closely with the architect and engineer design team to agree on the HVAC system philosophy and the proposed operation of the system. The amount of energy management and control, as well as system redundancy, are among important economic decisions that should be agreed on during this design process. Compromises made during the design development should make economic sense, be mutually beneficial, and be understood by affected members of the project team. It is best from a cost perspective to resolve HVAC issues as early in the design process as possible to avoid design delays and more costly changes later in the design process. This avoids delays in the design process and minimizes the effect of changes on other engineering disciplines. All parties involved must believe in the HVAC system philosophy to avoid costly and time-consuming retrofits during construction or at commissioning, validation, or after the facility begins operating.

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Hemicellulases

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Introduction

During the past decade, the industrial use of hemicellulases, especially xylanases, has grown remarkably. Hemicellulases form a group of enzymes that degrade different hemicelluloses in annual plants and woody materials. Originally, hemicellulases were studied for use in the total hydrolysis of waste liquors containing hemicelluloses or for fractions derived from lignocellulosics. Today, hemicellulases are used for partial or restricted hydrolysis of fibrous materials containing hemicelluloses. Thus, hemicellulases have found uses in processing of hemicellulose-containing plant materials in the food, feed, and pulp and paper industries. The market for hemicellulases has been growing since the beginning of the 1990s due to a better understanding of the role of hemicelluloses in processing of the raw materials and to increased knowledge of these enzymes. Several commercial hemicellulases with different properties are now available from enzyme producers.

Hemicelluloses are natural components in cereals, and their properties and performance in food and feed raw materials can be improved by the action of enzymes. Exogenous enzymes offer alternatives to chemical additives in baking, where the application of microbial enzymes is expected to increase. Most of the added enzyme types are already present at low levels in the grain raw material. Commercial enzyme preparations principally consist of the same enzyme types but are usually of microbial origin. Enzymes in food and feed applications may also be used for upgrading the quality of the raw material. In the pulp and paper industry, the partial hydrolysis of fiber hemicelluloses by hemicellulases leads to improved processes and decreased chemical consumption (i.e., cleaner technology). The role of hemicellulases has been clearly demonstrated in improving the bleachability of pulps, whereas in other applications their impact is less significant.

Most of the enzymes studied in applications have been hemicellulase mixtures. For a successful application of hemicellulases a good understanding of both the substrate and the enzyme complex is needed. A reason for the sometimes fluctuating results on the effects of enzymes in various applications is that the preparations tested may contain various activities, including enzymes other than hemicellulases. Commercial enzyme preparations may vary considerably in composition and ratio of various activities, depending on the source.

This chapter highlights progress in the use of hemicellulases and their properties and exploitation in different areas (e.g., food, feed, pulp and paper) where the applications are commercially used.

Structure and Composition of Hemicelluloses

Hemicelluloses are heteropolysaccharides consisting of several polymers and varying in their monosaccharide composition, glycosidic linkage composition, substitution pattern, and degree of polymerization (1). They are often reported to be chemically associated (e.g., cross-linked) to other polysaccharides, proteins, or lignin. The structural characteristics affect physical properties, such as solubility, viscosity, crystallinity, and porosity and thus the enzymatic degradability.

The definition of the term hemicellulose is not very clear. It mainly refers to plant cell wall polysaccharides that can be extracted by alkaline solutions. Some hemicelluloses are also soluble in water. Hemicelluloses are usually classified according to the main sugar residue in the backbone, for example, xylans (o-xylene), mannans (o-mannose), galactans (o-galactose), and glucans (o-glucose), the first two being the main groups of hemicelluloses. Depending on the origins, hemicelluloses are substituted, to varying degrees, by side groups (1). In cereals in particular, the term pentosan is often used as a synonym for hemicelluloses, especially for xylans. Hemicelluloses present in cereals and woody materials are discussed in the following. Pectic substances consisting of polygalacturonic acid, arabinans, galactans, and arabino-
galactans, and noncellulosic β-glucans, which sometimes are included in hemicelluloses, are not considered in this review.

Hemicelluloses in Cereals

In cereals, hemicelluloses consist of arabinoxylans, arabinoxogalactans, and xyloglucans, of which arabinoxylans are most abundant. The content of arabinoxylans in cereals is reported to be 1.4–8.5%, with water-soluble fractions contributing 1.5–4.3%. Wheat and rye brans have the highest content of arabinoxylans. The main component of aleurone cell walls of cereals is arabinoxylan, and the cell walls of grain endosperms also contain significant amounts of arabinoxylan.

The arabinoxylans of annual plants have a linear backbone of (1,4)-β-D-xylopyranosyl units, which is mainly substituted with terminal α-L-arabinofuranosyl substituents attached to carbon 2 and/or carbon 3 of the xylosyl residues (1). Thus, xylans may be mono- or double-substituted by arabinose. The xylan backbone often contains other substituents as well, such as 4-O-methyl-α-D-glucoronic, acetic, or ferulic acids. The ratio of arabinose to xylose may be used as a simple indication of the degree of substitution in arabinoxylans and varies between 0.54 and 0.70. The structure of arabinoxylans in annual plants varies among different species and parts. Thus, even arabinoxylans in aleurone and endosperm have different structures.

Hemicelluloses in Woody Materials

Wood is composed mainly of cellulose, hemicellulose, and lignin. Depending on the wood species, about 20–30% of dry weight of wood is hemicellulose. Hemicelluloses are associated with cellulose and lignin in woods. Not only the relative amounts but also the chemical composition of these polysaccharides in softwood and hardwood vary. Other polysaccharides, such as pectic compounds, are also present in wood in minor quantities. The distribution of carbohydrates in the wood fibers varies depending on the wood species and growing conditions (2). In general, in softwoods the xylan content of the innermost cell wall layer (the warty layer), is very high, but otherwise the xylan is relatively uniformly distributed throughout the fiber walls. In hardwoods, the outermost layers of fiber walls are rich in xylan. In softwoods the glucomannan content increases steadily from the outer to inner cell walls.

In wood, the two most common hemicelluloses are xylans and glucomannans. The hardwoods contain mainly xylan, whereas the amount of glucomannan in softwoods is approximately double compared with that of xylan. Hardwood xylan is composed of β-D-xylopyranosyl units, which may contain 4-O-methyl-α-D-glucuronic acid and acetyl side groups. 4-O-methylglucuronic acid is linked to the xylan backbone by O-(1,2) glycosidic bonds, and the acetic acid is esterified at the carbon 2 and/or 3 hydroxyl group. The softwood xylan is arabinono-4-O-methylglucuronoxylan, in which the xylan backbone is substituted at carbon 2 and 3 with 4-O-methyl-α-D-glucuronic acid and α-L-arabinofuranosyl residues, respectively. Softwood galactoglucomannan has a backbone of β-(1,4)-linked β-D-glucopyranosyl and β-D-mannopyranosyl units, which are partially substituted by α-D-galactopyranosyl and acetyl groups (2).

Hemicellulases

Due to the complex structure of hemicelluloses, several different enzymes are needed for their enzymatic degradation and modification (Fig. 1). The two main glycanases that depolymerize the hemicellulose backbone are endo-1,4-β-D-xylanase and endo-1,4-β-D-mannanase. Small oligosaccharides are further hydrolyzed by 1,4-β-D-xilosidase, 1,4-β-D-mannosidase, and 1,4-β-D-glucosidase. The side groups are removed by α-L-arabinosidase, α-D-glucuronidase, and α-D-galactosidase. Esterified side groups are liberated by acetyl xylan esterase and acetyl glucomannan esterase (3).

Hemicellulases are produced by many species of bacteria and fungi, as well as by several plants. Many microorganisms produce a multiple pattern of these enzymes to degrade the plant materials efficiently. After identification of the most desirable enzymes for a potential application, the production of the enzyme can be improved by genetic engineering. Today, most commercial hemicellulase preparations are produced by genetically modified Thermotoga maritima or Aspergillus strains.

Most microbial hemicellulases studied are active in slightly acidic conditions between pH 4 and 6 and at temperatures below 70 °C. More thermophilic, acidic, and alkalophilic hemicellulases are of great interest in many applications. Xylanases that are stable and function efficiently at high temperatures can be produced by thermophilic bacteria especially. Several xylanase genes, encoding proteins active at temperatures from 75 °C up to 95 °C (pH 6–8), have been isolated. The most thermophilic xylanase so far described are produced by species of the extremely thermophilic bacterium Thermotoga maritima (4). Only a few ther-

![Figure 1. Enzymes participating in the hydrolysis of xylans (a) and glucomannans (b): Ph, phenolic groups; Ac, acetyl; Ara, arabinose; MeGlcA, methyl-glucuronic acid; Xyl, xylose. Gal, galactose; Glc, glucose; Man, mannos.](image-url)
mophilic mannanases have so far been characterized (5,6). Xylanases and mannanases with alkaline pH optima have been detected especially in alkalophilic Bacillus species.

**Xylanases**

Since xylan is the most abundant hemicellulose in most raw materials, a major part of the published work on hemicellulases deals with the properties and mode of action, as well as applications of xylanases (7–9). Endoxylanases (1,4-β-D-xylanohydrolases, EC 3.2.1.8) catalyze the random hydrolysis of 1,4-β-D-xylosidic linkages in xylans (Fig. 1). Most xylanases are rather small proteins (molecular mass around 20 kDa) with a basic isoelectric point (pl 8–10). Another group of xylanases has also been identified. These xylanases, are somewhat larger (molecular mass over 40 kDa) with acidic isoelectric point (pl 3–5). This grouping of xylanases is in consonance with the classification of glycanses based of hydrophobic cluster and sequence analysis (10). The xylanases belonging to the two identified xylanase groups (glycosyl hydrolase families 10 and 11, formerly F and G) differ from each other also with respect to their catalytic properties (11). Xylanases with high Mr/low pl (family 10) seem to exhibit greater catalytic versatility than the low Mr/high pl xylanases (family 11) and thus they are, for example, able to more efficiently hydrolyze highly substituted xylans.

The three-dimensional structures of different low molecular mass xylanases belonging to family 11 have been determined (12–14). These enzymes have very similar structures; they are ellipsoidal, well-packed molecules having diameters between 30 and 45 Å, and do not contain any separate substrate-binding domain. Some bacterial xylanases, however, have been found to contain either a cellulose-binding domain (15) or a xylan-binding domain (16). Only one crystal structure of family 10 xylanases has been solved (17). Figure 2 shows the three-dimensional structures of a catalytic domain of the xylanase A (family 10) of Streptomyces lividans and the xylanase II (family 11) of Trichoderma reesei.

Most of the xylanases characterized act randomly and are able to hydrolyze different types of xylans, showing only differences in the spectrum of end products. The main products formed from the hydrolysis of xylans are xylobiose, xylotriose, and substituted oligomers of two to four xylosyl residues. The chain length and the structure of the substituted products depend on the mode of action of the individual xylanase (11). Some xylanases, however, have a rather strict substrate specificity. A unique xylanase that requires a glucuronic acid substituent in the xylan backbone is produced by Bacillus subtilis (18). On the other hand, xylanases that require at least 24 unsubstituted xylose residues for action are produced by Talaromyces emersonii (19).

**Mannanases**

Endomannanases (1,4-β-D-mannan mannanohydrolase, EC 3.2.1.78) catalyze the random hydrolysis of 1,4 mannopranosyl linkages within the main chain of mannan and various polysaccharides consisting mainly of mannose, such as glucomannans, galactomannans, and galactoglucomannans (Fig. 1). Mannanases are generally larger proteins than xylanases (Mr 30–90 kDa) and have acidic isoelectric points. They also seem to be a more heterogeneous group of enzymes than xylanases (i.e., no clear groups based on biochemical properties have been identified). According to protein sequences, the known mannanases belong to three different glycosyl hydrolase families (families 5, 26, and 44) (10).

The mannanase of T. reesei, has been found to have a multidomain structure similar to several cellulolytic enzymes, that is, the protein contains a catalytic core domain that is separated by a linker from a cellulose-binding domain (20). The first three-dimensional structure of mannanase was recently published (21).

The main hydrolysis products from galactomannans and glucomannans are mannobiose, mannotriose, and various mixed oligosaccharides. The hydrolysis yield is dependent on the degree of substitution, as well as on the distribution of the substituents (22). The hydrolysis of glucomannans is also affected by the glucose to mannose ratio. Some mannanases are able to hydrolyze not only the 1,4 bond between two mannose units but also the bond between the adjacent glucose and mannose units (23,24). Several endoglucanases are also able to hydrolyze galactoglucomannans.

**Other Hemicellulases**

Enzymes needed for further hydrolysis of the short oligomeric compounds produced by endoenzymes from hemicelluloses are β-xylosidase (1,4-β-D-xylosidase xylodrolase, EC 3.2.1.37), β-mannosidase (1,4-β-D-mannosidase mannohydrolase, EC 3.1.1.25), and β-glucosidase (Fig. 1). β-Xylosidases and β-mannosidase catalyze the hydrolysis of xylo- and manno-oligosaccharides, respectively, by removing successive xylose or mannose residues from the nonreducing termini. Exoglycanases are generally larger proteins than endoglycanases, with molecular weights above 100 kDa, and they are often built up by two or more subunits.

Side groups that are still attached to oligosaccharides after the hydrolysis of xylans and mannans by xylanase or mannanase, respectively, restrict the action of β-xylosidase and β-mannosidase. The hydrolysis stops at a substituted sugar unit. The β-xylosidase of T. reesei is unable to hydrolyze the linkage adjacent to a substituted xylose unit carrying a 1,3-linked α-L-arabinofuranosyl substituent, whereas the linkage is easily hydrolyzed if the substituent is 1,2-linked α-L-arabinofuranosyl or 4-O-methyl-α-D-glucuronic acid (25). Similarly, the 1,6-linked α-D-galactopyranosyl side group restricts the action of β-mannosidase of A. niger, which is able to only slowly hydrolyze the linkage adjacent to the substituted mannose unit.

The side groups connected to xylan and glucomannan main chains are removed by α-glucuronidase, α-arabinosidase (α-L-arabinofuranoside arabinofuranohydrolase, EC 3.2.1.55), and α-D-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) (Fig. 1). Acetyl substituents bound to hemicellulose are removed by esterases. There are clearly different types of side group cleaving enzymes. Some of them are able to hydrolyze only substituted short-chain oligomers, which first must be produced by the backbone depolymerizing endoenzymes (xylanases and man-
Evaluation of Hemicellulases

Enzyme activity is usually determined using isolated natural substrates. Isolation of a substrate from complex raw materials, however, often leads to chemical and structural modification of the substrate. Thus, in hemicelluloses, some of the side groups may be removed during chemical extraction, or the average degree of polymerization may change. Also, the physical status of the substrate in the activity assay may be different from that in the practical application. Thus, the solubility of the substrate in the activity assay is usually high, whereas in fiber-bound matrix the substrate is usually insoluble and/or may be also chemically linked to other components. Although the substrates in activity assays are not identical to the practical raw materials, they are chosen to correspond to the conditions under which the enzyme will be used. The main advantage and justification for the use of isolated substrates is that they give reproducible assay figures. Furthermore, they are more readily available and consistent and give the opportunity to compare different types of enzymes at various places.

It is well recognized that comparison of enzyme activities is complicated by the utilization of different analysis methods (27). Hence, in practical applications, the action of enzymes must be compared on the actual substrate. In these tests, the liberation of sugars and/or oligosaccharides into solution is the direct measurement of the action of the enzyme. This is usually carried out either by reducing sugar analysis or by HPLC. Several indirect methods to correlate the activity with the actual effect in the practical use, such as viscosity determination, have been developed. However, the most reliable method for understanding the effects of different enzymes is to compare the enzymes and dosages in the final application.

APPLICATION OF HEMICELLULASES

The major applications of hemicellulases in the food, feed, and pulp and paper industries are described in Table 1.

Food

Enzymes have long been recognized as useful tools for improving the processing behavior or properties of cereal foods, and their use is continuously increasing. The main enzymes used are amylases acting on starch, but hemicellulases (26).

nanases). Others are capable of also attacking intact polymeric substrates. Most accessory enzymes of the latter type, however, prefer oligomeric substrates. The synergism between different hemicellulolytic enzymes is also observed by the accelerated action of endoglycanases in the presence of accessory enzymes. Some accessory enzymes, such as an arabinosidase and an esterase of Pseudomonas fluorescens subs. cellulosa and an acetyl xylan esterase of Trichoderma reesei have been found to contain a multidomain structure with a separate cellulose binding domain (26).

Table 1. Benefits of Using Hemicellulases in Different Industrial Areas

<table>
<thead>
<tr>
<th>Application area</th>
<th>Effect</th>
<th>Benefit</th>
</tr>
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<tr>
<td>Food</td>
<td>Dough rheology, Loaf volume, Staling rate</td>
<td>Improved handling, Quality and yield</td>
</tr>
<tr>
<td>Feed</td>
<td>Nutritive value, Feed conversion rate</td>
<td>Improved storage, Cost savings</td>
</tr>
<tr>
<td>Pulp and paper</td>
<td>Bleachability</td>
<td>Chemical savings, quality</td>
</tr>
</tbody>
</table>
Hemicellulases have also been used in cereal processing. The main area in which hemicellulases are used is baking, where dough handling and bread quality can be improved by the action of enzymes.

**Hemicellulases in Cereal Processing.** Endogenous and added enzymes both have an important effect on the quality of cereal foods, especially in baking. Although enzymes act on molecular level, they can induce remarkable changes in both the microstructure and the functional properties of cereal foods. Progress has been made recently in the analysis of enzymes present in flours. The presence of endogenous enzymes has been suggested to be the cause of the variation in liquid loss among wheat doughs made from different cultivars (28). Differences in α-arabinofuranosidase, β-xylosidase, and endoxylanase levels have been detected in various European wheat varieties. Although present only at low levels, these enzymes may contribute to the bread-making performance of flours (29).

Enzymes have been shown to differ in their abilities to both solubilize insoluble substrates and further hydrolyze the released fragments. The chemical composition, architecture, and physical properties of the surrounding tissue of the cereal cell walls are all of importance if selective solubilization of the polysaccharides is attempted. Physical inaccessibility of the substrate may retard or hinder the reactions, and several enzymes may be needed. As compared with xylanases, less is known about the other enzymes acting on the hemicelluloses. The role of ferulic acid in cereals is interesting. Ferulic acid is esterified to arabinoxylans from barley cell walls (30).

**Baking.** Flour, water, yeast, and salt are the basic components of bread and other baked foods. It is, however, a common practice to improve the processing of dough and/ or the quality of the baked products by adding other ingredients. Process criteria are especially related to dough handling (machinability) and process yield (water retention, proofing times). The product quality is mostly evaluated on the basis of appearance (volume, color) and eating quality (crumb elasticity, flavor) (31). Various bread improving components, such as emulsifiers, oxidizing agents, and enzymes, have been shown to influence bread quality, especially when lower-quality flours with a low gluten content are used.

A small amount of arabinoxylans (pentosans) is present in wheat and rye flour. Insoluble arabinoxylan especially hinder the development of wheat gluten, which is vital for the formation of the loaf structure. White flour contains 2.5–3%, whole-meal wheat flour about 5%, and rye as much as 8% arabinoxylan, which has an important role in the water binding of dough. Arabinoxylans can bind nearly 10 times their weight of water and may account for approximately one-third of the water-binding capacity of dough. By controlled enzymatic hydrolysis of xylans, the dough becomes easier to handle and the resulting bread has a larger loaf volume and an improved crumb structure (32).

To understand the reactions of hemicellulases, several reports have concentrated on studying the mechanisms of action of xylanases and other hemicellulases. The release of high molecular weight water-soluble arabinoxylans from water-inextractable cell wall material has been shown. The specific viscosities of extracts from xylanase-treated doughs were 40–65% higher than those from untreated dough, because the average apparent viscosity of the extracted arabinoxylans remained high (33).

It has been suggested that the effect of xylanase on bread volume improvement can be ascribed to redistribution of water from the xylan phase to the gluten (continuous phase). Water distribution is of crucial importance in the dough and bread. The transfer of water from one component to another, due to the decreased water-binding capacity of a partially hydrolyzed polymer, is suggested to be a major reason behind changed functional properties (34). The increase in gluten volume fraction gives the gluten more extensibility, which eventually results in better oven spring (31).

Xylanases have also been found to improve wheat dough fermentation stability (35), counterbalance the variable flour quality in wheat bread baking (33), retard starch, and improve the texture of high-fiber bread (36). They are also useful in the manufacture of low-fat biscuits (37).

**Animal Nutrition and Feed.** A part of the energy content in many cereals is locked up in the form of nonstarch polysaccharides, undigestible for several animals. Therefore, selected enzymes, such as hemicellulases, can be added to break down these polymers, leading to increased metabolizable energy. In addition, degradation of hemicelluloses often liberates starch and protein, masked by the cell walls in the cereals, increasing their utilization. In some cereals, a large part of arabinoxylans is soluble and causes high viscosity in the small intestine of some animal species. Addition of xylanases decreases viscosity and improves feed utilization.

**Hemicellulases in Animal Nutrition.** Animal feed is predominantly composed of plant material, and cereals form the major part of diets for both poultry and pigs. Some of these raw materials, such as rye and barley, are known to be less suitable than maize and wheat. This is emphasized by their gross energy and metabolizable energy in the target species. Thus, barley, with a gross energy similar to that of wheat, has, in practice, an approximately 10% lower energy availability than wheat (38). This has previously limited its value as a feed component as compared with the more digestible materials. The decreased nutritive value has been associated with the presence of arabinoxylans in rye and wheat. Feed utilization and digestion can often be increased by the addition of external enzymes to the feed.

Endogenous enzymes play a crucial part in an animal’s own digestion of feed components. External enzymes added directly to feeds act as supplements to the normal digestive enzymes already found in animals. The external enzymes carry out their work on the feed materials present in the animal’s digestive tract. They can therefore be con-
considered as an extension of the animal’s own digestive enzyme system, especially in the case of young animals whose digestive systems are not fully mature. Enzymes can be added to animal feeds either to degrade soluble fiber, which has an antinutritional effect, or to supplement the animal’s own digestive enzymes. In the latter case, supplementation of the feed with amylase or protease is the current practice. \(\beta\)-Glucanases are also used, especially for the hydrolysis of barley \(\beta\)-glucans.

The content of arabinoxylan, \(\beta\)-glucan, and other nonstarch polysaccharides in feed raw materials does not remain constant, and varies with variety and also with the climatic conditions under which the raw material is grown. Prediction of the enzyme response has proved to be complicated. Different methods, such as viscosity measurements or determination of arabinoxylan level, have been studied as possible predictors of the nutritive quality with varying success (39). At present, however, no rapid predictive test exists that can be applied to raw material evaluation, and in vivo methods are still the best.

The desired pH optimum of the enzyme preparations are around 6–6.5, prevailing in the small intestine, the area in which the majority of the enzyme action of xylanases is believed to occur. The stability of the enzyme both in processing and in feed is important. Enzymes must be resistant to the heat processing of the feeds and also to the transition in the digestive system to the point of action. In practical feed-mill conditions, the addition of steam in pelleting is responsible for the loss of enzyme activity. Commercial preparations may be protected by being absorbed to a carrier or coated with a steam-resistant material that hinders access of the steam to the enzyme product. The intrinsic thermal resistance of an enzyme may thus be improved by a protective system. As the majority of xylanases used in feed applications have very similar intrinsic thermal stabilities, the thermal stability of different products is mainly a function of the efficiency of the carrier and/or coating technology used to protect the enzyme product. The loss of activity can be to some extent avoided by using dried enzymes, which are applied after pelleting (38).

**Poultry Feed.** Wheat has been found to have a variable energy content, depending on local growth conditions. While there has been found to be no correlation between the level of total nonstarch polysaccharides in wheat, a significant negative correlation between the level of water-soluble nonstarch polysaccharides and the average metabolizable energy (AME) has been demonstrated. It has been observed that supplementation of a wheat-based broiler diet with xylanase preparations improved the AME value of the wheat used by 7–10% (40). An increased release of proteins and nonstarch carbohydrates from the cells has been verified by chemical and microscopic analysis (41). The addition of xylanase enzymes can therefore be used to improve the nutritive value of wheat-based diets. This is now common practice in Western Europe and other areas of the world where wheat is available as a feed cereal.

Enzyme supplementation of wheat-containing diets is, however, cost sensitive, and the dosages used may not always be those that give the maximum effect. In practice, addition levels vary from 300 to 500 g of enzyme preparation per ton of feed for broilers (42). Too-high enzyme dosages have, in some cases, led to an imbalance between protein and energy.

Due to the inherent variations in biological systems, small fluctuations in results from place to place have been observed (38). This should be contrasted with the more traditional enzyme applications, where the response is much more predictable and can even be mathematically modeled. It has been observed that wheat with a lower AME than average responds more to the enzyme supplementation, thus introducing another source of variation in the response. Despite this, however, xylanases are now routinely used in wheat-containing diets for broilers, and considerable efforts are being made to understand their mode of action and predict their effects. Similar results have also been obtained with rye-based diets (43). The effects obtained with commercial enzymes may sometimes be due to the action of several enzymes, as the commercial preparations often contain several polysaccharide-degrading activities.

As compared with broilers, laying hens and turkeys are more mature animals with a more developed digestive tract, better able to cope with the nonstarch polysaccharides in cereals and vegetable sources. It has been observed that the advantage of the extra energy liberated by the enzyme supplementation of the feed can be gained by changing the feeding strategy. As the enzyme effect has been found to decrease during the growth of the birds, aging, a feeding strategy where enzyme dose is reduced as the feeding program continues, may provide a more cost-effective route for improvement of feed efficiency and performance.

**Pig Feed.** Pig feeds containing very high levels of wheat are also less common, as the formulation of pig feeds tends to be multicereal based. According to some experiments, however, increasing levels of xylanase supplementation, up to 600 g of the commercial enzyme per ton of feed (containing 20–35% barley and 30–35% wheat), resulted in improvement of performance, as measured by feed conversion ratio and live weight gain (38).

Pig feeds normally contain more by-products than poultry feeds because of the overall higher digestive efficiency of the pig compared with poultry. These by-products can also be upgraded by enzyme supplementation and, in some instances, offer higher potentials than the materials from which they are derived. The presence of noncereal feed raw materials in the complex diet (e.g., rapeseed meal, sugar beet pulp, sunflower meal) has been found to diminish the enzyme effect, as the substrates present were not degradable by the xylanase used. This implies that attention must be paid to the raw material composition of pig diets if the maximum enzyme effect is to be achieved (38).

**Pulp and Paper**

In the pulp and paper industry, hemicellulases are mainly used to improve the bleachability of pulps. In this application, xylanases are the most important group of enzymes, although mannanases may also facilitate the bleaching of certain types of pulps (44). In addition, xylan-
ases have been present in enzyme preparations used to improve the drainage (water removal) or deinking of recycled fibers. The mechanisms in these applications are, however, not clear, and they are obviously not based solely on the action of hemicellulases.

**Pulping and Bleaching Processes.** The main aim in chemical pulping is to remove lignin and to separate the wood fibers from each other to render them suitable for the paper-making process. In the pulping process, the lignified middle lamella located between the wood fibers is solubilized by various chemicals. Today, the predominant pulping method is the kraft process, where the cooking liquors are incinerated and the cooking chemicals recycled.

In bleaching, the primary goal is to remove the low amount of residual lignin present in the pulp after cooking without decreasing the molecular weight of cellulose. Lignin in unbleached pulps typically represents only about 1% of the dry weight. During pulping, however, lignin is chemically modified and condensed, resulting in poorly degradable structures. Cooking and bleaching are separate process phases, differing from each other with respect to the selectivity of the chemicals used. In the bleaching processes, lignin is sequentially degraded and extracted in several phases. Bleaching sequences are generally composed of at least five phases. Previously, the bleaching of chemical pulps was carried out with elemental chlorine and chlorine dioxide. In Europe today pulp is bleached by elemental chlorine free (ECF) or totally chlorine free (TCF) bleaching processes, where oxygen, ozone, or peroxide is used. Extensive modifications of hemicelluloses take place during pulping processes. During conventional kraft cooking, part of the hemicelluloses is first solubilized in the cooking liquor. At the start of the kraft process xylan in wood is partly solubilized by the alkaline cooking liquid and many of the side groups are cleaved off. It has recently been observed that the majority of the 4-O-methylglucuronic acid side groups are converted in the early phases of the kraft cook to hexenuronic acid (45). In the later phases of the process, when the alkalinity of the cooking liquor decreases, part of the solubilized xylan is relocated onto the cellulose fibers (46). Although glucomannan is the main hemicellulose in softwood, the bulk of glucomannans are dissolved and degraded during kraft pulping. Reprecipitation of glucomannan has not been reported to take place to the same extent as xylans. Thus, the relative amount of xylan is increased in pine kraft pulp as compared to pine wood (2).

In addition to xylan, lignin is also partially reabsorbed on the fibers. Lignin has been reported to be linked to hemicelluloses, forming lignin–carbohydrate complexes (LCC). Furthermore, hemicelluloses seem to physically restrict the passage of high molecular mass lignin out of the pulp fiber cell wall, and thus the removal of hemicelluloses, especially xylan, can be expected to enhance the extractability of residual lignin from pulps.

**Modification of Fibers by Hemicellulases.** The effect of hemicellulases in bleaching is based on the modification of pulp hemicelluloses, enhancing the removal of lignin in chemical bleaching. It has been proposed that the action of xylanases is due to the partial hydrolysis of reprecipitated xylan or to removal of xylan from the lignin-carbohydrate (LC) complexes. Both mechanisms would lead to enhanced diffusion of entrapped lignin from the fiber wall. In softwood kraft fibers, removal of xylan by xylanases was found to uncover lignin (47). Thus, it can be expected that removal of xylan improves the extractability of lignin by exposing lignin surfaces.

The partial hydrolysis of xylan or glucomannan may also degrade and improve the extractability of LCC. Both softwood and hardwood kraft pulps have been reported to contain LCC in which carbohydrates and lignin may be connected to each other by ether or glycosidic linkages (48). However, no direct evidence for the type of linkage(s) existing between hemicelluloses and lignin has yet been presented. The action of xylanases on both reprecipitated and LC xylan in enhancing bleachability suggests that it is probably not only the type but also the location of the xylan that is important in the mechanism of xylanase-aided bleaching.

**Hemicellulases in Bleaching Processes.** The use of xylanases in different bleaching sequences uniformly leads to a reduction in chemical consumption (8,44). The benefits obtained by enzymes are dependent on the type of bleaching sequence used, as well as on the residual lignin content (measured as the kappa number) of the pulp. Originally, xylanases were studied to reduce the consumption of elemental chlorine. Later, xylanases were combined with various ECF and TCF bleaching sequences to improve the otherwise lower brightness of pulp or to decrease the bleaching costs. In chlorine bleaching, an average reduction of 25% in active chlorine consumption in prebleaching, or a reduction of about 15% in total chlorine consumption has been reported both in laboratory scale and in mill trials. As a result, the concentration of chlorinated compounds, measured as adsorbable organic halogen (AOX), in the bleaching effluent during mill trials was reduced by 15–20% (8). Today, xylanases are industrially used both in ECF and TCF sequences. In ECF sequences the enzymatic step is often implemented due to the limiting chlorine dioxide production capacity. The use of enzymes allows bleaching to higher brightness values when chlorine gas is not used. In TCF sequences, the advantage of the enzymatic step is due to improved brightness, maintenance of fiber strength, and savings in bleaching costs.

The amount of enzyme needed for the bleaching is also a key parameter with respect to both enzyme cost and yield loss. Generally, it seems that although the hydrolysis (the solubilization of carbohydrates) increases as a function of the enzyme dosage used, no further benefits can be obtained to the bleachability after a certain limit (49). Thus, to maximize the positive effect of the enzyme on the pulp kappa number and brightness, and simultaneously to minimize the yield loss, laboratory-scale experiments are needed to optimize the enzyme dosage.

Enzymatic pretreatment has been shown to be fully compatible with existing industrial equipment, which is a considerable advantage of this method, especially when
compared with some other competing technologies. Xylanases are typically mixed with water before being added to the unbleached pulp by a shower bar and allowed to react in the high-density storage tank for 1 or 2 h before the subsequent chemical bleaching steps. Presently a number of mills in North America and Scandinavia use enzymes continuously.

The approximate price of xylanase in 1997 was less than $2/ton pulp. Estimations for the capital cost of enzyme delivery and pH adjustment varied from $10,000 to $1,000,000 in 1995. The potential economic benefits of enzyme bleaching are significant to the pulp and paper industry. Calculation of the relative economic benefits in an ECF sequence reveals that the reduction of the chlorine dioxide consumption leads to savings of at least $2/ton pulp (Table 2). The costs of oxygen-based chemicals (ozone, peroxide) are even higher and the respective savings even more pronounced.

FUTURE OUTLOOK

Today, hemicellulases are used in three major industrial sectors: in food as baking aids, in feed for increasing the nutritional value, and in the pulp and paper industry for saving chemical costs. Most applications are based on the action of xylanases, and several commercial xylanase preparations with different properties are available. The future developments of hemicellulases aim at more efficient and stable enzymes. Obviously, enzymes with different pH and higher-temperature optima and stability are desired for most applications. Thermal stability has been identified as one of the key properties in all industrial sectors. The use of monocomponent enzyme products, alone or in optimized combinations for targeted use, will also become more common. Tailored side group cleaving enzymes will be available, in addition to the present major endoglycanases. Already most of the industrial enzymes are produced by strains developed by genetic engineering. These techniques are expected to lead to more cost-efficient enzyme products.

BIBLIOGRAPHY


Table 2. Cost Savings with Xylanases in Bleaching

<table>
<thead>
<tr>
<th>Chemical savings</th>
<th>Chlorine dioxide</th>
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<td>Add</td>
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<tr>
<td>Net savings</td>
<td></td>
<td>$2.3–2.5 /ton pulp</td>
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Source: From Roehm Enzymes, Finland.
HEMICELLULOSE CONVERSION

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INTRODUCTION

Hemicellulose is a plant cell wall polysaccharide that is localized mostly in cell wall middle lamella. It is in close association with cellulose, lignin, and pectic materials. This close association contributes to the rigidity and flexibility of the plant cell wall. Hemicellulose is the third-most abundant polymer in nature. In some plants, it comprises up to 40% of the total dry material. Unlike cellulose, hemicellulose exhibits a wide diversity in both structure and constitution. The degree of polymerization of hemicellulose is usually less than 200. The type and amount of hemicellulose vary widely, depending upon plant material, type of tissue, stage of growth, and so on. For those reasons, it is difficult to obtain a typical sugar composition of a typical hemicellulose.

Hemicelluloses are heteropolysaccharides that are composed of various hexoses (e.g., glucose, mannose, and galactose), pentoses (D-xylose and L-arabinose), uronic acids, acetic acid, and other minor sugars. Thus, by definition, hemicelluloses are short branched-chain heteropolysaccharides of mixed hexosans and pentosans that are easily hydrolyzed (1). The most common form of hemicellulose is composed of xylose polymer (xylan) that is found in large quantity in annual plants and deciduous trees and in smaller amounts in conifers. Xylan of grasses and cereals is generally characterized by the presence of L-arabinose, which is linked as a single unit side chain to a D-xylose backbone. Hydrolysis of hemicellulose in annual plants, agricultural wastes, and hardwood yields glucose, D-xylose, L-arabinose, and other minor sugars. Table 1 summarizes the amounts of hemicellulose in different plant materials; Table 2 summarizes the sugar composition of selected biomass materials after the hydrolysis.

HEMICELLULOSE HYDROLYSIS

Hemicellulose can be hydrolyzed to its sugar constituents either enzymatically or chemically. Chemical hydrolysis of hemicellulose is much easier to accomplish than the hydrolysis of cellulose due to the heterogeneous nature of hemicellulose, its chemical composition, and its low degree of polymerization. Most acids are good hydrolytic agents. The most common method of acid hydrolysis uses dilute mineral acids. Frequently during acid hydrolysis, xylose is

OUTLINE

Introduction

Hemicellulose Hydrolysis

Bioconversion of Hemicellulose Sugars

Ethanol

Conversion of Xylose to Ethanol by Yeasts

Conversion of Xylose to Ethanol by Bacteria

Conversion of Xylose to Ethanol by Mycelial Fungi

Xylitol

2,3-Butanediol

Bibliography

See also Enzymes, Pulp and Paper Processing; Hemicellulose conversion.

HEMICELLULOSE HYDROLYSIS

Hemicellulose can be hydrolyzed to its sugar constituents either enzymatically or chemically. Chemical hydrolysis of hemicellulose is much easier to accomplish than the hydrolysis of cellulose due to the heterogeneous nature of hemicellulose, its chemical composition, and its low degree of polymerization. Most acids are good hydrolytic agents. The most common method of acid hydrolysis uses dilute mineral acids. Frequently during acid hydrolysis, xylose is

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**Table 1. Biomass Constituents**

<table>
<thead>
<tr>
<th>Type of material</th>
<th>Hemicellulose (%)</th>
<th>Cellulose (%)</th>
<th>Lignins (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monocotyledons</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stems</td>
<td>25 – 50</td>
<td>25 – 40</td>
<td>10 – 30</td>
</tr>
<tr>
<td>Leaves</td>
<td>80 – 85</td>
<td>15 – 20</td>
<td>–</td>
</tr>
<tr>
<td>Fibers</td>
<td>5 – 20</td>
<td>80 – 95</td>
<td>–</td>
</tr>
<tr>
<td><strong>Woods</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hardwood (angiosperms)</td>
<td>24 – 40</td>
<td>40 – 55</td>
<td>18 – 25</td>
</tr>
<tr>
<td><strong>Papers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newspaper</td>
<td>25 – 40</td>
<td>40 – 55</td>
<td>18 – 30</td>
</tr>
<tr>
<td>Wastepaper</td>
<td>10 – 20</td>
<td>60 – 70</td>
<td>5 – 10</td>
</tr>
<tr>
<td>Waste fibers</td>
<td>20 – 30</td>
<td>60 – 80</td>
<td>2 – 10</td>
</tr>
</tbody>
</table>

*Source: From Ref. 2.*

**Table 2. Hemicellulose Neutral Carbohydrate Content of Agriculture Residues**

<table>
<thead>
<tr>
<th>Plant Residues</th>
<th>Xylose</th>
<th>Arabinose</th>
<th>Glucose</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cobs</td>
<td>65.1</td>
<td>9.6</td>
<td>25.3</td>
<td>–</td>
</tr>
<tr>
<td>Leaves</td>
<td>59</td>
<td>9.4</td>
<td>29.1</td>
<td>2.5</td>
</tr>
<tr>
<td>Stalks</td>
<td>70.5</td>
<td>9.0</td>
<td>14.5</td>
<td>5.9</td>
</tr>
<tr>
<td>Husks</td>
<td>53.5</td>
<td>12.3</td>
<td>32.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Pith</td>
<td>71.5</td>
<td>9.8</td>
<td>15.7</td>
<td>3</td>
</tr>
<tr>
<td>Fibers</td>
<td>63.8</td>
<td>6.6</td>
<td>26.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>57.9</td>
<td>9.1</td>
<td>28.1</td>
<td>5</td>
</tr>
<tr>
<td>Soybean</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stalks and leaves</td>
<td>59.9</td>
<td>6.6</td>
<td>6.1</td>
<td>27.4</td>
</tr>
<tr>
<td>Hulls</td>
<td>26.6</td>
<td>12.7</td>
<td>21</td>
<td>39.7</td>
</tr>
<tr>
<td>Sunflower</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stalks</td>
<td>60.6</td>
<td>2.2</td>
<td>32.6</td>
<td>4.6</td>
</tr>
<tr>
<td>Pith</td>
<td>10.7</td>
<td>11.8</td>
<td>63.5</td>
<td>14</td>
</tr>
<tr>
<td>Flax Straw</td>
<td>64.6</td>
<td>12.8</td>
<td>1.2</td>
<td>21.4</td>
</tr>
<tr>
<td>Sweet clover hays</td>
<td>49.3</td>
<td>21.9</td>
<td>8.9</td>
<td>9.9</td>
</tr>
<tr>
<td>Peanut hulls</td>
<td>46.3</td>
<td>5</td>
<td>46.6</td>
<td>2.1</td>
</tr>
<tr>
<td>Sugarcane bagasse</td>
<td>59.5</td>
<td>14.5</td>
<td>26</td>
<td>–</td>
</tr>
</tbody>
</table>

*Source: From Ref. 3.*

degraded rapidly to furfural and condensation byproducts. Some degradation products are inhibitory to microorganisms. The toxic effect of those chemicals on cell growth and ethanol formation of *Pichia stipitis* and *Saccharomyces cerevisiae* are shown in Tables 3 and 4 (4). The use of a very dilute acid (less than 0.2% acid by weight), shorter reaction time, a lower reaction temperature, and rapid removal of hydrolytic agents are preferred to prevent the decomposition of sugars, particularly xylose. In recent years, a great deal of research has focused on the dilute acid hydrolysis of agricultural residues and wood products to obtain hemicellulose sugars (5,6).

**BIOCONVERSION OF HEMICELLULOSE SUGARS**

When cellulosic materials are subjected to hydrolysis, a mixture of monosaccharides is produced. The predominant sugars released are glucose, xylose, and to a lesser extent, arabinose, galactose, and mannose. When microorganisms are exposed to this sugar mixture, the phenomena of diauxic growth and differential rates of sugar utilization are often observed. The differential utilization of sugars by a given microorganism is dictated by sugar uptake, utilization rates, and by the degree of catabolite repression, which affects specific enzyme activity and biosynthesis. As a rule, microorganisms prefer glucose over galactose, followed by xylose and arabinose.

Because hemicellulose is abundant in nature and renewable, extensive research has been undertaken to convert hemicellulose-derived carbohydrates, particularly xylose, into useful products. In this article, the emphasis is on the utilization of hemicellulose-derived xylose into ethanol, xylitol, and 2,3-butanediol. Other products such as solvents (acetone and butanol) (7) and single-cell proteins (8) have been reviewed.
**Table 3. The Effect of Potential Inhibitors on *Pichia stipitis* Growth and Ethanol Production from Xylose**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (g/L)</th>
<th>% Inhibition (Growth)</th>
<th>% Inhibition (EtOH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furfural</td>
<td>2</td>
<td>99</td>
<td>95</td>
</tr>
<tr>
<td>HMF</td>
<td>5</td>
<td>98.6</td>
<td>91.4</td>
</tr>
<tr>
<td>Acetate</td>
<td>10</td>
<td>36</td>
<td>69</td>
</tr>
<tr>
<td>HBA</td>
<td>0.75</td>
<td>70</td>
<td>84</td>
</tr>
<tr>
<td>SGA</td>
<td>0.75</td>
<td>62</td>
<td>80</td>
</tr>
<tr>
<td>Vanillin</td>
<td>0.5</td>
<td>88</td>
<td>89</td>
</tr>
</tbody>
</table>

Source: From Ref. 9.

*a* HMF, hydroxymethylfurfural; HBA, hydroxybenzaldehyde; SGA, siringaldehyde.

**Table 4. The Effects of Potential Inhibitors on *S. cerevisiae* Growth and Ethanol Production from Glucose**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (g/L)</th>
<th>% Inhibition (Growth)</th>
<th>% Inhibition (EtOH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furfural</td>
<td>2</td>
<td>90</td>
<td>89</td>
</tr>
<tr>
<td>HMF</td>
<td>5</td>
<td>89</td>
<td>95</td>
</tr>
<tr>
<td>Acetate</td>
<td>10</td>
<td>48</td>
<td>27</td>
</tr>
<tr>
<td>HBA</td>
<td>0.75</td>
<td>53</td>
<td>37</td>
</tr>
<tr>
<td>SGA</td>
<td>0.75</td>
<td>61</td>
<td>54</td>
</tr>
<tr>
<td>Vanillin</td>
<td>0.5</td>
<td>51</td>
<td>30</td>
</tr>
</tbody>
</table>

Source: From Ref. 9.

*a* HMF, hydroxymethylfurfural; HBA, hydroxybenzaldehyde; SGA, siringaldehyde.

**ETHANOL**

Ethanol is the most studied metabolic product from xylose by fermentation. Ethanol can be used as a “neat” fuel substitute for gasoline. It can also be used as an oxygenated fuel extender by converting ethanol to ethyl tertiary-butyl ether (ETBE). Using ethanol as fuel and fuel extender provides energy security and diversity, improves global competitiveness, reduces trade deficits, revitalizes the agricultural industry, and promotes energy independence. It also improves the environment by improving urban air quality and by reducing the threat of global warming. Currently the production of ethanol from biomass is not competitive. Many research efforts were made to achieve the economical production of ethanol from biomass (for review see Ref. 9). In order to increase ethanol production efficiency from biomass, full and efficient utilization of hemicellulose carbohydrates is essential.

**Conversion of Xylose to Ethanol by Yeasts**

Yeasts are facultative organisms that have the ability to produce energy for their own use from suitable organic compounds under either aerobic or anaerobic conditions. Under aerobic conditions, sugars are metabolized to carbon dioxide and water for the production of energy and cell constituents. Under anaerobic conditions, most sugars are metabolized to ethanol by a process known as alcohol fermentation. Although many facultatively fermentative yeasts utilize xylose as carbon source for growth, the ability of those yeasts to produce ethanol from xylose is limited (10).

Yeast strains that utilize xylose often produce xylitol from xylose extracellularly as a normal metabolic activity, but only a few can produce significant quantities of ethanol. The prominent strains that produce ethanol from xylose include *Kluveromyces maxianus* (11), *Pachysolen tannophilus*, *Candida shihatae*, and *Pichia stipitis*. However, the efficient production of ethanol from xylose is limited by the regulation of dissolved oxygen as well as by the imbalance of cofactors in the metabolic pathway during xylose utilization. In recent years, there have been many efforts carried out to improve yeast strains to produce ethanol from xylose more efficiently.

**Metabolic Pathway.** Ethanol fermentation by yeasts from xylose can be divided into four distinctive steps. The first step is the reduction of xylose to xylitol, mediated by NADPH/NADH-linked xylose reductase (XR). This is followed by the oxidation of xylitol to xylose, mediated by NAD-linked xylitol dehydrogenase (XDH). Xylulose-5-phosphate, the key intermediate, is generated from the phosphorylation of xylulose by xylulose kinase. Xylulose is then channeled into the pentose phosphate pathway for further metabolism (Fig. 1). In contrast, a great majority of bacteria convert xylose into its ketoisomer, xylulose, mediated by xylose isomerase (commonly known as glucose isomerase) as the initial step in xylose metabolism.

**Ethanol Yield and Carbon Balance.** Starting with 6 molecules of xylose, 10 molecules of ethanol can be produced using the combination of two pathways (Fig. 1). The net equation for the reactions leading to the production of ethanol from xylose is

\[
6 \text{ xylose to } 10 \text{ ethanol } + 10 \text{ CO}_2
\]

These values are the theoretical maximum yields (same as for glucose fermentation) that represent the maximum value, which can be achieved only if no sugar is assimilated to cell mass or oxidized through the TCA cycle. Of the total carbon contained in xylose, two-thirds goes to ethanol, and
the other one-third is lost as CO₂. On a weight basis, the yield of ethanol from both xylose and glucose is 51%.

Yeast That Ferment Xylose to Ethanol. Following are the general characteristics of yeasts that ferment xylose to ethanol.

1. Oxygen requirement (12).
   a. Oxygen is required for the efficient uptake of xylose.
   b. Under low dissolved oxygen (DO) conditions, the electron transport system is not able to oxidize NADH efficiently, this causes the imbalance of NADH/NAD⁺ that leads to the accumulation of xylitol.
   c. An increase in DO will enhance cell growth and xylose fermentation.
   d. When oxygen is in excess, TCA cycle activity is enhanced, which results in excessive cell growth and causes the reassimilation of ethanol produced.
   e. The reassimilation of ethanol leads to the accumulation of acetaldehyde and acetic acid.
   f. The optimal DO gives the lowest xylitol accumulation with the highest ethanol yield.

2. Enzyme cofactor imbalance
   a. Xylose reductase and xylitol dehydrogenase have different cofactor requirements.
b. Different nitrogen sources (organic vs. inorganic) affects the level of xylitol dehydrogenase activity (13).

c. Different DOs affect the balance of cofactors.

3. Low ethanol tolerance compared with that in \textit{S. cerevisiae}
\begin{itemize}
  \item Fermentation is inhibited at an ethanol concentration of 42 to 45 g/L.
  \item Xylitol, glycerol, arabitol, and acetate are the common byproducts.
  \item Higher sensitivity to potential fermentation inhibitors than glucose-fermenting yeast strains during glucose fermentation (Tables 3 and 4).
  \item Specific ethanol productivity from xylose is at least one order of magnitude lower than that in glucose fermentation by \textit{S. cerevisiae}.
\end{itemize}

\textbf{Natural Yeast Strains.} \textit{P. tannophilus}, \textit{C. shehatae}, and strains of \textit{P. stipitis} are three of the most intensively studied naturally occurring yeast species that produce a significant quantity of ethanol from xylose.

\textit{Pachysolen tannophilus}. \textit{P. tannophilus} is an unusual yeast that was originally isolated by Wickerham (14) from wood sulfite liquor. This yeast was the first naturally occurring yeast species that was found to produce a significant quantity of ethanol from xylose (15). More detailed studies of this yeast were reported by Slininger et al. (16). As described in the literature, ethanol production from xylose by \textit{P. tannophilus} exhibited the following characteristics:

\begin{enumerate}
  \item A small amount of oxygen was required for optimal ethanol production.
  \item A lag phase of about 20 h was observed before the accumulation of ethanol.
  \item Ethanol yield was reported to be 0.34 g ethanol per g xylose consumed (68\% of the theoretical value).
  \item Optimal temperature for cell growth and ethanol production was 32 °C.
  \item Optimal pH for cell growth was 2.5–4.5, and optimal pH for ethanol production was 2.5.
  \item Ethanol is reassimilated under high DO conditions.
  \item Ethanol production was subjected to substrate (55 g/L xylose) and product (30 g/L ethanol) inhibition.
  \item Xylitol was accumulated during fermentation.
\end{enumerate}

\textit{Candida shehatae}. The ability of \textit{C. shehatae} to produce ethanol from xylose was first reported by du Preez and van der Walt (17) in 1983. Since then, many studies have been conducted relating to the properties of this yeast. As described in the literature (18–20), ethanol production from xylose by \textit{C. shehatae} exhibited the following characteristics:

\begin{enumerate}
  \item A small amount of DO is required for maximal ethanol production.
  \item Cell growth was inhibited at an ethanol concentration of 37.5 g/L, and cell viability was significantly lower in the presence of up to 50 g/L ethanol.
  \item Glucose and mannose are the preferred substrate in a sugar mixture.
  \item Ethanol yield can be as high as 84\% of the theoretical value (0.43 g ethanol per g xylose consumed).
  \item The maximum ethanol production rate was 0.48 g/g/h at 50 g/L xylose.
  \item Xylitol was accumulated under very limited DO conditions due to the accumulation of NADH.
  \item Under high DO conditions, the ethanol produced was reassimilated.
\end{enumerate}

\textit{Pichia stipitis}. \textit{P. stipitis} is the most effective natural yeast for the conversion of xylose to ethanol. This yeast species shares many characteristics with its close relative \textit{C. shehatae}. Toivola et al. (21) performed a systemic screening program with type strains of some 200 yeast species and identified \textit{P. stipitis} as one of the yeast species that produces ethanol from xylose. There are many studies that explored the properties of this yeast in relation to its oxygen requirement, ethanol tolerance, enzyme cofactors balance, and so on. As described in the literature (22,23), ethanol production from xylose by \textit{P. stipitis} exhibited the following characteristics:

\begin{enumerate}
  \item Trace oxygen was required to sustain cell growth and maintenance.
  \item \textit{P. stipitis} has a higher ethanol (64 g/L) tolerance than other xylose-fermenting yeasts.
  \item Compared with \textit{C. shehatae}, \textit{P. stipitis} has a lower specific ethanol productivity but a higher ethanol yield.
  \item Up to 57 g/L of ethanol can be accumulated by \textit{P. stipitis}.
  \item Xylitol production by \textit{P. stipitis} was lower than other xylose-fermenting yeasts.
  \item The optimum temperature for ethanol production was 25 °C.
  \item The optimum pH range for growth and fermentation was 4–7.
  \item Ethanol was reassimilated at high DO levels, and acetate was formed when ethanol was metabolized.
\end{enumerate}

\textbf{Genetically Modified Yeasts.} Attempts to modify the xylose-fermenting pathway in \textit{S. cerevisiae} using the xylose isomerase gene from various bacterial sources have not been successful (24) in spite of previous reports showing the ability of glucose-fermenting yeast strains, \textit{S. cerevisiae} (25) and \textit{Schizosaccharomyces pombe} (26), to produce ethanol from xylose or from xylose in the presence of xylose isomerase (glucose isomerase) (27) (Fig. 2). Other approaches of improving yeast strains through genetic recombination have met with some encouraging results.

\textit{Saccharomycyes cerevisiae}. Kotter and Ciriacy (28) studied xylose utilization of a \textit{S. cerevisiae} transformant that expressed two key enzymes (xylose reductase and xylitol dehydrogenase) derived from \textit{P. stipitis} in xylose metabo-
lism. Under fermentative conditions, the transformant converts only half of available xylose to xylitol and ethanol. The acquired ability to ferment xylose was interpreted to be a result of the duel cofactor dependence of XR and the generation of NADPH by the pentose phosphate pathway. The limitation of xylose utilization in the transformant was likely caused by an insufficient capacity of the non-oxidative pentose pathway, as indicated by the accumulation of sedoheptulose-7-phosphate and the absence of fructose-1,6-diphosphate and pyruvate accumulation. Recombinant Yeast Saccharomyces 1400 (pLNH33). The recombinant yeast strain Saccharomyces 1400 (pLNH33) was developed using the high-ethanol-tolerance Saccharomyces yeast 1400 (29) as the host and cloned with the xylose reductase and xylitol dehydrogenase genes from P. stipitis, and the xylulokinase gene from S. cerevisiae. Saccharomyces 1400 (pLNH33) was shown to ferment both sugars in a 1:1 mixture of glucose (52.8 g/L) and xylose (56.3 g/L) (Fig. 3) to ethanol under microaerobic condition in a relatively high yield (84% of theoretical value) (30). A final ethanol concentration obtained was 50 g/L after 48 h of incubation. This recombinant was also shown to produce ethanol from glucose and xylose that were derived from corn fiber during simultaneous saccharification and fermentation process in the presence of a fungal cellulase (31). Conversion of Xylose to Ethanol by Bacteria A wide range of bacterial species utilizes D-xylose and L-arabinose as carbon and energy sources. In most case, the direct isomerization of aldopentoses to their corresponding ketoses is the first step in pentose metabolism. For example, D-xylose is converted into D-xylulose, and L-arabinose is converted into L-ribulose. L-Ribulose can be converted into D-xylulose by epimerase. D-xylulose is the key intermediate for further metabolism. For this reason, most bacterial species can readily utilize L-arabinose as well. Bacterial species, particularly those belonging to Klebsiella and Erwinia, and Escherichia coli, are well known for their ability to metabolize hexoses and pentoses to produce either neutral compounds (butanediol, acetoin, and ethanol) or mixed acids and ethanol. Research on the production of ethanol from pentoses by bacteria has revolved around the improvement of such bacteria through genetic recombination.
Klebsiella oxytoca M5A1. Pyruvate decarboxylase and alcohol dehydrogenase genes encoding the ethanol pathway from Zymomonas mobilis were transferred into K. oxytoca (32). The transformant, K. oxytoca M5A1, was able to divert pyruvate from the normal fermentative pathway to ethanol production. K. oxytoca M5A1 was able to produce ethanol from both glucose and xylose in excess of 40 g/L with an efficiency of 0.48 g of ethanol per g xylose and 0.5 g of ethanol per g glucose. The maximal volumetric productivity for both sugars was similar (ca. 2 g/L).

Klebsiella planticola. Multicopy plasmids containing the Z. mobilis pyruvate decarboxylase gene were inserted into K. planticola. The transformant was shown to produce 31.6 g/L ethanol from a mixture of sugars (79.6 g/L) that included xylose (33). The yield of acids (formate, acetate, lactate) and butanediol was reduced significantly.

Escherichia coli. Pyruvate decarboxylase of Z. mobilis was transferred into E. coli (34). The transformant was able to produce 39.2 g/L ethanol from 80 g/L xylose with an indicated yield of 96% of the theoretical value. The maximal volumetric productivity was 0.7 g ethanol per liter per hour in batch fermentation (35).

Zymomonas mobilis. Two operons encoding xylose assimilation enzymes (xylose isomerase and xylulokinase) and pentose phosphate pathway enzymes (transaldolase, and transketolase) were constructed and introduced into Z. mobilis (36). The transformant, CP4 (pZB5), was able to not only grow but also ferment xylose anaerobically into ethanol. This transformant also acquired the ability to ferment glucose and xylose simultaneously with a slower rate of xylose than glucose utilization. Anaerobic fermentation using equal amounts of glucose (25 g/L) and xylose (25 g/L) in mixture gave an ethanol yield of 25 g/L ethanol with 95% of the theoretical yield within 30 h.

Conversion of Xylose to Ethanol by Mycelial Fungi

Several filamentous fungi belonging to the genera Fusarium, Mucor, Rhizopus, and Monilia are known to ferment...
both glucose and xylose to ethanol (37). The early work on xylose metabolism in Fusarium lini indicated the potential for ethanol production from xylose by this fungus (38). Overall, the slow rate of ethanol production from xylose by fungi is undesirable because it would add to capital costs in industrial application. However, because additional ethanol can be obtained from xylose, the use of mold for this purpose is an option.

**XYLITOL**

Xylitol is a five-carbon sugar alcohol that occurs naturally in small quantities (less than 0.9 g/100g) in many fruits and vegetables that constitute part of human diet. It is the only so-called second-generation polyol sweetener that is allowed to have specific health claims in some world markets. Unfortunately, it is one of the most expensive polyol sweeteners. Availability and cost of production are the obstacles impeding the increased use of xylitol. On an industrial scale, xylitol is produced through chemical reduction of xylose derived from hemicellulosic hydrolyzate. Birch hydrolyzate is the most common substrate. The chemical process is expensive because of the high temperature and high pressure required for hydrogenation of xylose. Furthermore, extensive steps for separation and purification add to the cost.

Xylitol can be formed as a metabolic intermediary of d-xylose utilization by a wide range of microorganisms. It is converted from xylose either by NADPH-dependent aldehyde reductase, or from xyulose by NADH-dependent xylitol dehydrogenase (Fig. 1). Many natural yeast strains have the ability to produce a significant quantity of xylitol from xylose extracellularly as a normal metabolic activity. The prominent strains that produce xylitol include Candida sp. (39), Candida boidinii (40), Candida guilliermondii (41), Candida parapsilosis (42), Candida tropicalis (43), and Debaryomyces Hansenii (44). However, xylitol is an expensive substrate for xylitol production. Recent advances in obtaining xylose-rich hemicellulose hydrolyzates from lignocellulosic materials can provide ample substrate for the biological production of xylitol inexpensively. As a result, xylitol can be potentially produced from such materials as an option for effective utilization of lignocellulosic biomass.

Usually the weight yield of xylitol produced from xylose by naturally occurring yeast species is around 75 to 80%. Two of the reasons for the relatively low yield are the growth of cells and the need for energy maintenance. Ideally, xylitol overaccumulation would occur if the pathway from xylose to xylitol was cut off, but this is unrealistic because of the redox balance. The next option is to reduce the activity of xylitol dehydrogenase. Therefore, the mutation-selection technique, based on differential xylose-xylitol utilization, can be applied to obtain high-xylitol-producing organisms. Based on this principle, a mutant strain from a proficient xylose-utilizing Candida yeast was selected; it had xylitol weight yields as high as 90% from xylose (45).

The production of xylitol from hemicellulosic materials has been considered as an alternative approach in the utilization of the xylose fraction of biomass substrates and the production liquid of fuels and chemical feedstocks. Over the years, many studies have been conducted utilizing the hemicellulose portion of agricultural residues for xylitol production. The materials studied include sugarcane bagasse (39,46), Eucalyptus wood (47), and rice straw (48).

**2,3-BUTANEDIOL**

Butanediol (butylene glycol) is a colorless and odorless liquid with a high boiling point (180–184 °C) and a low freezing point (60 °C). The heating value of butanediol (27,198 J/g) is similar to that of ethanol (29,055 J/g) and methanol (22,081 J/g). It can be dehydrated to methylethyl ketone (MEK) and used as an octane booster for gasoline or as a high-grade aviation fuel. MEK can be dehydrated to 1,3-butadiene and dimerized to styrene. Therefore, butanediol has a diverse industrial usage particularly as a polymeric feedstock in addition to its use for manufacturing butadiene or antifreeze (49).

Among many isomers, 2,3-butanediol is the only one that can be produced by microorganisms. Bacterial species of Klebsiellae are known to metabolize hexoses and pentoses derived from hemicellulose to produce 2,3-butanediol, acetoin, and ethanol as metabolic products. Other groups of enterobacteria such as Erwinia also utilize hemicellulose-derived carbohydrates to produce mixed acids and 2,3-butanediol. Research on the production of butanediol and its immediate precursor, acetoin, from renewable biomass has revolved around the microorganisms Klebsiella oxytoca (K. pneumonia) and Enterobacter aerogenes (Aerobacter aerogenes).

Butanediol-producing bacteria utilize all the major sugars (hexoses and pentoses) to produce butanediol in high yield and high concentration (up to 10%, w/v) under optimum conditions (e.g., DO, pH, etc.) (49,50). Three enzymes, α-acetolactate synthetase (ALS), α-acetolactate decarboxylase (ALDC), and acetoin reductase (AR), are directly responsible for the accumulation of 1 mole of butanediol from 2 moles of pyruvate (51). In K. terrigena and E. aerogenes, these enzymes are encoded by the gene budB (ALS), budA (ALDC), and budC (AR), respectively. The three genes are organized in an operon and activated by transcriptional products of the budR gene (52). The expression of the operon is optimal under anaerobic conditions at low pH environments and in the presence of a low concentration of acetate. The metabolic pathway leading to butanediol formation is shown in Figure 4.

DO has a profound effect on butanediol production and end-products distribution. Oxygen supply is important because it determines the flow of carbon source via the respiratory pathway versus the butanediol-producing fermentative pathway. The role of oxygen supply on biomass and butanediol accumulations has been studied extensively. Butanediol yield may be optimized by minimizing the available oxygen. However, with a small oxygen transfer rate, the cell yield will be low, causing the total reaction rate to slow down. In the presence of excessive oxygen, metabolism leads to the oxidation of NADH to NAD+ . A high NAD+/NADH ratio leads to excessive biomass accu-
Figure 4. The metabolic pathway leading to the production of 2,3-butanediol and the role of acetic acid. ALS, \(\alpha\)-acetolactate synthetase; ALDC, \(\alpha\)-acetolactate decarboxylase; AR, acetoin reductase.

There is a general agreement among the investigators that the oxygen supply rate is perhaps the most critical criterion for the performance of butanediol fermentation (see Ref. 53 for detail).

In addition to DO, the pH environment also exerts great influence on the product distribution and yield. In enterobacteria, the type and ratio of fermentation products formed depend on two biochemical constraints: the need to maintain redox balance, and the requirement for maintaining the pH of the medium in a physiological range. Therefore, in a different pH environment, different relative amounts of end products are formed, resulting in an increased proportion of neutral compounds and decreased pH of the medium (53). Under a neutral or high-pH environment, bacteria tend to utilize a carbon source for cell growth and organic acid production. It has been shown that when the pH is lower than 6, over 50% of the pyruvate was channeled into butanediol production. Under this condition, enzymes of the butanediol pathway can constitute as much as 2.5% of the total protein in \(E.\) aerogenes (54).

Zeng et al. (55), investigated the effect of pH on growth and product formation from glucose by \(E.\) aerogenes in a continuous culture operation and found the pH range of 5.5–6.5 is optimal for butanediol and acetoin production. A similar pH optimum was also observed in \(K.\) oxytoca.

In general, the biomass concentration increases steadily with increased pH. At high pH, butanediol concentration decreases and there is a concomitant increase of acetic acid production. Acetic acid has a dual role in the regulation of butanediol formation. It serves as activator for butanediol accumulation at low concentration. At a concentration of 10 g/L or higher, it inhibits butanediol production (56). The strength of acetic acid inhibition depends on the concentration of its undissociated form, HAc, and the concentration of HAc is in turn determined by the pH.

The production of butanediol from lignocellulosic materials has been considered as an alternate approach in the conversion of biomass substrates to liquid fuels and chemical feedstocks. Over the years, there have been many studies utilizing agricultural residues for butanediol production. The materials studied include hardwood hemicellulose fraction (57,58), wheat and barley straws (59), corn stover (59), and corn cobs (60).

**BIBLIOGRAPHY**

HUMAN AND PRIMATE CELL LINES

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KEY WORDS
Adenovirus
Bioreactor
Carcinoma
Cytokine
Diploid
Glycosylation
Immortalization
Oncogene
Transformation
Vaccine

OUTLINE
Introduction
Human Cell Lines
Human Diploid Cells
Human Carcinomas
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INTRODUCTION

Before the advent of monoclonal antibodies and genetically manipulated cell lines, the major use of primate and human cells was as a substrate for viral vaccine manufacture (e.g., polio, rubella, and measles in human diploid cells MRC 5 or WI 38 [1]).

It was not until the mid 1970s that it was considered that continuous cell lines may also prove useful for vaccine production. At the same time, there was a great deal of interest in the use of interferon in clinical studies. The major source was from primary human leukocyte cultures, but the amount produced was very limited.

Exploitation of animal cell lines in the field of biotechnology as opposed to bacteria, filamentous fungi, and yeast has occurred relatively recently because of complex culture media, slow population doubling times, and low productivity levels of cells. The ability of animal cells to glycosylate proteins in an active form, unlike bacteria, has led to new developments in manufacturing, purification, and recombinant DNA technology in the engineering of cells for production of therapeutic proteins. Strict safety standards must be set for any biological manufacturing process involving eukaryotic protein production, research into the adaptation of suitable cell lines, the latest bioreactor development, immortalization methods, and cell products are discussed in this article.

HUMAN CELL LINES

Human Diploid Cells

In the 1950s there was an urgent need to produce a vaccine to combat polio, which led to the use of primary monkey kidney cells for growing the virus. This was successful until it was discovered after a large amount of vaccine had been released that monkeys were infected with wild simian viruses (e.g., SV40) [2].

The unreliability of primary cell culture led to the development of human diploid cells, such as MRC 5 and WI 38 [1], and human fetal lung fibroblasts, which were not infected by SV 40 and are now the preferred substrate.

Human diploid cells are particularly susceptible to viruses pathogenic to humans (e.g., polio, echo, arbo, rhino, measles, rubella, and coxsackie A9) and are very valuable for their isolation and development of vaccines [1–3].

These two cell lines are nontumorigenic, essential when considering using human diploid cells for live virus vaccines in humans [4]; have a finite life span (because they have no malignant characteristics) [5]; and begin to senesce after passage [5]. These cells must be cryopreserved to prevent cross-contamination, genetic drift, microbial contamination, and maintenance of low passage stocks [6–9].

MRC 5 and WI 38 are free of latent and oncogenic viruses and are genetically stable; therefore, they are the permitted cell lines used for producing products such as vaccines for human use [5], although MRC 5 is preferred because of its higher replication rate and better tolerance to environmental changes [1].

Human Carcinomas

Useful in the study of cancer and drug therapy, human carcinomas are also useful models for the study of normal and pathophysiological events in vivo [10]. Two human colon cell lines particularly useful are CaCo 2 and HT 29 because they undergo enterocyte differentiation in vitro [11]. They have been widely used for studies on the barrier properties of the human intestine for drug transport and the cytoprotective activity of potential drugs and their pharmaceutical formulations [12].

A rotating wall vessel (RVV) designed to simulate cell cultivation conditions on earth in the microgravity of space has been set up using these cell lines [13]. Reduced shear forces allow the cells to grow in three-dimensional form, to differentiate and to mimic the in vivo tissues. This type of bioreactor could provide an environment for cell cultures, enabling novel cell aggregate configurations that may be
usefully applied in the field of pathogenesis of infection and tissue engineering.

PRIMATE CELL LINES

Vero

The Vero cell line was established in 1962 to overcome the problem of using primary monkey kidney cells that were infected with the SV 40 virus (14–16). The use of primary tissue for poliovirus vaccine became more difficult because of international agreements on the conservation of wild animals. The Institute Merieux in France extensively tested the Vero cell line, demonstrating that Vero cells show no tumorigenicity, contain no aberrant viruses, and sustain efficient proliferation of various vaccine viruses (16). As a result, killed polio vaccine using Vero cells instead of primary monkey kidney cells has been available in France since 1984. Production is carried out on microcarriers in large-scale bioreactors.

Vero is a classical cell line that is still used as a capable substrate for virus reproduction of a wide variety of completely unrelated viruses, such as arboviruses, hemorrhagic fever viruses, rubella, measles, herpes, and growth of rickettsiae. (Unfortunately, Vero cells do not culture wild-type influenza viruses, which still need to be cultured on primary monkey kidney cells for identification.) Vero cells have been one of the most powerful basic resources for the entire field of animal virology in the past quarter of a century and will be a major cell substrate for virology and biotechnology in the years to come.

Other primate cell lines, such as COS 1 and COS 7 African green monkey kidney cells transformed with the SV 40 virus, have been found along with Vero cells to express HIV-1-like particles once transfected (17). This important development offers an alternative to the use of live virus vectors for the production and evaluation of AIDS vaccines based on noninfectious particles.

Table 1 summarizes some of the versatile human and primate cell lines that are currently being used in the biotechnology industry and the products they have to offer.

IMMORTALIZATION

Primary cell lines can be maintained in vitro for a limited period. As cells approach senescence (34), they are unable to synthesize DNA. They reach a crisis point where cell division ceases and the cells die, this is known as apoptosis, or programmed cell death.

The ability to develop immortalized cells in vitro is a powerful tool for the biological investigator. The immortalization of cells establishes continuous cell lines that play an important role in the study of the biology of cell growth, differentiation, and senescence. Large-scale culture of immortal cell lines enables the production of large quantities of DNA or any cell product for research or for the pharmaceutical industry.

The development of the biotechnology industry and the requirement for eukaryotic cells in the post-translational processing of protein products of cells has placed a great deal of pressure on the scientific and regulatory communities to provide continuous cell lines from specific cell lineages that are safe to use and with phenotypic properties relevant to their tissue of origin.

Table 2 provides the most up-to-date examples of the in vitro methods used in the immortalization of primary cultures of various cell types.

BIOREACTORS AND PRODUCTS

Bioreactors are used to obtain the maximum quantity of protein required. Fermenters are used for cell lines that grow in suspension or for high-density production of cells (e.g., HIV-1 and HIV-2 in human T lymphoblastoid cells). Fixed-bed bioreactors are advantageous because they provide close cell-to-cell contact, which is important for virus transfer (18). Porous glass spheres in fixed-bed systems retain nonadherent cells and provide large quantities of HIV.

For attached cell lines (e.g., 293, human embryokidney) (29), solid-support bioreactors have been developed either by a stirred-tank reactor using microcarriers (55) or hollow-fiber culture systems (56). For example, the polio vaccine is prepared in Vero cells grown on microcarriers in 1,000-L tanks (16).

Medium replacement is important when growing cells on a solid support or in suspension to sustain growth and protein production. A constant supply of growth factors must be supplied while reducing buildup of inhibitory metabolites such as lactate and ammonia (29). Serum-free formulations must be considered when dealing with extracellular protein purification from mammalian cells. Other environmental aspects to be considered for efficient management of bioreactors are low shear forces, high transfer rates of oxygen and carbon dioxide, and a controlled microenvironment.

Examples of new bioreactors are the Technomouse, which combines the principle of nutrient and oxygen perfusion via silicone membranes and organlike cultivation of human cells (long-term primary tissue culture is possible) (56). The VERAX process (57) uses a fluidized bed of macroporous collagen carriers, which is particularly suitable for the production of secreted recombinant products, such as Aujeszky herpesvirus in Vero cells.

Products

Interferon was the first cytokine to be made from cultured animal cells, and it is now prepared routinely at more than 95% purity, showing that scale-up to 10,000 L is possible and economically viable (26). Originally produced from human white blood cells, only small amounts could be obtained. NAMALWA, a transformed B-lymphoblastoid cell line, produces plentiful amounts of interferon and is safe to use on humans. The final product (Wellferon) is of high purity, correctly glycosylated, and contains at least six distinct α-interferons, unlike that produced from Escherichia coli by recombinant DNA technology (27).

Fibronectin, an effective factor for the growth of anchorage-dependant cells, is produced by HUH 6 (Table 1), a human hepatoblastoma cell line. At more than 90%
Table 1. Human and Primate Cell Lines

<table>
<thead>
<tr>
<th>Cell line name</th>
<th>Cell line type</th>
<th>Product</th>
<th>Use</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>U 937</td>
<td>Human T-cell lymphoblast</td>
<td>Supports growth of HIV-1 and HIV-2</td>
<td>Vaccine production and research</td>
<td>18–22</td>
</tr>
<tr>
<td>THP 1</td>
<td>Human T-cell lymphoblast</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J M, C 8166</td>
<td>Human T-cell lymphoblast</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J HAN</td>
<td>Human T-cell lymphoblast</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPMI 2650</td>
<td>Human carcinoma</td>
<td>Growth factors autocrine-stimulator, TGF-α, and TGF-β</td>
<td>Regulation of cell growth and productivity in vitro of human epithelial cells</td>
<td>23</td>
</tr>
<tr>
<td>HUH clone 5</td>
<td>Human hepatoblastoma</td>
<td>Fibronectin</td>
<td>Attachment factor in serum-free media</td>
<td>24</td>
</tr>
<tr>
<td>NAWALWA</td>
<td>Human lymphoblastoid</td>
<td>IPSF, IIα</td>
<td>Improves immuno-globulin productivity from human hybridomas</td>
<td>25</td>
</tr>
<tr>
<td>NAMALWA</td>
<td>Human lymphoblast</td>
<td>Interferon α when infected with Sendai virus and sodium butyrate</td>
<td>Treatment of hairy cell leukemia potentiates cytotoxic T cell and natural killer cell response</td>
<td>26,27</td>
</tr>
<tr>
<td>293</td>
<td>Human embryo kidney</td>
<td>Supports growth of adenovirus vectors that carry reporter genes into neuronal cells</td>
<td>Gene therapy in cystic fibrosis Structure and function analysis</td>
<td>28</td>
</tr>
<tr>
<td>293 transformed with human parathyroid calcium receptor</td>
<td>Human embryo kidney</td>
<td>Recombinant protein (similar to human plasma factor X)</td>
<td>Structural studies and clinical evaluation and growth of viruses</td>
<td>29–31</td>
</tr>
<tr>
<td>HepG 2</td>
<td>Human hepatocellular carcinomas</td>
<td>Human plasma proteins, e.g., transferrin, plasminogen, α-fetoprotein, albumin (PLC/PRF/5-albumin not produced)</td>
<td>Experimental models for investigation of plasma protein biosynthesis and synthesis of hepatitis B surface antigen HbsAg (not HepG2)</td>
<td>32,33</td>
</tr>
<tr>
<td>PLC/PRF/5 Hep3B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vero</td>
<td>African green monkey kidney</td>
<td>Aujeszky virus and oral polio virus</td>
<td>Vaccine production</td>
<td>16</td>
</tr>
</tbody>
</table>

TFG = transforming growth factor.

SAFETY CONSIDERATIONS

Any cell line used for the propagation of a specific product for the pharmaceutical industry must be vigorously controlled. Fully authentic cell banks provide that safety measure and are stored in liquid nitrogen for indefinite periods. We only need to recall the first cell line to be isolated, HeLa in 1950, deemed to be the single most important tool of biomedical research in the past 50 years. It was sent all over the world, resulting in mass cross-contamination and millions of dollars of medical research being lost.

Guidelines have been set for the acceptability of using human diploid cells for the production of live virus vaccines in humans (58) to ensure that there are no extraneous microbial contaminants or transforming factors (59,60). The increasing use of animal cells for the production of recombinant proteins and vaccines means that standardization in this field is ever more important to ensure the provision of safe and reliable therapeutic diagnostic reagents (61,62).

A further major concern is the long-term risk of malignancy represented by heterogeneous contaminating DNA, especially if it were to contain potentially oncogenic sequences (e.g., hepatitis B vaccine produced by recombinant DNA techniques). The World Health Organization (WHO) has published strict guidelines on the use of cell lines for vaccine production (63).

FUTURE TRENDS

Gene Therapy

Recently, treatment of human genetic disorders has been mediated by gene transfer technology, especially for monogenic disorders such as cystic fibrosis. Research has been carried out into the feasibility of using a defective nonreplicating recombinant adenovirus vector to transfer a reporter gene (e.g., β-galactosidase) into neuronal cells (28).

Human embryonal kidney cells 293 were used as a packaging cell and cotransfected with the vector, which could then be inserted into cystic fibrosis patients. Gene therapy replaces the specific gene sequence into a target cell (28).

If this is successful, this may lead to an improvement in controlling monogenic diseases in which only a minority of cells are required for genetic alteration in order to replace a deficient enzyme.

Bioartificial Liver

Liver failure is the major cause of mortality in the United States, with 30,000 deaths per year. There are no artificial devices available, transplants are effective but donors are limited.
### Table 2. In Vitro Methods

<table>
<thead>
<tr>
<th>Cell line type</th>
<th>Method of transformation</th>
<th>Uses</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human umbilical vein endothelial cells</td>
<td>Low-level radiation, oncogenes, SV40, spontaneous somatic cell hybridization with a human osteosarcoma</td>
<td>Von Willebrand factor, prostacyclin (e.g., ECV304 and EA.hy926)</td>
<td>35–39</td>
</tr>
<tr>
<td>HPEC A1, human placental cells</td>
<td>Plasmic pRNS1 lipofection</td>
<td>Endothelial characteristics after 80 cell divisions</td>
<td>40–43</td>
</tr>
<tr>
<td>Human B lymphocytes</td>
<td>Epstein-Barr virus</td>
<td>Express normal B-cell markers, e.g., HLA class I and II antigens, tissue typing, Genetic research</td>
<td>44,45</td>
</tr>
<tr>
<td>Human dermal fibroblasts</td>
<td>SV40 T antigen</td>
<td>Oncogenic transformation, aging, senescence</td>
<td>48</td>
</tr>
<tr>
<td>Monkey kidney epithelium</td>
<td>Genetic engineering, oncogenes, polyoma large T, H-ras</td>
<td>Alternative to primary cell culture</td>
<td>49–51</td>
</tr>
<tr>
<td>Human embryo kidney 293</td>
<td>Sheared human adenovirus 5 DNA</td>
<td>Sensitive to human adenoviruses; isolation of transformation defective host range mutants of adenovirus type 5 and titration of adenovirus type 21</td>
<td>52,53</td>
</tr>
<tr>
<td>293N3S</td>
<td>Produced by passaging 293 in nude mice</td>
<td>Large-scale production of viruses in bioreactors</td>
<td>54</td>
</tr>
</tbody>
</table>

A temporary support to support biotransformation and detoxification functions is required to sustain patients waiting for a donor or until the liver has repaired itself.

Development of a hollow fiber reactor using hepatocyte entrapment with rabbit and rat models has been successful, and scale-up is now being developed for human clinical trials (64).

**CONCLUSION**

The products and cell lines of human and primate origin discussed in this article have contributed to the general acceptance of using animal cells as the substrate for production of therapeutic proteins.

Many proteins are now obtained by expressing the gene concerned in Chinese hamster ovary cells by recombinant DNA technology, but human and primate cell lines are used as well. Many proteins will always be made more simply in bacteria, yeast, or insect cells and in the future by chemical synthesis. But until then, where glycosylation of proteins is necessary and chemical refolding of bacterial products is not always possible for full pharmaceutical activity, mammalian cells will still be used, especially with the constant improvement in protein-free and serum-free formulations and improvement in higher cell densities through bioreactor research.

**REFERENCES**


INTRODUCTION

The creation of hybridomas capable of producing monoclonal antibodies (MAbs) was accomplished by Kölliker and Milstein in 1975 (1). Two decades later, the number of MAbs used in research, purification, and immunoassay applications is large and has sustained continued growth (2-5). The medical applications for MAbs have not lived up to expectations, lagging behind other biologicals, because only a relatively small number have received approval by the FDA (6-8) (Fig. 1), four for therapeutic and five for in vivo diagnostic use (Table 1). However, this could change rapidly in the near future. In 1996, of the 12 biologics approved by the FDA, four were MAbs (9). Two MAbs were approved in 1997 (10), one of which (Zenapax) was the first approved humanized antibody. Three Biologic License Applications (BLAs) for MAbs were also filed in 1997 (11-13), and one was filed in May 1998 (14), for a total of six under review by FDA at that time. In addition, of the 350 biopharmaceutical products in development, more than 70 are MAbs, and several are in pivotal clinical trials near completion (15-18) (Fig. 2). Thus, the number of fillings of BLAs with the FDA and international regulatory agencies for MAb-based products could see significant growth in the future, to be followed by corresponding approvals.

MAbs are routinely produced in vivo and in vitro. For in vivo production, mice (mostly BALB/c) are the preferred species. The abdominal cavity of pristane-primed mice is used to incubate injected hybridoma cells, which grow and secrete MAb that concentrates in the ascitic fluid. The MAb-rich ascites is collected by repeated needle aspiration (tapping) of the animals (19,20).

With the advent of mammalian cell culture scale-up for the production of recombinant proteins during the 1980s (21-26), concomitant with the high expectations regarding the potential uses and demand for MAbs (27-31), the cultivation of hybridoma cells was intensively studied. This led to the development of a variety of bioreactors, culture media, and culture processes (31-44), many of which, when properly combined and used, easily generate required quantities of high-quality material at relatively low cost.

Genetically engineered MAbs, such as chimeric, humanized, MAb fragments and fusion proteins, are produced in mammalian and insect cells, microorganisms, and transgenic animals and plants (45-57).

Production of human MAbs using human cells still presents important challenges, although progress has been made (58-60). More significant advances have been made with the use of bacteriophage display technique and the development of transgenic mice, which express the human genes for antibody production (61,62). These animals when inoculated with antigens produce only human antibodies. Their esplenocytes can be isolated and fused with murine myelomas to produce stable hybridomas that produce human MAbs, although containing murine glycosylations instead of human. The time and procedures needed to generate these hybridomas are the same as for conventional murine hybridomas.

As clearly seen in Table 1, very different processes for MAb production have been made to work in cost-effective, validated fashion. The choice will depend on the intended application, scale required, personnel experience, and available infrastructure. With the available technology, even with the present limited level of understanding of murine hybridoma physiology, setting up a culture process for MAb production should be a relatively straightforward exercise.

HYBRIDOMA GROWTH AND MAB PRODUCTION

The performance of a process involving cultivation of hybridomas or any other cells will be the result of the com-
Table 1. Monoclonal Antibodies Approved by the FDA (by May 1998)

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Indication</th>
<th>Approval date</th>
<th>Production method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orthoclone OKT®3</td>
<td>Ortho Biotech, (Raritan, NJ)</td>
<td>Reversal of acute kidney transplant rejection</td>
<td>June 1986</td>
<td>Ascites (63)</td>
</tr>
<tr>
<td>ReoPro</td>
<td>Centocor (Malvern, PA)</td>
<td>Antiplatelet prevention of blood clots in the setting of high-risk PTCA</td>
<td>December 1994</td>
<td>Extended perfusion (spin-filter) (44)</td>
</tr>
<tr>
<td></td>
<td>Eli Lilly (Indianapolis, IN)</td>
<td>Prevention of cardiac ischemic complications in patients undergoing PTCA</td>
<td>November 1997</td>
<td></td>
</tr>
<tr>
<td>CEA-Scan</td>
<td>Immunomedics (Morris Plains, NJ)</td>
<td>Metastatic colorectal cancer imaging</td>
<td>June 1996</td>
<td>Ascites</td>
</tr>
<tr>
<td>MyoScint</td>
<td>Centocor (Malvern, PA)</td>
<td>Myocardial necrosis imaging agent</td>
<td>July 1996</td>
<td>Extended perfusion (spin filter)</td>
</tr>
<tr>
<td>Verluma</td>
<td>Boehringer ingelheim (Ridgefield, CT) NeoRx (Seattle, WA)</td>
<td>Small cell lung cancer imaging agent</td>
<td>August 1996</td>
<td></td>
</tr>
<tr>
<td>ProstaScint</td>
<td>Cytogen (Princeton, NJ)</td>
<td>Recurrent prostate cancer imaging agent</td>
<td>October 1996</td>
<td>Extended perfusion (hollow fiber) (64)</td>
</tr>
<tr>
<td>Rituxan</td>
<td>Idec Pharmaceuticals (San Diego, CA) Genentech, Inc. (S. San Francisco, CA)</td>
<td>Low-grade non-Hodgkin’s lymphoma</td>
<td>November 1997</td>
<td>Suspension culture</td>
</tr>
<tr>
<td>Zenapax</td>
<td>Hoffman-La Roche, Inc. (Nutley, NJ)</td>
<td>Prevention of acute graft rejection in kidney transplant</td>
<td>December 1997</td>
<td>Suspension culture</td>
</tr>
</tbody>
</table>

Source: Based on Ref. 9.
Note PTCA = percutaneous transluminal coronary angioplasty

Combination of three main factors: the cell line, the culture medium, and the bioreactor conditions.

The Hybridoma Cell

Preparation of Hybridomas. Hybridomas are prepared by fusing splenocytes (antibody-producing spleen B lymphocytes) from antigen-treated animals and myelomas (1). Splenocytes do not propagate indefinitely in vitro but myelomas do, so when both types of cells are fused, the hybridomas formed are able to propagate and produce antibodies. Many detailed protocols for preparation of hybridomas have been described in the literature (2,65–69).

There are two preferred and widely used murine myelomas that descend from a transplantable murine (BALB/c) plasmacytoma, MOPC 21, that was adapted to in vitro culture and renamed P3K (70). These two myelomas are P3-X63-Ag8.653 (71) and NSO/1 (65), and both have lost the ability to produce the heavy and light chains of the original MOPC 21 antibody (IgG1). A third nonproducing cell, Sp2/0-Ag14 (72), was obtained through several steps (73) and is itself a hybridoma derived from the fusion of myeloma P3-X63-Ag8X and splenocytes from a BALB/c mouse (74). The use of any of these cells guarantees that the antibodies produced by hybridomas derived from them will correspond only to those specified by the genes from the splenocytes used in the fusion. Monoclonality, however, will only be ensured when the proper procedures are used to clone and subclone the hybrids produced (68,75–79). Cloning and subcloning are also required to select stable clones that retain the antibody class and the specific productivity (see “Hybridoma Stability”).

Preparation and Characterization of Hybridoma Banks. After clone selection, it is important to ensure the availability of homogeneous and healthy cell stocks, and this is accomplished by preparing cell banks. For industrial applications, cell banks are prepared at several stages: research, master cell bank (MCB), manufacturer’s working cell bank (MWCBI), and end-of-production cells (EPC).
MCBs are intended for the preparation of MWCBS, which in turn, are prepared to ensure a supply of homogeneous cell seed for manufacturing purposes and for the life of the product, and EPC banks are for quality control and validation purposes (80,81). The successful preparation and maintenance of cell banks requires very healthy, exponentially growing cells, the use of fresh medium and appropriate quality and quantity of cryopreservative (usually dimethylsulfoxide [DMSO]) at time of freeze, and storage in monitored liquid nitrogen freezers. Detailed procedures and protocols for preparation, freezing, and storage of hybridoma stock banks are well described in the literature (82–84). Characterization of the banks is required to ensure identity, consistent performance, and lack of contaminating adventitious agents. The characterization includes recoverability of the cells upon thawing, identification of cell species, growth and production kinetics of the cells, and testing for the presence of endogenous and adventitious infectious agents (Table 2).

Hybridoma Stability. Somatic variation (antibody class shift) resulting in structural MAb variants produced by hybridomas have been reported (85–87). This phenomenon occurs as a normal evolutionary event of the humoral immune response, but once stable hybridoma clones are selected its frequency is low. The most frequent instability problem in hybridomas seems to be a decrease in MAb yield that correlates with the emergence of nonproducing cells (88–94). Nonproducing hybridomas arise with the loss of chromosomes 12 for the heavy and 16 and 6 for the light, \(\alpha, \beta, \) and \(\gamma\) chains, respectively (95–98). Loss of chromosomes occurs with higher frequency shortly after fusion, so it is very important to done and subclone to ensure the selection of stable clones. Interspecies hybrids such as murine/human or murine/rabbit have increased chromosome segregation compared to murine/murine, which results in more frequent instability problems (35,99).

For extended cultivation processes, it is important to evaluate the performance of the cells beyond the intended incubation time and to compare the quantity and quality of the MAb produced during the runs and to prepare EPC banks as mentioned above (100).

The Culture Medium

The culture medium has to provide, at a minimum, an energy source and all nutritional requirements of the cells.

**Table 2. Characterization of Hybridoma Banks**

<table>
<thead>
<tr>
<th>Test</th>
<th>MCB</th>
<th>MWCBS</th>
<th>EPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterility</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mycoplasma</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Growth and production kinetics</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Authenticity</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Species-specific viruses</td>
<td>+a</td>
<td></td>
<td>+a</td>
</tr>
<tr>
<td>Retroviruses</td>
<td>+a</td>
<td></td>
<td>+a</td>
</tr>
<tr>
<td>Adventitious viruses in vitro assay</td>
<td>+a</td>
<td></td>
<td>+a</td>
</tr>
<tr>
<td>Adventitious viruses in vivo assay</td>
<td>+a</td>
<td></td>
<td>+a</td>
</tr>
</tbody>
</table>

Source: Ref. 80 and 81.

*aRetrovirus testing is not required for murine hybridomas (81).*

**Energy Sources.** Hybridoma cells use glucose and glutamine as the preferred sources of energy. Evidence suggests that glucose and glutamine use by mammalian cells in culture is under reciprocal regulation (101–103). Hybridomas are derived from tumor cells (myelomas) and as such metabolize glucose mainly through glycolysis (104). Many hybridomas generate lactate in quasi-stoichiometric levels compared to the glucose used; the lactate accumulates in the medium, and in later stages of the culture it can be used by the cells (105,106). Glucose utilization rates range from less than 1 to more than 10 pmol/cell/day (43); the sugar is required by hybridomas and growth does not occur in its absence.

Glutamine is also essential for growth and maintenance of cultures, and its utilization rate, which ranges from less than 1 to more than 5 pmol/cell/day (43), depends on the concentration. Glutamine addition has been shown to increase the longevity of cultures, which translates into higher MAb yields (107–109). Recent studies have shown that glucose and glutamine consumption can be redirected to minimize lactate and ammonia production by using a rational medium design and a feeding strategy. Application of such approaches in fed-batch culture has led to very high levels of cells and MAb (110).

Pyruvate is both produced and consumed by hybridomas at different rates (line dependent), but its addition to the medium is not required for growth or MAb production.

**Nutritional Requirements**

**Amino Acids.** Mouse cells, like other animal cells, are unable to synthesize certain (essential) amino acids, and these have to be provided in the medium. Nonessential amino acids can be synthesized by the hybridomas, but are frequent components of some media formulations (i.e., Ham’s F-12, and others [111]). Addition of these amino acids results in lower energy requirements by the cells because anabolic amino acid metabolism is eliminated. Depending on the cell line, this supplementation may also result in higher specific growth rates.

**Lipids.** Lipids are essential structural elements of all cell membranes, and one type, the phospholipids, are the most abundant components of membranes (112). Starting from ethanolamine and fatty acids, hybridomas synthesize phosphatidyl ethanolamine (113), from which they can then derive other phospholipids (114,115). Hybridomas respond differently to lipid supplements, and specific effects on cell and MAb yields have been reported (116).

**Vitamins and Inorganic Ions.** These components are required by hybridomas and mammalian cells in general. Sufficient amounts are included in most media formulations (111), and limitations are not frequently observed under normal non-high-density growth conditions.

**Oxygen.** Hybridoma specific oxygen consumption rates have been reported to range from 1 to 40 pg/cell/h (117–119). Oxygen is one of the main limiting factors in the maintenance of viable populations in high-density perfusion cultures. In most instances, hybridoma cells will be unaffected by a wide range of oxygen concentrations. However, high oxygen concentrations (i.e., 100% or more of air saturation) can inhibit growth rates and induce cell lysis.
in later culture stages, presumably through the production of damaging oxygen radicals. In some hybridomas, the optimal concentrations of dissolved oxygen for growth and MAb production are different (118,120).

**Carbon Dioxide.** Most hybridomas will initiate growth well in an air atmosphere without additional CO$_2$. However, CO$_2$ produced by the cells has a significant effect on cell and MAb yields.

These formulations have been modified often to include the reduced risk for introducing adventitious agents of animal origin, such as retroviruses and prions, which is a significant concern if the MAb product is intended for therapeutic applications (131–133).

**Basal Media.** Most media used in mammalian cell culture are based on the balanced salt solution. For hybridoma work, Dulbecco's Modified Eagles Medium (DMEM) and a 1:1 mixture of DMEM and Ham's F-12 (111) are commonly used and contain the nutritional requirements discussed. These formulations have been modified often to increase cell and MAb yields.

**Supplements.** A universal supplement used in cell culture is animal serum. Serum contains nutrients and many factors that directly or indirectly stimulate hybridoma growth.

Basal media supplemented with serum (usually 10% or less fetal bovine serum [FBS]) will allow exuberant growth of murine hybridomas in either stationary (i.e., T flasks) or suspension (shake flasks or spinners), and cultures in simple batch mode without optimization will yield, in most cases, from 1 to 3 x 10$^6$ cells/mL and from 10 to over 100 mg/L of MAb. If needed, serum depleted of immunoglobulins can be used to minimize their contamination of the product (121).

Serum can be replaced by combinations of supplements such as insulin, transferrin, ethanolamine, and selenium, which are employed in serum-free formulations (122,123). Proteins and other contaminants are reduced when utilizing serum-free formulations and eliminated with protein-free formulations. Studies on serum-free and protein-free media are abundant in the literature (122-129), and several convenient commercial formulations are available (Table 3). Addition of basic proteins and spermine, a low molecular weight polyamine, to serum-free media has been found to stimulate IgM production by a human hybridoma line (130).

Most murine hybridomas adapt readily to one or more of these media, with growth kinetics, cell, and product yields often as good or better than those in serum-containing media. In addition, when done properly, the cells respond well to freezing in serum-free and protein-free media (123,128,130), as shown in Table 4. Additional advantages in using serum-free and protein-free media include the reduced risk for introducing adventitious agents of animal origin, such as retroviruses and prions, which is a significant concern if the MAb product is intended for therapeutic applications (131–133).

**Bioreactors**

A large variety of in vitro systems are used to grow hybridomas, including multiwell plates, Petri dishes, T flasks, multiplate containers, hollow fiber cartridges, roller bottles, shake flasks, spinner flasks, airlift fermentors, and stirred-tank fermentors (30–44). The bioreactors provide the vessel and the physicochemical environment, which includes, among the most important factors, pH, temperature, agitation, gas exchange, redox potential, and osmolality.

**Effect of pH.** Hybridomas can tolerate pH extremes (i.e., 6.4 to 8) if adapted gradually. Uptake and utilization of nutrients are affected by pH: for instance, glucose and glutamine utilization rates increase when the pH is alkaline (Fig. 3). Cultures evolve toward acidity because of the production of lactic acid and CO$_2$. To control the pH, sodium bicarbonate is frequently used as a buffering agent and is initially added to media in concentrations of grams per liter. Organic buffers such as 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 2-morpholinoethanesulfonic acid (MES), 3-morpholino-propanesulfonic acid (MOPS) or others (134) are also used at concentrations of 10 mM or higher.
Table 4. Growth and MAb Production by Three Hybridomas Cultivated in 2-L Spinner Flasks Using Serum-Free and Protein-Free Media

<table>
<thead>
<tr>
<th>Media</th>
<th>Line A</th>
<th>Line B</th>
<th>Line C</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF</td>
<td>1.2</td>
<td>1.9</td>
<td>1.6</td>
</tr>
<tr>
<td>PF</td>
<td>1.5</td>
<td>1.9</td>
<td>1.5</td>
</tr>
<tr>
<td>SF</td>
<td>0.038</td>
<td>0.04</td>
<td>0.041</td>
</tr>
<tr>
<td>PF</td>
<td>0.022</td>
<td>0.043</td>
<td>0.033</td>
</tr>
<tr>
<td>SF</td>
<td>18.3</td>
<td>17.3</td>
<td>16.9</td>
</tr>
<tr>
<td>PF</td>
<td>31.5</td>
<td>16.1</td>
<td>21</td>
</tr>
<tr>
<td>SF</td>
<td>74</td>
<td>62</td>
<td>90</td>
</tr>
<tr>
<td>PF</td>
<td>108</td>
<td>76</td>
<td>106</td>
</tr>
</tbody>
</table>


Note: All hybridomas were derived from BALB/c splenocytes. Line A was derived from fusion with P3-X63Ag8.653 myeloma (71) and produces IgM. Lines B and C were derived from fusion with P3NS1/1-Ag4-1 myeloma (65) and produce IgG1 and IgG2a, respectively.

Data correspond to averages obtained in 8 and 6 consecutive passages, respectively in serum-free (SF) medium containing bovine transferrin and insulin and protein-free (PF) medium containing chelated iron to replace transferrin. The adapted cells had been frozen in corresponding media formulations supplemented only with 10% DMSO as cryoprotectant, and were grown as described upon thawing.

Figure 3. Effect of pH on the utilization of glucose and glutamine and the production of lactate and ammonia by line XMMLY-B. The hybridoma was grown in chemostat culture at a dilution rate of 0.017 h⁻¹. Data were collected at each pH after reaching steady states. (a) Glucose concentration (■), lactate concentration (●), ratio of lactate produced to glucose utilized (▲). (b) Glutamine concentration (■), ammonia concentration (●), ratio of ammonia produced to glutamine utilized (▲).

Maximum specific growth rates are usually observed at pH values between 6.8 and 7.3, but growth and MAb production are frequently optimal at different pH values. A production process may consist of a growth phase in which the pH is controlled at a certain value, followed by a production phase where the pH is adjusted to the value that maximizes product secretion.

The quality of the secreted MAb can be affected by the pH in the medium either by facilitating chemical changes or, when low, by providing a favorable environment for the action of released acidic proteases (106,135–137). Thus, the effect of pH on growth, productivity, MAb yields, and MAb quality should be determined to maximize yields and product consistency.

Effect of Temperature. Optimal temperature for myeloma and hybridoma growth is 37 °C, and the cells recover from excursions to slightly higher temperatures. Lower temperatures cause decreases in growth and MAb production, but the cells are not affected and recover promptly when placed again at 37 °C. Myelomas and hybridomas can be maintained at room temperature for several days, which is convenient for shipments of cells in culture. For permanent storage a liquid nitrogen freezer is essential. A properly frozen hybridoma stock, upon thawing and placed in culture with fresh medium, will usually resume growth within 48 hours.

Effect of Agitation and Gas Bubbles. Concerns about shear sensitivity of mammalian cells prompted the development of airlift fermenters for use with mammalian cells (139) and hybridoma growth (140,141) that are used for commercial production of MAbs (Table 1). However, experience has shown that each line is different (142), shear sensitivity depends on culture conditions (143–145), most hybridomas are not especially sensitive to shear stress in
culture, and some can stand very harsh agitation. Bubble-cell interactions cause damage, but shear-protective agents such as the polyol Pluronic F-68, which is frequently added to cell cultures, can minimize the damaging effects of bubble disengagement (144–145).

**Redox Potential.** Although the oxidation-reduction potential (redox potential) is routinely measured on-line in microbial fermentations, the same is not frequently done in cell cultures. Little understanding of correlations between the redox potential on growth or MAb production exist (146), but optimal values may be defined and applied in the future.

**Effect of Osmolarity.** Hybridomas, like other mammalian cells, grow uninhibited in osmolalities ranging from 260 to 300 mOsmol/kg H₂O (approximate osmolarities of 260 to 300 mmol/L [147]) and commercial basal media are designed to have these values by adjusting the NaCl content. High osmolarity has been reported to inhibit growth rate and cell yields (148), but also to induce higher MAb secretion at later stages of cultivation (149–153). Some amino acids and analogs act as osmoprotective agents for hybridomas and can even increase specific antibody productivity (154).

**Growth Kinetics**

Requirements for hybridoma growth are a viable inoculum and proper extracellular environment, including essential nutrients and suitable physicochemical conditions.

When all these conditions are met, hybridomas, like all other eukaryotic cells in culture, have kinetics of growth similar to microbial cells and also obey the exponential growth law (155,156). Hybridomas are not anchoragedependent and grow as well in nonagitated cultures, such as multiwell plates, Petri dishes, T flasks and hollow fiber bioreactors as do they in agitated containers, including roller bottles, shake flasks, spinner flasks, or fermenters. Hybridoma cells grow monodispersed, and in batch cultures, in most media, they reach concentrations of several million per milliliter, with viabilities of well over 90% throughout the growth phase.

When growth data are plotted as viable cell counts against time, a typical batch growth curve of hybridoma may contain the six phases observed in microbial culture: lag, accelerating growth, exponential growth, decelerating growth, stationary, and decline (156). A lag period is generally observed when starting cultures using cells thawed from frozen stocks. Upon thawing, the cells go through an initial period of adaptation, but once exponential growth is attained no lag period will occur upon subculturing provided that the cells are passaged to fresh medium while still growing exponentially. If a culture is maintained beyond the exponential phase, a lag often is observed upon passage, and such cells are said to have been overgrown. The longer cells are maintained in the spent medium beyond the exponential phase, the more likely that a lag period will occur in the next culture (157). Ultimately, there will be a point of overgrowth past which all cells will die either by apoptosis or by necrosis (158), induced by starvation or toxic metabolite buildup (159–164), and the culture can no longer be recovered.

During exponential growth, doubling times for most hybridomas are between 14 and 23 h, corresponding to maximum specific growth rates ranging from 0.05 to 0.03 h⁻¹.

Hybridomas have been grown in chemostat cultures at dilution rates ranging from critical (Dc), which is equivalent to maximum specific growth rate (156), to less than 10% of Dc. Viability decreases with dilution rate (Fig. 4a), and a common observation with all hybridomas is that an intrinsic minimum specific growth rate of approximately 0.02 h⁻¹ exists for all of them (164,165) (Fig. 4b). A minimum specific growth rate was also reported for other cells and attributed to nutritional limitations (166). Death of hybridomas at low dilution rates may be partly by apoptosis induced by nutritional limitation(s) and reduced protein synthesis. A practical implication of this phenomenon in a continuous culture process is that the fraction of dead cells will increase with decreases in the dilution rate (purge rate in high-density perfusions [156]). The ratio of viable/dead cells will have effects on MAb concentrations and homogeneity and on the longevity of production runs, all of which impact product yields.

**MAb Production Kinetics**

MAbs are produced by viable cells (167). Patterns observed for MAb production by hybridomas are the same as those seen with microbial cells for product formation (168), and most can be defined either as growth-associated, partly growth-associated, non-growth-associated, or negatively growth-associated (169–178).

Figure 5 shows the growth and MAb production of two hybridomas in batch cultures. One clearly has growth-associated production (a), and the second (b) has a non-growth-associated pattern with more than 90% of the MAb produced after growth has ceased.

Specific productivities of several hybridomas at different dilution rates in continuous chemostat cultures are shown in Figure 6. The pattern of line XMMEN-A was non-growth-associated, whereas that of all other hybridomas tested in the group was growth-associated. The specific pattern is very important in defining the productivity profile of continuous cultures, and depending on the system and its mode of operation, the bleed or harvest rate will have to be adjusted for maximum productivity (156). In high-density cultures, productivity can also be affected by other factors, external or autocrine (179), which may not be obvious or predictable in low-density cultures and that need to be evaluated under operation conditions.

The validity and usefulness of applying the microbial growth and non-growth-associated concepts to the production of proteins by animal cells, including hybridomas, has been questioned (180). However, it is clear that for two cell lines with different productivity profiles (as shown in Fig. 5), the optimal harvest times will be very different, and this will have a significant impact on the corresponding operations.

**MAb Production Processes**

**In Vitro.** MAb yields by most hybridomas in simple batch cultures without optimization are in the 10 to 100
mg/L range. Production of milligram quantities can be easily accomplished using T flasks, spinner flasks, or roller bottles. Gram quantities require either more of these containers and more labor or the use of larger vessels such as fermenters. Productive batch cultures need a minimum of conditions, which include an exponentially growing inoculum, a buffered medium with an initial pH adjusted between 7.2 and 7.5, and the temperature of incubation regulated at 37°C ± 0.5°C. Growth kinetics, specific MAb productivities, and other characteristics of several hybridomas grown with these conditions in batch suspension cultures and in different vessels are compiled in Table 5.

Fed-batch culture of hybridoma growth continues to evolve, and very high MAb yields, in the order of grams per liter, have been reported recently (110,181–184). This may become the process of choice in future commercial operations.

High-density continuous processes can be done at many scales with available preassembled equipment. The systems vary in their complexity, instrumentation, and requirements for infrastructural support. Some are easy to set up and operate, but others require special training and experience. All demand attention in order to be used routinely in successful ways, but they are reliable and productive when understood and operated properly (39–44).

In Vivo. Production in ascites has the advantage of high concentration, and it is widely used. It is commonly used in the production of small quantities of MAb for research and diagnostic purposes; two of the approved MAbs for medical use are made using ascites technology (Table 1). Contract suppliers can provide ascites at different scales from a few milligrams to kilograms per year. Facilities designed for production of up to kilogram quantities of MAbs under cGMP are operated for contract manufacturing by companies such as Charles River in Massachusetts and North Carolina and Genzyme Transgenics. Although clearly not the preferred method of production for therapeutic MAbs, as explicitly stated by the Committee for Proprietary Medicinal Products (185), validated and cost-effective ascites processes exist, and several MAbs in advanced clinical trials are made in ascites.

Emerging in vivo processes involve the use of transgenic animals. Reports of high yields in the milk of goats and other animals coupled with effective separation and purification techniques may make this a competitive technology for certain applications (186). However, for regulatory and strategic reasons, it is advisable to adopt in vitro processes for production of pharmaceutical-grade MAbs. The use of animals for production of MAbs for therapeutic applications will have to deal with, among other complications, the growing concerns of potential bovine spongiform encephalopathy or other prion contaminations (133,187,188).

Production of antibodies in plants has also been done (50) and may represent a viable alternative for some applications.

MAb Recovery and Purification

MAbs are secreted by hybridomas, and when using protein-free media, the cultures contain high MAb/total protein ratios. In high-density, hollow-fiber perfusion cultures, MAb levels corresponding to more than 40% of the total protein have been reported (43). For some applications, a cell-free harvest of this relative composition may
Figure 5. Growth curves and MAb production by two hybridoma lines (a, XMMME-C, and b, XMMEN-A) with growth-associated and non-growth-associated productivity patterns. Total (○), viable (△), and MAb concentration per mg/L (□).

Figure 6. Growth-associated and non-growth-associated productivity patterns of several hybridoma cells grown in continuous chemostat cultures. Symbols same as in Figure 4.

be used as such without further processing to remove other proteins present (although contaminating cellular proteases may degrade the MAb upon prolonged incubation).

MAb recovery traditionally starts with cell removal by centrifugation or filtration. New processes aim at obviating separation steps by applying the entire culture to a fluidized bed of chromatography beads (189). Purification of MAbs involves usually two to three steps, which may include combinations of precipitation or column processes such as ion-exchange separation; affinity separations (protein A or dye ligand); or hydroxylapatite, hydrophobic, mixed-bed, and gel filtration (190–192).

PROCESS CHANGES

Process development is a multidisciplinary endeavor aimed at maximizing yields and minimizing costs while maintaining product consistency. Traditionally, there has been reluctance in industry to publish results on the development of processes for biopharmaceuticals, including those for MAbs (34). This reluctance reflects the need to maintain advantage over competitors.

Significant advances have taken place in the understanding of hybridoma physiology and in the development of highly productive MAb processes. Through the years, a wealth of information from academia and industry became available in publications and through other sources. The development and implementation of an MAb production process benefits today from this information, and systematic approaches to follow have been described (34,193).

Many approved biologicals are manufactured by obsolete or suboptimal processes. For instance, OKT3, the first therapeutic MAb approved, is made with ascites technology. It was introduced into the market 12 years ago and continues to be manufactured by the same process. There are several reasons for this apparent conservatism in the biopharmaceutical industry.

First, there is the pressure to expedite manufacturing for introduction of a biological product into the market. A recent example of this pressure was that confronted by Chiron Corporation for the production of Betaseron (recombinant interferon-β). After approval of the product in
that culture conditions may significantly affect the prop-

April 1993 for the treatment of relapsing-remitting multi-
ple sclerosis (194), the company had an initial shortage of
manufacturing capacity. It was forced to establish a com-
puterized lottery system to supply between 12,000 and
20,000 patients the first year until capacity was brought
up in line with the demand (195). Obviously, with such
pressing needs, there is little incentive to introduce process
changes that may produce further delays.

A second and very important cause has been the diffi-
culty of introducing process changes after pivotal phase III
clinical testing or after commercialization of a product.
Regulatory approval of process changes could only be ob-
tained after the results of extensive and costly testing in
toxicity and efficacy provided guarantees that the
changes were not altering significant properties of the
product (196). Thus, in several instances, introduction of
process changes has either been abandoned or postponed
as long as the difficulties and costs implied could not be
offset by the potential gains.

More recently, the established dogma in the production
of biopharmaceuticals that “the process defines the prod-
uct” has lost its absolutism. This has been an evolutionary
process guided by caution. New powerful techniques are
being used for characterization of biologics, and experience
with the clinical use of these products has helped
redefine the relevance of microheterogeneity and the poten-
tial problems with contaminants such as residual DNA,
proteins, and endogenous retroviruses (197–198). As a re-

Note: N/D = no data.

Table 5. Batch Culture of Hybridoma Lines in Suspension

<table>
<thead>
<tr>
<th>Line</th>
<th>Parental myeloma</th>
<th>MAb type</th>
<th>Bioreactor</th>
<th>Maximum specific growth rate (h⁻¹)</th>
<th>Doubling time (h)</th>
<th>Specific MAb productivity (b pg/viable cell/day)</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>XMMN-A</td>
<td>P3-X63Ag8.653</td>
<td>IgM</td>
<td>2-L spinner</td>
<td>0.05</td>
<td>14</td>
<td>15</td>
<td>13</td>
<td>N/D</td>
<td></td>
</tr>
<tr>
<td>XMMN-A</td>
<td>P3-X63Ag8.653</td>
<td>IgM</td>
<td>40-L stirred fermenter</td>
<td>0.05</td>
<td>14</td>
<td>20</td>
<td>15</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>XMMN-A</td>
<td>P3-X63Ag8.653</td>
<td>IgM</td>
<td>40-L airlift fermenter</td>
<td>0.018</td>
<td>37</td>
<td>24</td>
<td>15</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>A-72</td>
<td>P3-X63Ag8.653</td>
<td>IgM</td>
<td>2-L spinner</td>
<td>0.05</td>
<td>14</td>
<td>15</td>
<td>14</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>A-78</td>
<td>P3-X63Ag8.653</td>
<td>IgM</td>
<td>2-L spinner</td>
<td>0.05</td>
<td>14</td>
<td>13</td>
<td>14</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>XMMLY-B</td>
<td>P3NSI/Ag4-1</td>
<td>IgG1</td>
<td>2-L spinner</td>
<td>0.043</td>
<td>16</td>
<td>52</td>
<td>12</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>B-17</td>
<td>P3NSI/Ag4-1</td>
<td>IgG1</td>
<td>2-L spinner</td>
<td>0.033</td>
<td>21</td>
<td>76</td>
<td>10</td>
<td>N/D</td>
<td></td>
</tr>
<tr>
<td>B-17</td>
<td>P3NSI/Ag4-1</td>
<td>IgG1</td>
<td>40-L stirred fermenter</td>
<td>0.023</td>
<td>29</td>
<td>&gt;80</td>
<td>20</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>XMMME-C</td>
<td>P3-X63Ag8</td>
<td>IgG2a</td>
<td>2-L spinner</td>
<td>0.043</td>
<td>16</td>
<td>40</td>
<td>2</td>
<td>N/D</td>
<td></td>
</tr>
<tr>
<td>XMMME-D</td>
<td>P3NSI/Ag4-1</td>
<td>IgG1</td>
<td>2-L spinner</td>
<td>0.038</td>
<td>18</td>
<td>90</td>
<td>5</td>
<td>N/D</td>
<td></td>
</tr>
<tr>
<td>XMMCO-E</td>
<td>P3NSI/Ag4-1</td>
<td>IgG2b</td>
<td>2-L spinner</td>
<td>0.035</td>
<td>20</td>
<td>90</td>
<td>5</td>
<td>N/D</td>
<td></td>
</tr>
<tr>
<td>XMMLY-F</td>
<td>P3NSI/Ag4-1</td>
<td>IgG2a</td>
<td>2-L spinner</td>
<td>0.046</td>
<td>15</td>
<td>70–90</td>
<td>50–75</td>
<td>40</td>
<td></td>
</tr>
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<td>Sp2/0</td>
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<td>XMMID-G</td>
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<td>40-L stirred fermenter</td>
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July 1993 for the treatment of relapsing-remitting multiple sclerosis (194), the company had an initial shortage of manufacturing capacity. It was forced to establish a computerized lottery system to supply between 12,000 and 20,000 patients the first year until capacity was brought up in line with the demand (195). Obviously, with such pressing needs, there is little incentive to introduce process changes that may produce further delays.

A second and very important cause has been the difficulty of introducing process changes after pivotal phase III clinical testing or after commercialization of a product. Regulatory approval of process changes could only be obtained after the results of extensive and costly testing in toxicity and efficacy provided guarantees that the changes were not altering significant properties of the product (196). Thus, in several instances, introduction of process changes has either been abandoned or postponed as long as the difficulties and costs implied could not be offset by the potential gains.

More recently, the established dogma in the production of biopharmaceuticals that “the process defines the product” has lost its absolutism. This has been an evolutionary process guided by caution. New powerful techniques are being used for characterization of biologics, and experience with the clinical use of these products has helped redefine the relevance of microheterogeneity and the potential problems with contaminants such as residual DNA, proteins, and endogenous retroviruses (197–198). As a result, regulatory agencies have gradually adopted a less-stringent view on the inalterability of the process and the dedication of facilities and have shifted toward a more flexible position based on the ability to demonstrate equivalence of the products made by different processes and at different sites. New regulations and guidelines encourage process improvements when capable of producing equivalent product (199–201). However, it should be kept in mind that culture conditions may significantly affect the properties of MAbs (202,203), and efforts should be made to identify these effects as early as possible in order to guide the selection of the most advantageous technology for a process (204).

CONCLUSIONS

With the present accessibility to relevant, practical information, availability of hybridoma-specific culture media, alternative bioreactor technology, and multiple purification methods, in most cases a single MAb production process can be easily implemented.

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HYBRIDOMA, ANTIBODY PRODUCTION

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**INTRODUCTION**

Oxygen (O2) plays a primary role in cell metabolism and viability. By serving as the terminal electron acceptor in oxidative phosphorylation, O2 is important for energy production. Oxygenation is a major consideration in the design of bioreactors for cultivating cells. Although mammalian cells do not consume O2 rapidly (the specific O2 uptake rate, qO2, is typically 0.05–0.5 × 10−3 mmol cell−1 h−1) (1, 2), O2 can be a limiting factor in part because of its poor solubility in aqueous media. Oxygen demand by cells is regulated by environmental conditions such as O2 tension (pO2), pH, cell type, cell density, and nutrient depletion, all of which may vary throughout the culture period. To accommodate increasing O2 demands as cultures increase in cell density and proliferative state, a number of bioreactor configurations have been developed (3): agitation, use of microcarriers for adherent cells, and sparging can effectively enhance the driving force for O2 transfer.

Although much attention has been focused on overcoming extracellular resistances to O2 transfer, intracellular events further complicate the development of optimal oxygenation strategies. During aerobic metabolism, the reduction of molecular O2 to water may yield three potentially damaging reactive oxygen species (ROS): the superoxide anion (O2•−), the hydroxyl radical (OH•), and hydrogen peroxide (H2O2). ROS are also produced independently of respiration by O2-consuming reactions in the cytosol (4) as well as in the culture medium. ROS can damage lipids, proteins, sugars, and DNA. Such damage, collectively known as oxidative stress, can lead to damage of the cell membrane and induction of cell death if it is not countered by radical-scavenging defense systems. This dual nature of O2 should be considered when developing mam-
malian cell culture strategies, and provides an impetus to use controlled hypoxic conditions to optimize reactor productivity (5). The extent of hypoxia can be characterized by the extent to which \( q_\text{O}_2 \), is decreased relative to that for cells without \( O_2 \) limitation. Moderate hypoxia not only will decrease \( q_\text{O}_2 \), but may also lower ROS concentrations and increase metabolic efficiency (6). Insights into the physiological responses of cells to hypoxia will facilitate the use of \( p_\text{O}_2 \) as a manipulatable parameter during cell culture, as well as provide increased understanding of the consequences of an interruption in the \( O_2 \) supply.

It would not be possible for a cell to respond to \( O_2 \) deprivation and \( O_2 \) toxicity without an inherent ability to sense alterations in \( p_\text{O}_2 \). Indeed, cells have evolved complex regulatory mechanisms to ensure metabolic efficiency and protection against oxidative damage. As the \( O_2 \) supply changes, cells in vivo and in vitro adapt through multiple physiological responses, ranging from altered energy metabolism and production of antioxidants to changes in growth and differentiation. \( O_2 \) deprivation elicits homeostatic responses on the systemic, local, and intracellular levels. Adaptation at the systemic level involves a widespread increase in \( O_2 \) delivery to the tissues through regulation of breathing, red blood cell mass (erythropoiesis), and vasodilation. Locally, the tissue response to hypoxia entails proliferation of capillaries (angiogenesis). Adaptation to \( O_2 \) deprivation at the cellular level involves a shift from oxidative phosphorylation and gluconeogenesis to anaerobic glycolysis, as well as the expression of stress-specific proteins. Whether at the organismal or cellular level, many of the effects noted here are mediated through a cascade of events that are initiated by the sensing of \( p_\text{O}_2 \) and propagated by a signal which ultimately leads to hypoxic regulation of specific gene expression. The proteins coded for by these genes facilitate cellular survival at low \( p_\text{O}_2 \) and act to restore normal \( p_\text{O}_2 \).

This article focuses on the current understanding of \( O_2 \)-dependent responses of animal cells, particularly those exposed to hypoxic conditions. The effects of \( p_\text{O}_2 \) on cells in a wide range of culture systems are addressed first. Subsequently, the molecular mechanisms by which hypoxia mediates its effects—\( O_2 \)-sensing, signal transduction, and gene expression—are examined. Mechanisms used by cells in vivo have provided the groundwork for uncovering many of the mechanistic details that underlie the hypoxic responses of cultured cells. Although different cells exhibit similar coping mechanisms during periods of \( O_2 \) deprivation, their specific adaptation to hypoxia is individually tailored according to cell type and function, as well as the extent of hypoxia.

CELL RESPONSES TO \( p_\text{O}_2 \) AND HYPOXIA

Upon first encountering \( O_2 \) deprivation, cells respond rapidly by altering several properties, such as reducing proliferation, shifting to anaerobic glycolysis, and inhibiting protein synthesis. Survival under sustained hypoxia requires a second phase of adaptive responses, which are characterized by extensive cellular and metabolic reprogramming. The selective expression of stress proteins and other specific proteins underlies these secondary processes. Upregulated expression of genes involved in glycolysis and downregulated expression of those involved in gluconeogenesis or the TCA cycle is a prime example of this adaptive mechanism. Immediate and adaptive responses to changes in \( p_\text{O}_2 \) are discussed in the following sections.

Growth, Viability, and Differentiation

The effects of dissolved oxygen concentration (DO) on cell growth in batch culture have been evaluated by many investigators (7–12). The DO value for maximal cell concentration in batch culture is typically about 50% of air saturation (50% DO) but may vary considerably with cell type. Lower values of DO, 5–35% (13) and 0.5% (6), have been reported for maximal steady-state viable cell concentrations in continuous hybridoma culture. Besides differences between cell lines, poor batch growth at low DO could result from added stress from low initial cell concentrations, glucose depletion, or insufficient time for the cells to adapt to low DO.

Steady-state cell viability has been shown to increase as the DO is decreased to 0.1–0.25% DO in continuous culture (6,13). Enhanced viability at lower DO may reflect less oxidative stress imposed on the cells. The greater production of ROS associated with progressively greater DO could damage cellular components and induce cell death. Mammalian cells are able to tolerate the presence of ROS because of the action of antioxidants. However, damage ensues when antioxidant defenses are overwhelmed by ROS. Indeed, in a murine hybridoma culture, increased DNA fragmentation correlated with increasingly hyperoxic culture conditions from 200% to 476% DO (14). Although maximum cell growth in batch culture usually occurs below 100% DO, some cell lines have been adapted to grow at near-maximal rates at DO values as high as 200% DO (15). CHO cells have been adapted to 380% DO (16) and, more recently, a murine hybridoma was adapted for growth at 150% DO by gradually exposing the cells to higher DO levels in continuous culture (17). In both cases, resistance to \( O_2 \) toxicity has been attributed to increased activity of antioxidant enzymes during the adaptation process.

An obvious way to avoid oxidative stress is to reduce the amount of \( O_2 \) available to the cells, as was demonstrated for hybridomas (6). Low \( p_\text{O}_2 \) stimulates the proliferation of other cell types, particularly fibroblasts seeded at low density (18–20). The proliferation rate and lifetime (i.e., number of population doublings before senescence) of WI-38 and IMR-90 lung embryo fibroblast cells were increased by reducing the gas-phase \( O_2 \) concentration from 20% to 2% (19). Based on these findings, Falanga and Kirshner (21) set out to establish fibroblast cultures from single cells, which normally fail to proliferate under 20% \( O_2 \). Low \( p_\text{O}_2 \) stimulated and maintained single-cell proliferation, which is consistent with fibroblast proliferation during hypoxic situations in vivo (e.g., during wound healing).

I increased clonal growth of cells under \( O_2 \) concentrations less than that of the ambient atmosphere suggest that \( p_\text{O}_2 \) may play a role in directing developmental processes. In other words, \( p_\text{O}_2 \) in tissues may regulate not only cell growth and death but also cell differentiation. This hy-
Antioxidants and ROS

Mammalian cells have evolved a line of defense to limit the damage caused by ROS generated during aerobic metabolism. This antioxidant defense system includes glutathione, vitamin E, and enzymes such as superoxide dismutase (SOD), catalase, glutathione-S-transferase (GST), and glutathione peroxidase (GPX). In addition, extracellular antioxidants such as albumin, urate, and bilirubin also protect cells against oxidative damage.

High pO₂ in culture may increase the production of ROS to levels that overwhelm inherent antioxidant defenses. The associated detrimental effects can be overcome by stepwise adaptation to increases in pO₂. Adaptation of hybridoma cells to 100% DO from 10% was associated with an increase in the specific activities of the antioxidant enzymes GPX, GST, and SOD (17). Similarly, adaptation of CHO cells to growth under 99% O₂ was associated with increased specific activities of SOD, catalase, and GPX (16). Thus, enhanced tolerance likely is caused in part by upregulation of the synthesis of these enzymes. For example, a cis-acting regulatory sequence that is responsive to ROS, the antioxidant responsive element (ARE), has been found to activate the genes encoding the enzymes GST and NAD(P)H:quinone reductase (36). However, the activity of some antioxidant enzymes may decrease again above 100% DO (17), possibly through inactivation by ROS.

Decreased colony formation at higher pO₂ in semisolid culture suggests that hematopoietic colonies are susceptible to oxidative stress. Indeed, addition of antioxidants such as monothioglycerol and β-mercaptoethanol to high-pO₂ cultures yields colonies similar in size and number to those in low-pO₂ cultures (22,23,37,38). H₂O₂ and superoxide concentrations have been found to increase throughout the duration of bone marrow cell cultures (39). Progenitor cell production was increased by adding catalase, an H₂O₂ scavenger, or mannitol, an hydroxyl radical scavenger. In contrast, even though superoxide levels were decreased, the effects of SOD addition were negligible.

Rather than adding antioxidants or upregulating their synthesis through adaptation, an alternate strategy to reduce ROS damage is to decrease ROS generation by operating at low pO₂. Although low pO₂ can be beneficial in promoting proliferation, as already stated, and suppressing apoptosis (see following), it may actually sensitize cells to injury during reoxygenation. Involvement of ROS in reoxygenation injury has been demonstrated by decreased damage in tissues treated with antioxidants during reoxygenation (40). ROS involvement is also shown by increased glutathione levels during reoxygenation of CHO cells from severe hypoxia or anoxia (41). Glutathione levels increase more extensively after more severe hypoxic exposure and increase to a much greater extent after an abrupt increase in pO₂ than during gradual reoxygenation. Sensitization to oxidative damage could not be attributed to a decrease in glutathione levels during hypoxia. In fact, there is evidence to contradict a contribution of decreased antioxidant levels to sensitization to oxidative stress. Some antioxidant enzymes, including SOD, GST, and catalase, are part of a family of stress-related proteins that may be induced during hypoxia (42-44). Upregulated expression of these enzymes during O₂ deprivation may anticipate their role in detoxifying ROS produced during reoxygenation (45).

It has been suggested that intracellular ROS are mediators of apoptosis and may even trigger cell death if present at elevated levels (46). In support of this hypothesis, the features of cells undergoing apoptosis closely resemble those of cells stressed by oxidative damage in terms of their morphology, physiology, biochemical, and gene expression (47). Intracellular ROS may induce cell death in several ways, from direct damage of DNA to protein oxidation. If oxidants induce apoptosis, a natural extension would be that antioxidants inhibit apoptosis. Indeed, antioxidants such as N-acetylcysteine (48), N-acetylcarnitine (49), idebenone (50), and trolox (analog of vitamin E) (51) have been shown to inhibit apoptosis. Antioxidant protection against cell death is not confined only to direct oxidant-induced apoptosis but also to receptor- and growth factor-mediated forms of apoptosis. This suggests that oxidation may be a common mechanism in the progression of many different forms of apoptosis. Studies correlating oxidative damage with the induction of apoptosis have led to the
identification of another potential antioxidant, the Bcl-2 protein. The antioxidant role of Bcl-2 is suggested by its ability to protect cells from H$_2$O$_2$-induced and menadione (a quinone compound that generates O$_2^-$)-induced oxidative death (52,53). Overexpression of the Bcl-2 protein has also been shown to suppress apoptosis triggered by glucocorticoids, irradiation, chemotherapeutic agents, growth factor deprivation, and tumor necrosis factor alpha (TNF-α) (47,54). For these reasons, Bcl-2 is thought to inhibit cell death by decreasing the generation of ROS, which would in turn inhibit intracellular oxidation reactions critical to progression of the apoptotic program. However, Bcl-2 may have other functions that are unrelated to its putative antioxidant properties. Under hypoxic conditions with low levels of ROS, Bcl-2 is still able to protect cells from apoptotic death (55–57).

**Stress-Related Proteins**

Redirection of protein synthesis in response to hypoxia is part of a more general mammalian cell response to stresses such as temperature elevation and substrate deprivation. Such adverse conditions elicit expression of specific sets of stress proteins, namely, heat shock proteins (HSPs), glucose-regulated proteins (GRPs), oxygen-regulated proteins (ORPs), and hypoxia-associated proteins (HAPs). Stress proteins play an important role in adaptation to a new environment, as well as increased resistance to the same or other stresses. Which sets of proteins are upregulated varies with the cell type and the type of stress involved. However, recruitment of a specific family of proteins is not restricted to a single stimulus, as would be implied by its name.

The best-characterized of the stress proteins are HSPs, in particular the HSP70 family, the members of which are produced by many cell types as a protective response to high temperature, glucose deprivation, hypoxia, ischemia, the presence of heavy metal salts, and reoxygenation (58). These proteins function as chaperones that decrease protein denaturation and facilitate protein folding, processing, and trafficking. They are believed to help regulate biological processes including embryogenesis, differentiation, growth, and metabolism (59). GRPs have been less extensively characterized, but like HSPs, their response is not stress specific in regards either to their stimuli for upregulation or to their protection against other stresses (60). Investigations into stress protein induction in response to hypoxia and ischemia have implicated the upregulation of HSPs, GRPs, and ORPs. It should be noted that many of the ORPs have in fact been identified as a subset of the GRPs (61).

The effects of O$_2$ deprivation and subsequent reoxygenation on the potential coordinated regulation of HSPs and GRPs have been examined in CHO cells (61). The data indicate that while GRP induction is a sustained response to prolonged O$_2$ deprivation, HSP induction is only transient. Furthermore, when cells are reintroduced to an O$_2$-containing environment, the GRPs are repressed whereas the HSPs are again temporarily induced. Recently, an antioxidant role for both the constitutive and inducible forms of HSP70 has been confirmed (62). Overexpression of the HSP70s in rat cardiomyocytes confers a marked increase in tolerance to oxidative challenges including exposure to H$_2$O$_2$, hydroxyl radical, menadione, and hypoxia and reoxygenation. This finding is consistent with reports that oxidative stress induces expression of a number of HSPs, and that preexposure to mild hyperthermia or moderate oxidative stress significantly increases resistance of lymphocytes challenged with high doses of ROS (63). Interestingly, HSP32 (heme oxygenase-1) is also expressed in response to prolonged (~12 h) hypoxia (64).

Redirected protein synthesis is further demonstrated by the selective expression of ORP150 in cultured rat astrocytes exposed to hypoxia, but not in cells exposed to heat shock, H$_2$O$_2$, Co$^{2+}$, 2-deoxyglucose, or tunicamycin (65). Interestingly, the human analog of ORP150 is expressed in response to a wider range of conditions—exposure to hypoxia, 2-deoxyglucose, or tunicamycin, but not to heat shock—in the HeLa and human astrocytoma U373 cell lines (66). The wider expression range may be a characteristic of established cell lines because ORP150 induction is also restricted to hypoxia in human mononuclear phagocytes. As discussed earlier, many stress proteins upregulated by exposure to hypoxia are also involved in the response to other stresses. The term HAPs has been coined to describe a unique set of endothelial cell stress proteins that are distinct from HSPs, GRPs, and most ORPs. Like ORP150 in primary cells, these HAPs are preferentially upregulated by exposure to hypoxia, while other stresses such as heat and glucose deprivation do not induce them (67). Moreover, HAPs do not offer any cross-protection against other stresses. The glycolytic enzyme, GAPDH, has been identified as a HAP. GAPDH expression in the nuclear fraction of endothelial cells is upregulated mostly at the transcriptional level, and suggests a nonglycolytic role for this enzyme (68,69). In contrast, the GAPDH mRNA content in rat cardiomyocytes remains unchanged during hypoxia (64).

Although the molecular mechanisms underlying the regulation of stress proteins by hypoxia and reoxygenation are still unclear, it is known that their expression is, at least in part, regulated at the transcriptional level. Thus far, most insight into these regulatory mechanisms has been gained from studies of the HSP genes. Transient exposure of cultured myogenic cells to hypoxia has been shown to transcriptionally activate the HSP70 gene through binding of a nuclear transcription factor, heat shock transcription factor (HSTF), to distinct cis-acting regions, heat shock elements (HSEs), within the 5’ promoter (70). Furthermore, it was found that heat shock and hypoxia induce the HSP70 gene through the same regulatory HSTF–HSE complex. However, only one of two functional HSEs is required for heat shock inducibility, whereas both are needed for hypoxic induction (71). The activation of HSTF is highly dependent on cellular redox potential. Thioredoxin-reducing agents inhibit HSTF activity and the expression of HSP genes in HeLa cells challenged by heat or other stresses (72), which is consistent with a report that the antioxidant N-acetyl-L-cysteine decreases the induction of HSP32 mRNA and protein in rat cardiomyocytes exposed to hypoxia (64).
Cell Metabolism

O₂ is a substrate for ATP generation and a modulator of the oxidation-reduction potential of cell culture media. Thus, pO₂ is a major determinant of the metabolic profile of a cell, affecting both nutrient consumption (oxygen, glucose, and glutamine) and waste production (lactate and ammonia) (73). Steady-state metabolic rates, including qO₂, are not greatly affected by pO₂ between 10% and 100% DO (15–150 mm Hg). pO₂ dependence above 10 mm Hg is reflected primarily by changes in the oxidative phosphorylation ratios: cytoplasmic [ATP]/[ADP][Pi] and intramitochondrial [NAD⁺][NADH] (74). As pO₂ and the respiration rate decrease, the rate of ATP synthesis declines. The resulting excess in ATP consumption over synthesis decreases the [ATP]/[ADP][Pi] ratio, which stimulates respiration (74). If pO₂ is held steady at the lower value, the [ATP]/[ADP][Pi] ratio will decline until a new steady state is attained. The rapid turnover of ATP allows these changes to occur within a few seconds and, hence, respiration appears unaffected by changes in pO₂. Moreover, a decrease in pO₂ may also lower the [NAD⁺][NADH] ratio, which also enhances respiration (74). However, below a pO₂ of about 10 mm Hg, changes in the [ATP]/[ADP][Pi] and [NAD⁺][NADH] ratios are not sufficient to maintain baseline respiration rates (74), and steady-state qO₂ decreases with pO₂ in a Michaelis–Menten-type manner with K₉₀ < 1 mm Hg for hybridomas (6,13). Prolonged exposure to severe hypoxia (80% decrease in qO₂) or anoxia has been shown to suppress CHO-K1 cell O₂ uptake, as evidenced by a transiently (~24 h) lower value of qO₂ at the same pO₂ value during reoxygenation (41).

To compensate for reduced aerobic metabolism during O₂ deprivation, the flux through glycolysis increases, with most of the glucose being converted to lactate. This phenomenon, referred to as the Pasteur effect, has been observed by many investigators (5,9,10,13,73,75). Glutamine metabolism is also O₂ dependent. Glutamine is an essential nitrogen and carbon source for the biosynthesis of metabolic intermediates and for energy production via the TCA cycle; ammonia and alanine are its major nitrogen-containing by-products. When excess O₂ is available, cultured cells typically obtain most of their metabolic energy from glutamine oxidation (13). In contrast, when O₂ is limiting, cells favor glucose over glutamine for energy production. Because glutamine metabolism is diverted from providing metabolic intermediates to providing energy at low pO₂, glutamine is then recruited to a greater extent for cell mass production. This is illustrated by an increase in glutamine utilization and a diminishing yield of NADH from glutamine at very low DO (6,13). The greater glutamine consumption rate also makes more nitrogen available for biosynthesis, which is consistent with higher specific production rates for ammonia and alanine (13,73). Similarly, Ozturk and Palsson (13) reported a large increase in the specific consumption rate of most amino acids under anoxic conditions in continuous hybridoma culture.

Interestingly, the metabolic alterations observed under hypoxic conditions may resemble those occurring under hypoxia. A shift to anaerobic metabolism has been reported in hyperoxic cultures of CHO cells (76), hybridomas (17), WI-38 cells (9), and human lymphocytes (77). Hypoxia has also been reported to increase the glutamine consumption rate (17). At first thought, it is counterintuitive that there would be an increasing reliance on glycolysis at high DO. However, the increased glucose consumption rate may result from O₂ toxicity. Enhanced glycolysis may compensate for impaired mitochondrial function under hypoxia, as well as provide additional energy to prevent and repair oxidative damage.

Although an increase in anaerobic metabolism to make up for energy deficits is useful for surviving O₂ limitation, the greatest defense against hypoxia would be a suppression of energy demand. This strategy has been perfected by cells from the highly anoxia-tolerant aquatic turtle. In response to anoxia, ATP demand is decreased by 90% in aquatic turtle hepatocytes and by 50% in brain cortical cells (78).

Protein Synthesis

Protein turnover accounts for a large fraction of the ATP demand during normoxia (78). Therefore, inhibition of protein synthesis and degradation under O₂-limiting conditions would conserve energy and contribute to maintaining the ATP supply-demand balance. Indeed, an important feature and one of the first signs of hypoxia in many cell types is a rapid and dramatic decline in overall protein biosynthesis (78–81), which has been attributed to a block in translation (78,80). Protein synthesis rapidly recovers to control levels on reoxygenation. Protein degradation is increased by ~30% under hypoxia in NHIK 3025 cervical carcinoma cells (79), but is decreased by 94% in aquatic turtle hepatocytes (78). The rapid reduction of protein synthesis that occurs when O₂ is limited does not appear to be in response to blockage of the electron transport chain or to the associated change in redox state, but rather has been attributed to the presence of an O₂ sensor that senses the lack of molecular O₂ or O₂ by-products under hypoxic conditions.

Most reports on the effects of DO on protein production by cultured cells have been limited to antibody production by hybridomas and lymphocytes. The effects of pO₂ on the specific antibody production rate (qAb) are cell line specific, and the optimal DO level for cell growth is often different than that for antibody production. Over the range from 1% to 100% DO, qAb may be greater at low DO (7,75), greater at intermediate DO (6), or largely unaffected by DO (13,17,82,83). qAb may be decreased below 1% DO (6,84). However, other investigators have reported no change in qAb from anoxic exposure in continuous or perfusion culture (13,85). It must be emphasized that these conclusions are limited to the conditions tested. For example, although qAb for the CC9C10 hybridoma cell line was similar for 10–100% DO, qAb was 50% greater at 125% DO (17). Also, cells grown in serum-free media (SFM) appear to be more sensitive to low DO. Three hybridoma cell lines cultured in SFM exhibited a 50–85% lower qAb for DO = 7.5% while the same cell lines grown with serum showed no decrease in qAb even at 3% DO (82).

Much less has been reported regarding the effects of pO₂ or hypoxia on recombinant protein production. The limited
data available suggest that $q_p$ is generally greater at higher DO, but the DO range over which the transition occurs is cell line dependent. Chotigueat et al. reported an increase in steady-state follicle-stimulating hormone (FSH) production with DO by CHO cells in perfused microcarrier culture; $q_{FSH}$ was threefold greater at 60–90% DO than at 10% DO (86). Similarly, Wang et al. reported 50–240% greater $q_{EPO}$ by L-929 cells at 75% DO in batch microcarrier culture as compared to that at 7.5–30% DO, with a larger increase in $q_{EPO}$ at 75% DO for cells cultured in SFM (87). In contrast, $q_{TGF-β}$ by BHK cells was unchanged at 7.5–75% DO in batch microcarrier culture and then increased with DO up to 225–300% DO for cells in serum-containing and serum-free media, respectively. At the other end of the DO spectrum, Lin et al. examined the effects of O2 deprivation on tPA production by CHO cells in perfused microcarrier culture (5). $q_{TGF-β}$ did not decrease from that at 33% DO during exposure to mild hypoxia (40–60% decrease in $q_{TGF-β}$), but was affected by more severe O2 deprivation. $q_{TGF-β}$ decreased by 80% during the first day of severe hypoxia (80% decrease in $q_{TGF-β}$), and then recovered to control levels during the next 2 days of severe hypoxia. Upon reoxygenation, $q_{TGF-β}$ transiently (3 days) increased to twice the control value. During anoxic exposure, $q_{TGF-β}$ gradually decreased by 80% over 2 days and then remained at that value even for 2 days after reoxygenation (5).

The attraction in using mammalian cells for recombinant protein expression lies with their ability to carry out complex posttranslational processing, which is often an important determinant of a protein’s activity in vivo. Few studies have addressed the effect of O2 or hypoxia on protein glycosylation. Regoeczi et al. (88) examined the in vivo glycosylation of serum proteins obtained from rats placed in a hypobaric chamber at 380 mm Hg (80 mm Hg pO2). The only significant difference in transferrin glycosylation was a greater proportion of molecules fucosylated (fucosylation index) in hypoxic vs. control animals (28% vs. 24%; $p = .02$). In contrast, the IgG fucosylation index was lower in hypoxic animals (10% vs. 20%; $p = .012$). Chotigueat et al. (86) examined the effects of DO on the isoelectric focusing (IEF) gel profile of FSH produced by CHO cells in perfused microcarrier culture. FSH acidity increased with DO, as evidenced by an increase in the fraction of FSH isoforms with $pI \leq 4.5$ from 65% at 10% DO to 88% at 90% DO. The shift of FSH isoforms to lower $pI$ was attributed to more extensive sialylation at higher DO values, which is consistent with a fivefold greater sialyltransferase activity in cells at 90% DO vs. that at 10% DO. Lin et al. (5) examined the specific activity and gel electrophoresis profiles of tPA produced by slowly growing CHO cells in perfused microcarrier culture. Exposure to hypoxia or anoxia and subsequent reoxygenation did not significantly affect tPA specific activity or site occupancy (fraction of tPA molecules glycosylated at three sites (68 kDa) vs. two sites (65 kDa)). However, during reoxygenation from anoxia, an extra tPA band appeared at 70 kDa. This band, which may have resulted from disrupted signal peptide cleavage or altered glycosylation, disappeared after 3 days at 33% DO.

**Growth Factors and Their Receptors**

The effects of O2 on hematopoietic cell growth and differentiation may be caused by differences in the production of growth factors or their receptors with pO2. Oxygen-regulated production of the cytokine erythropoietin (EPO) and of several angiogenic growth factors has been well established. There is evidence for specific effects of pO2 on individual cell types. For example, macrophages produce cytokines in an O2-dependent manner (89), while lymphocytes and macrophages that are cocultured under 5% O2 cooperate to produce a factor that stimulates proliferation of erythroid precursor cells (90). There is also evidence that cord blood mononuclear cells produce more interleukin 6 (IL-6) under 5% compared to 20% O2 (91).

Changes in growth factor receptor expression in response to low pO2 may exhibit different patterns than those for the respective ligands. For example, while low pO2 upregulates the transcription and synthesis of transforming growth factor β1 (TGF-β1) in dermal fibroblasts, culture under 2% O2 for 24 h decreases receptor binding of [125I]TGF-β by 65% and TGF-β type II receptor mRNA levels by 90% (92). Fibroblasts cultured under 2% O2 also showed decreased receptor binding of [125I]EGF (epidermal growth factor) that was reversible after 12 h in culture under 20% O2 (92). For both receptors, decreased ligand binding was in large part the result of a lower number of binding sites. Exposure to hypoxia has also been shown to reversibly decrease [125I]EGF binding to and the number of available EGF receptors (EGFR) on hepatocytes, without affecting total EGFR protein content (93). Interestingly, there was no effect of hypoxia on [125I]insulin binding.

**MOLECULAR MECHANISMS MEDIATING OXYGEN EFFECTS**

The metabolic and cellular changes that accompany hypoxia are primarily the result of differential gene expression. The mechanisms underlying these gene-based responses involve hypoxia sensing, signal transduction, and ultimately expression of the appropriate gene.

**Oxygen Sensing**

Despite the advances being made toward identifying O2-regulated cellular functions, the nature of the O2 sensor itself and the path of the signal from the sensor to the machinery that controls protein expression and function remain unclear. Among the molecules that can bind to and react with molecular O2 are heme proteins. By analogy to hemoglobin, heme proteins are thought to change conformational state between oxygenated and deoxygenated forms, thus regulating their function depending on pO2. Involvement of a ferroheme protein in the O2-sensing process, originally suggested by Goldberg et al. (94), is supported by several lines of evidence. Although much of the insight into mechanisms of O2-sensing stems from studies
on EPO-producing hepatocytes and on carotid body type I cells, it seems that a heme-based O2 sensor system is present in most cell types (95).

The response to hypoxia can be mimicked, both in magnitude and direction, by exposure to cobaltous ions or iron-chelating agents (96) or blocked by carbon monoxide, hallmarks of the involvement of a heme-containing protein. Expression of hypoxia-inducible genes (Table 1) is generally enhanced in cells treated with desferrioxamine (DF), an iron chelator. Iron chelators prevent synthesis of functional heme proteins by inhibiting the incorporation of ferrous atoms into the heme molecule. Cells with a nonfunctioning O2 sensor are unable to detect O2 present in the environment and are likely to interpret their state as hypoxic. Furthermore, because iron catalyzes the conversion of H2O2 to OH• and OH− via the Fenton reaction, iron chelators may significantly alter levels of chemical messengers in the O2-sensing pathway and lead to an hypoxic-like response (97). The heme protein hypothesis is further substantiated by experiments in which cobalt and nickel substitute for iron in the heme moiety. These transition metals exhibit a characteristic low binding affinity for O2, thereby locking the heme protein in the deoxy conformation and sending an hypoxic signal. All the hypoxia-induced genes examined have been shown to be stimulated under such conditions (Table 1), while hypoxia-inhibited genes are also inhibited by Co2+ and DF. Conversely, the effects of hypoxia can be abrogated by using carbon monoxide (CO), which substitutes for O2, to lock heme in the oxy conformation (94). Studies on expression of the phosphoenolpyruvate carboxykinase (PCK) gene in primary rat hepatocytes (139) further confirm the CO–O2 sensor relationship, although in this case it acts conversely: PCK gene expression is inhibited under hypoxia, but is stimulated under elevated pO2 or in the presence of CO (Table 1). Parallel effects of CO on O2-sensing are evident in the carotid body, where ventilation is regulated (142). The current through K+ channels in type I cells is rapidly downregulated by lowering pO2. Addition of CO to hypoxic type I cells reverses the downregulation of K+ currents. Collectively, these data implicate a heme protein that changes conformation on binding O2 or CO as the primary O2 sensor.

It is likely that the O2 sensor is a membrane-bound, multisubunit cytochrome b protein that binds O2 and reduces it to ROS such as superoxide and H2O2, which then in turn leads to downstream signaling of gene transcription. Numerous findings have contributed to this hypothesis. Absorption spectra of hepatoma cells and carotid body cells coincide with those of type b-like cytochromes that undergo a shift in conformation in response to changing pO2 (143,144). Among this group of b-cytochromes are those that are part of the NADPH–oxidase enzyme complex, which generates H2O2 in an O2-dependent manner. During hypoxia, the H2O2-generating activity of the oxidase would be inhibited, leading to lower H2O2 levels (4). In confirmation, the rate of H2O2 production by hepatoma cells decreased as pO2 decreased (145). A regulatory role for H2O2 in the coupling of environmental pO2 to intracellular events has been demonstrated: adding exogenous H2O2 to hypoxic cultures negated the expected increase in EPO production, while adding antioxidants or catalase to hypoxia can be abrogated by using carbon monoxide (CO), which substitutes for O2, to lock heme in the oxy conformation and sending an hypoxic signal. All the hypoxia-induced genes examined have been shown to be stimulated under such conditions (Table 1), while hypoxia-inhibited genes are also inhibited by Co2+ and DF. Conversely, the effects of hypoxia can be abrogated by using carbon monoxide (CO), which substitutes for O2, to lock heme in the oxy conformation (94). Studies on expression of the phosphoenolpyruvate carboxykinase (PCK) gene in primary rat hepatocytes (139) further confirm the CO–O2 sensor relationship, although in this case it acts conversely: PCK gene expression is inhibited under hypoxia, but is stimulated under elevated pO2 or in the presence of CO (Table 1). Parallel effects of CO on O2-sensing are evident in the carotid body, where ventilation is regulated (142). The current through K+ channels in type I cells is rapidly downregulated by lowering pO2. Addition of CO to hypoxic type I cells reverses the downregulation of K+ currents. Collectively, these data implicate a heme protein that changes conformation on binding O2 or CO as the primary O2 sensor.

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O2-sensing also appears to involve the regulation of nucleotide cyclases, heme-containing proteins that catalyze the conversion of intracellular ATP/GTP to cyclic AMP/GMP (148). Hypoxia decreases cAMP and cGMP levels, which in turn may alter stimulation of protein phosphorylation pathways. It is not known whether these nucleotide cyclases actually sense extracellular pO2 levels, similar to the mechanism proposed for oxidases, or if they are regulated by a signal generated further downstream from the O2 sensor. For example, H2O2 can affect activation of guanylate cyclase and alter cGMP levels (4). In this way, during hypoxia, lower concentrations of H2O2 could contribute to modifying the phosphorylation status of certain proteins.

Contrary to these observations, it is also possible that heme proteins may not be involved in the hypoxic regulation of certain genes, such as endothelial GAPDH (69). If a heme protein is involved, it possesses different structural or functional properties from those involved in other hypoxia-responsive systems as regulation of endothelial GAPDH (e.g., by CO) differs from that of EPO or VEGF (see Table 1).

**Signal Transduction**

**Protein Phosphorylation.** A decrease in H2O2 concentration could transduce the hypoxic signal through several different pathways, most likely involving protein phosphorylation and redox potential. Protein phosphorylation is an important intracellular transduction mechanism because of its regulation of several transcription factors. A heme protein in the bacterium Rhizobium melliloti senses hypoxia and activates its kinase domain, leading to phosphorylation of transcription factors and subsequent gene expression (149). In eukaryotes, NADPH oxidase, the putative O2 sensor that operates in some cell types, becomes phosphorylated on activation (150), suggesting that the dependence of O2-sensing on a phosphorylation cascade is conserved between bacteria and mammals. Tyrosine phosphorylation has been implicated in the hypoxic activation of NF-κB DNA-binding activity (151) and in the transcriptional and posttranscriptional induction of the VEGF gene (152,153). The DNA-binding activity of hypoxia-inducible factor-1 (HIF-1; see next section) is also dependent on its phosphorylation status. Treatment with phosphatase or with kinase inhibitors prevented hypoxia-inducible binding and EPO gene expression, demonstrating the requirement for HIF-1 to be in a phosphorylated form (154).
Table 1. Oxygen-Regulated Gene Expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>Hypoxia</th>
<th>DF</th>
<th>CO²⁻</th>
<th>CO reversal¹</th>
<th>Regulation</th>
<th>References</th>
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<tbody>
<tr>
<td>Erythropoietin (EPO)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Y</td>
<td>HIF-1</td>
<td>94,97–102</td>
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<tr>
<td>Tyrosine hydroxylase (TH)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>HIF-1</td>
<td>103–105</td>
</tr>
<tr>
<td>Transferrin (TF)</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>HIF-1</td>
<td>106</td>
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<tr>
<td>Inducible nitic oxide synthase</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>HIF-1</td>
<td>107</td>
</tr>
<tr>
<td>Heme oxygenase 1</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>HIF-1</td>
<td>108</td>
</tr>
<tr>
<td>Xanthine oxidase I</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>109</td>
</tr>
<tr>
<td>Retrotranspon VL30</td>
<td>+</td>
<td></td>
<td></td>
<td>HIF-1</td>
<td></td>
<td>110</td>
</tr>
<tr>
<td>Erythropoietin inducible factor-1α</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>111</td>
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<tr>
<td>Erythropoietin inducible factor-1β</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
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<td>111</td>
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<tr>
<td>Interleukin 2 (IL-2)</td>
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<td></td>
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<tr>
<td>Interleukin 4 (IL-4)</td>
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<tr>
<td>Interferon-γ (IFN-γ)</td>
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<tr>
<td>Ornithine decarboxylase</td>
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<td>Interleukin 10 (IL-10)</td>
<td>-</td>
<td></td>
<td></td>
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<td>112</td>
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<tr>
<td>Vascular endothelial growth factor</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Y</td>
<td>HIF-1</td>
<td>96,114–118</td>
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<tr>
<td>Platelet-derived growth factor-β</td>
<td>+</td>
<td>+</td>
<td></td>
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<td>Transforming growth factor-β (TGF-β)</td>
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<td></td>
<td></td>
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<td>Acidic/basic fibroblast growth factor (a/bFGF)</td>
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<tr>
<td>Tumor necrosis factor-α (TNF-α)</td>
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<td></td>
<td></td>
<td>NF-IL-6 (P)</td>
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<tr>
<td>Endothelin</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>123</td>
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<tr>
<td>Interleukin 6 (IL-6)</td>
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<td></td>
<td></td>
<td></td>
<td>NF-IL-6</td>
<td>112,124,125</td>
</tr>
<tr>
<td>Interleukin 1 (IL-1)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>126</td>
</tr>
<tr>
<td>Interleukin 8 (IL-8)</td>
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<td></td>
<td></td>
<td></td>
<td>NF-IL-6 (P)</td>
<td>124,127</td>
</tr>
<tr>
<td>Placental growth factor (PLGF)</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td></td>
<td>96</td>
</tr>
<tr>
<td>Glucose transporters</td>
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<td></td>
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<tr>
<td>GLUT-1</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Y</td>
<td>HIF-1</td>
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<td>GLUT-3</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>129</td>
</tr>
</tbody>
</table>

| Metabolic Enzymes                   |         |    |      |              |                  |            |
| Lactate dehydrogenase-A (LDH-A)     | +       | +  | +    |              | HIF-1            | 129,132–135|
| Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | +       | +  | +    | N            | HIF-1            | 69         |
| Aldolase A                          | +       | +  |      |              | HIF-1            | 129,135,136|
| Enolase 1                           | +       |    |      |              | HIF-1            | 135,136    |
| Phosphoglycerate kinase 1 (PGK-1)   | +       | +  |      |              | HIF-1            | 132,136    |
| Phosphofructokinase L (PFK-L)       | +       | +  |      |              | HIF-1            | 132,136    |
| Pyruvate kinase                     | +       |    |      |              |                  | 136,137    |
| Hexokinase                          | +       |    |      |              |                  | 137         |
| Triose phosphate isomerase          | +       |    |      |              |                  | 138         |
| Phosphoenolpyruvate carboxykinase   | -       | -  |      | Y            |                  | 139,140    |
| Tyrosine aminotransferase           | -       |    |      |              |                  | 141         |

Note: DF, desferrioxamine; +, induced gene expression; −, suppressed gene expression; Y, yes; N, no; P, probable, but not proven; HIF-1, hypoxia-inducible factor-1-binding motif; NF-IL-6, nuclear factor–interleukin-6-binding motif.

¹Reversal of hypoxic effects by carbon monoxide (CO).

**Redox Potential.** Although hypoxia-mediated enhancement of DNA-binding activity of several transcription factors could reflect changes in the phosphorylation state of these proteins, it could also reflect changes in the cellular redox state. Cellular redox potential is governed in part by the ratio of reduced (SH) to oxidized (S–S) thiol groups that results from the surrounding pO₂. Under normoxic conditions, the equilibrium is maintained by H₂O₂ either through direct oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) or through formation of the hydroxyl radical, which can oxidize glutathione and protein thiol groups (4). Under hypoxic conditions, the de-
increasing H₂O₂ concentration is accompanied by a shift in the equilibrium toward the reduced forms of glutathione and protein thiols. This shift in redox state can regulate the activity of K⁺ channels and the DNA-binding activity of transcription factors (4).

Several studies have coupled changes in transcription factor activity with perturbations in the redox state of the cell. Transcription factors AP-1, NF-κB, and p53, as well as protein factors such as heat shock proteins (HSPs), are redox sensitive. An ubiquitous nuclear protein called Ref-1 serves as a redox factor that stimulates AP-1 DNA-binding activity through single conserved cysteine residues in the c-Fos and c-jun proteins that comprise the AP-1 transcription complex (155). AP-1 activity is enhanced by antioxidants and reducing agents (155,156), whereas it is inhibited by H₂O₂ (157) and sulfhydryl blockers (155). Furthermore, data suggest that the c-fos and c-jun family of genes are regulated at the transcriptional level as well, with exposure to hypoxia inducing mRNA expression (114). However, in these latter studies AP-1 activity was not assessed. In contrast to AP-1, NF-κB activity is induced by oxidative stress: H₂O₂ can activate NF-κB, while treatment with thiol reagents or other antioxidants abolishes its activity (156,158). A conflicting report has shown enhanced NF-κB activity under hypoxic conditions (151). Like AP-1, it seems that NF-κB also requires reduction of specific cysteine residues in its DNA-binding domain, since its association with DNA in vitro is inhibited by alkylating or oxidizing agents (159). However, there is currently no evidence linking modification of NF-κB thiol groups to regulation of its biological activity. This fact demonstrates the complex nature of regulation of cellular signaling through redox chemistry.

The functions of HSPs are multifold: they protect cells from stress from heat, ROS, and sulfhydryl reagents because they aid protein folding, processing, and transport. Expression of HSPs is dependent on binding of heat shock transcription factors (HFs) to their promoter region. The relevance of the cellular redox state in the heat shock response is demonstrated by the inhibition of HSF activity and HSP gene expression by thiol reducing agents (72). The induction of HSP expression with reoxygenation is consistent with these findings (61).

The role of redox status on the basic helix-loop-helix (bHLH) group of proteins, a broad group of transcription factors that share common helix-loop-helix regions required for dimerization, is also of considerable interest. Binding and activation of USF, a bHLH transcription factor, proceeds only when two cysteine residues in the HLH dimer interface are reduced (160). This mechanism also implicates redox regulation in the assembly of other transcription factors, in particular of the activation of HIF-1, a heterodimer consisting of two bHLH transcription factors, the HIF-1α and HIF-1β subunits (111). Of the factors mentioned previously, HIF-1 is probably the most relevant to low-pO₂ studies because it is an ubiquitous transcription factor (see Table 1) whose function is restricted to the hypoxic regulation of a broad range of physiologically relevant genes (161–163).

HIF-1 DNA-binding activity and the protein levels of HIF-1α and HIF-1β each increase exponentially as cells are exposed to decreasing pO₂ in vitro within the physiological range. A half-maximal response was seen between 1.5% and 2% O₂ in the gas phase, with a maximal response at 0.5% O₂ (124). Maintaining the proper redox ratio is essential for the hypoxic activation of HIF-1. Changing the redox state of cells through treatment with oxidizing agents impairs hypoxia signaling mechanisms by disrupting expression of HIF-1α and HIF-1 DNA-binding activity (164). HIF-1 must remain in the reduced state to enable binding to DNA. No cysteine residues were found in the basic DNA-binding domain of either HIF-1α or HIF-1β. However, one cysteine was found in the HLH region of HIF-1β and several in the PAS (Per-ARNT-Sim; named for the domain found in the Drosophila transcription factors Period and Single-minded and the mammalian aryl hydrocarbon receptor nuclear translocator protein) domain of each subunit (164). Interaction between the HLH and PAS domains is necessary for heterodimerization of HIF-1α and HIF-1β and for subsequent DNA binding. The redox-sensitive activation of HIF-1 has since been attributed primarily to the HIF-1α subunit (165).

Another factor that has been shown to activate transcription of specific target genes in hypoxic cells is C/EBPβ. C/EBPβ shows a structural feature akin to the AP-1 complex with a cysteine residue similarly positioned as in c-Fos and c-jun that is critical for DNA-binding activity (124,155). C/EBPβ protein binds to an hypoxia-inducible element called the NF-IL-6 site in a tissue-specific manner (124,125). Although this site is distinct from the HIF-1-binding motif, the cellular processes leading to gene transcription may still be related. Given the analogy between the actions of HIF-1 and C/EBPβ and the implication that redox potential plays a role in both HIF-1α- and C/EBPβ-mediated hypoxia-inducing mechanisms, the NF-IL-6 site becomes another important locus for induced gene expression under O₂ deprivation. The presence of tissue-specific factors regulates whether transcription can be driven by HIF-1α or C/EBPβ.

In addition to increased transcription rates, gene expression during hypoxia is also regulated by the stability of mRNA. Redox may also contribute to this posttranscriptional mode of gene regulation. Czyzyk-Krzeska (4) reported that binding of hypoxia-inducible protein factors to tyrosine hydroxylase mRNA stability elements requires that they be in the reduced state.

Thus, posttranslational signaling mechanisms that involve both phosphorylation and sulfhydryl group modifications contribute to the transcriptional and posttranscriptional response to hypoxia. HIF-1 activation best exemplifies these modes of regulation. In addition, activation of HIF-1 may depend on ligand binding. Association of HSP90 with HIF-1α allows this subunit to change configuration, thereby enabling dimerization with HIF-1β and subsequent DNA binding of the HIF complex (166). Considering this information, it now becomes logical to extend the previous hypothesis suggesting that the signaling cascade begins with a heme protein O₂ sensor and is propagated by an intermediate, such as H₂O₂. Intermediate molecules are likely to target certain transcription factors, such as HIF-1, and regulate their binding to the respective genes.
Gene Expression

Transcriptional Regulation. The identification of the aforementioned transcription factors has facilitated our understanding of O₂ regulation of genes and consequently advanced our understanding of the physiological adaptation to hypoxia. Despite a significant reduction in total mRNA synthesis when cells are deprived of O₂, transcription of several mammalian genes that encode for various hormones, cytokines, and enzymes is markedly increased. In particular, low pO₂ induces expression of genes controlling erythropoiesis, ventilation, angiogenesis, and energy metabolism. Furthermore, the commonality among the regulatory elements present in these genes suggest that they are part of a widespread O₂-sensing system. Although beyond the scope of this article, cellular adaptation to the other extreme, hyperoxia, also involves the upregulation of certain genes which, in this case, lead to detoxification of ROS (36).

EPO. Progress in understanding O₂-dependent gene expression has mostly stemmed from characterizing hypoxia-induced production of erythropoietin (EPO) because the effects are more dramatic for this cytokine than for the other O₂-sensitive genes studied thus far. EPO is the key hormone in erythropoiesis: synthesized primarily in the adult kidney and fetal liver, it travels to hematopoietic tissues where it binds to its receptor on erythroid progenitor cells, protecting them from apoptosis and allowing them to proliferate and differentiate into mature red blood cells. Under the hypoxic stress of anemia or reduced blood oxygenation, EPO production is as much as 1000-fold greater than normoxic levels (167). By increasing the number of red blood cells, EPO enhances the O₂-carrying capacity of the blood. Studies on the transcriptional control of the EPO gene have been greatly facilitated by the use of the cultured human fetal hepatoma cells Hep3B and HepG2 (168). Increased EPO production under hypoxia in these model systems mirrors the in vivo response. There is a close correlation between the level of hypoxia and the production rates of EPO protein and EPO mRNA in these cells.

Regulation of transcription rate involves interactions between trans-acting elements (transcription factors) and cis-acting elements within the promoters and enhancers of regulated genes. The best-studied mechanism of hypoxic regulation is the binding of HIF-1 to DNA, and is best exemplified by regulation of the EPO gene. The cis element most critical to hypoxic induction of EPO transcription is located in the 3' flanking region of the gene, 120 bp downstream from the polyadenylation site. The 3' enhancer includes three sequences that contribute to the hypoxic response: (1) a 5' portion containing the highly conserved HIF-1-binding site, (2) the middle portion, which does not bind specific proteins but is necessary for the hypoxic induction of transcription, and (3) a 3' portion containing highly conserved nuclear receptor-binding half-sites that resemble known steroid and thyroid hormone response elements. A variety of hormones act by binding to nuclear receptors. In particular, the binding of a specific nuclear receptor, orphan nuclear factor 4, to the EPO enhancer amplifies the transcriptional activation by HIF-1, demonstrates a marked impact on the hypoxic induction of the EPO gene and contributes to tissue specificity of this response (169).

The importance of the 3' enhancer had previously misled some to believe that it was solely responsible for hypoxic induction of the EPO gene. However, transfection studies with the enhancer revealed that mRNA synthesis of a reporter gene was only induced about 10-fold, which is well below the induced level of endogenous EPO mRNA (170). This discrepancy was resolved through identification of O₂-sensitive elements within the promoter region, 5' to the EPO gene (171). The 3' enhancer and 5' promoter act synergistically to achieve at least a 40-fold stimulation of transcription of the EPO gene under hypoxia, commensurate with endogenous EPO mRNA levels. Similar to the enhancer region, the EPO promoter contains nuclear hormone response half-sites. Alone, they do not contribute to hypoxic induction, but they are needed to amplify the induction signal. It has also been proposed that the EPO gene may be negatively regulated by higher pO₂. Other elements in the EPO promoter including GATA sites and ribonucleoprotein-binding sites may contribute to the inhibition of a constitutive activator protein and lead to repression of transcription under normoxia (167). During hypoxia, when the negative regulators are reduced, they could be displaced from their corresponding DNA-binding sites and allow transcription to proceed. This finding is consistent with studies that suggest that under high pO₂ the O₂ sensor generates greater amounts of ROS that lead to the inhibition of EPO gene expression (146).

More recently, other transcription factors have been implicated in transducing the signal from the HIF-1 site to the transcriptional initiator. The non-DNA-binding transcriptional activator P300/CBP binds to HIF-1α and enhances hypoxic induction by increasing the response of the EPO enhancer (172). P300/CBP is a multifunctional protein that, in addition to regulating several tissue-specific enhancers, plays a role in controlling the cell cycle and differentiation pathways. The HIF-1-binding site within the EPO enhancer also binds constitutively to a smaller protein complex consisting of the activating transcription factor-1 (ATF-1) and the cAMP-responsive element-1 (CREB-1) (173). During hypoxia, these two transcription factors are displaced by HIF-1. Thus, HIF-1-dependent induction of gene expression involves interactions between different transcription factors and sequences. It is likely that these interactions direct the specificity of the hypoxic response, that is, which genes or cells are affected.

Several findings have led to the conclusion that HIF-1-mediated induction of gene expression is not restricted to the EPO gene or to cells originating from the kidney or liver. Transfection experiments in many different cell lines using the EPO 3' enhancer coupled to reporter genes have demonstrated the universality of this O₂-sensing and signal transduction mechanism (174). Factors with similar affinity for specific DNA sequences within the enhancer were activated regardless of whether or not the cells were EPO producing (98,175). Furthermore, mutated EPO enhancers, which are known to prevent HIF-1 binding, abolished hypoxic induction of mRNA synthesis of the reporter genes. O₂ regulation of other genes in an HIF-1-dependent
fashion is confirmed by the observation that the key regulatory sites for many other O2-regulated genes contain the HIF-1-binding consensus sequence (see Table 1) with the core motif CGTG (161). Among those to be discussed next are the genes encoding TH, VEGF, GLUT-1, and several glycolytic enzymes.

**TH.** The most immediate response to hypoxia at the whole-body level is the regulation of ventilation. The sensitivity of type I cells in the carotid body to a decrease in pO2 is greater than in other O2-sensitive cells: inhibition of conductance through K channels, membrane depolarization, increase in intracellular calcium, and release of neurotransmitters occur rapidly. Neurotransmitters convey the hypoxic signal to the sinu nerve. Hypoxia stimulates the protein activity, as well as the fast induction of gene expression, of tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of the neurotransmitter dopamine. The molecular mechanisms by which hypoxia induces increased TH gene expression have mostly been explored in PC-12 (pheochromocytoma cell line) cells, which exhibit similar O2-dependent characteristics as the scarce type I primary cells (104). A decrease of O2 levels (to 15% O2 in the gas phase) produces an hypoxic effect. When PC-12 cells are exposed to 10% O2, TH mRNA levels reach a maximum within 6 h and are sustained at a high level for up to 53 h thereafter (4). The initial increase in TH mRNA is primarily attributed to a rapid induction of transcription, whereas maintenance of TH mRNA during long-term exposure to hypoxia involves mechanisms that enhance mRNA stability (see later section).

The cis elements required for hypoxic induction of TH are located in a promoter region containing HIF-1, AP-1, and AP-2 sites (105). As for the EPO gene, binding of the HIF-1 complex to its site is required for hypoxic induction, but other interactions are also involved. Binding of c-Fos/c-jun heterodimers to the AP-1 site within the TH promoter was shown to increase in hypoxic cells (105). Mutation of the AP-1 site, which prevented binding of these transcription factors, inhibited hypoxic induction. Another similarity to regulation of the EPO gene is the possibility that nuclear receptors may also play a role in cooperation with HIF-1 for TH (176). Moreover, as with EPO mRNA, decreases in H2O2 levels caused by the addition of antioxidants or catalase induced TH mRNA expression. This suggests that H2O2 serves as an intermediate during the hypoxic regulation of both the TH and EPO genes.

**Angiogenic Growth Factors.** Angiogenesis is a fundamental process not only in development but also in wound healing, recovery from ischemic injury, and the pathogenesis of tumor growth. The common denominator among these processes is the hypoxic microenvironment in which they usually occur. The formation of new capillaries increases blood flow to the tissues, allowing delivery of an adequate supply of O2. Control of vascular functions and blood vessel proliferation under hypoxic conditions has been associated with a group of O2-regulated genes that encode several growth factors important for stimulating growth and migration to sites of ischemic injury (see Table 1). Some of these genes have been reported to be inducible by hypoxia share homologous sequences and similar regulatory mechanisms to those operating in the EPO and TH genes.

Of the angiogenic cytokines, VEGF, which is believed to be the central mediator of blood vessel formation, has garnered the most attention. VEGF is produced by many different cell types and tissues, and its expression is significantly amplified by hypoxia. Hypoxic induction of VEGF mRNA has been demonstrated both in vivo and in cultured cells (115). The preponderance of data suggest that VEGF regulation employs similar O2-sensing, signal transduction, and gene expression mechanisms as both EPO and TH (Table 1). Transfection experiments using reporter genes localized the cis-acting element containing the well-conserved HIF-1-binding site to a 28-bp sequence, 900 bp upstream of the transcriptional start site (116,177). Minchenko et al. (178) previously reported that two O2-responsive elements existed in the 5' and 3' regions flanking the VEGF gene. AP-1- and AP-2-binding sites were also identified within the VEGF promoter (114,177).

The C/EBPα/DNA-binding factor has been found to mediate IL-6 induction by hypoxia in endothelial cells and vascular smooth muscle cells (124,125). Transfection of IL-6 promoter-reporter gene constructs revealed the NF-IL-6-binding site as the hypoxia-inducible element, suggesting that a similar mechanism may account for hypoxic induction of other genes that contain NF-IL-6 sites, such as TNF-α and IL-8.

**Genes Involved in Energy Metabolism.** To maintain viability and function, cells have carefully choreographed strategies of nutrient utilization. During periods of O2 deprivation, when the O2-dependent tricarboxylic acid cycle and oxidative phosphorylation are impaired, cells adapt by generating ATP primarily through anaerobic glycolysis. Because the glycolytic pathway produces much less ATP than oxidative metabolism, the glycolytic capacity of the cells must be enhanced under hypoxic conditions. The rate of glycolysis can be increased by transporting more glucose into the cell, by upregulating the activity and synthesis of glycolytic enzymes, and by upregulating the expression of gluconeogenic enzymes that consume large amounts of ATP. As discussed next, hypoxia governs expression of the genes involved in all three modes of adaptation.

In the first mode, glucose uptake increases to compensate for lower ATP yields. The expression of glucose transporters has been found to increase in response to several environmental stimuli. Most notably, hypoxia induces insulin-independent glucose transporter 1 (GLUT-1) protein and mRNA both in vivo and in various cell lines (179). The induction of GLUT-1 contributes to sustained energy levels under hypoxic stress in muscle cells, endothelial cells, adipocytes, HeLa cells, and HT 1080 (fibrosarcoma) cells. Increases in glucose uptake and lactate production are consistent with increases in glucose transport (128,180). It is likely that hypoxic regulation of the GLUT-1 gene, whose enhancer contains two phorbol ester response elements and a CAMP response element, depends on synergy between multiple cis-acting elements and transcription factors (95). In addition to GLUT-1, its isoform GLUT-3 is also induced by hypoxia in the HepG2 hepatoma cell line, whereas GLUT-2 expression is inhibited (129). The isoform-specific regulation of glucose transport coin-
cides with the decrease in the apparent $K_m$ for glucose observed under hypoxia: GLUT-3, which demonstrates the most dramatic induction by hypoxia, also has the lowest apparent $K_m$ for glucose (128,180). Increased steady-state levels of GLUT-1 mRNA in response to hypoxia are not only the result of increased transcription; GLUT-1 is also subject to regulation through mechanisms that stabilize mRNA.

The remaining two modes by which $pO_2$ alters glucose metabolism involve the regulation of a number of glycolytic and gluconeogenic enzymes. As expected, the response to hypoxia is opposite in direction depending on the pathway with which the enzyme is associated (see Table 1). In hypoxic surroundings, increased expression of rate-limiting glycolytic enzymes would shift metabolism toward glycolysis. However, in a well-oxygenated environment in selected tissues, enhanced expression of PCK would favor gluconeogenesis.

In general, the three groups of genes—those for glucose transporters, glycolytic enzymes, and gluconeogenic enzymes—exhibit patterns of expression in response to hypoxia that are consistent with their response to transition metal ions (e.g., cobalt) or iron chelators (e.g., DF), as has been shown for other $O_2$-regulated genes (Table 1). Some of these genes possess cis-acting elements, which convey this response and cross-compete with the EPO enhancer for binding to HIF-1 (132). This suggests that hypoxic regulation of EPO and certain metabolic genes are linked by a similar $O_2$-sensing and signal transduction mechanism. Indeed, the HIF-1-binding consensus sequence has been found in several of these genes (Table 1).

Because induction of LDH-A activity by hypoxia is one of the greatest among the glycolytic enzymes, its hypoxia-inducible elements have been studied in detail. Expression of the mouse LDH-A gene in response to hypoxia is driven by cooperation among three domains, which are arranged similarly to those observed in the EPO 3’ enhancer. There is (1) an HIF-1-binding site, which is required but not sufficient for hypoxic induction of gene expression, (2) an adjacent sequence, which is located in an analogous position as a critical region in the EPO enhancer, and (3) a CAMP response element (CRE) (133). At a minimum, in transfection experiments the HIF-1 site must be accompanied by either of the other two domains to confer hypoxic inducibility. Furthermore, the CRE-binding protein is highly homologous to P300, which binds to HIF-1α (see earlier discussion). Despite the substantial induction of LDH-A mRNA by hypoxia, no effect on its isozyme LDH-B was observed (129). Therefore, the distinctive pattern of response to hypoxia by the genes involved in energy metabolism is both isozyme- and isozyme-specific, as in the cases of GLUT-1 (and GLUT-3) and LDH-A, respectively. Finally, hypoxic induction of LDH and GAPDH, which are used as a measure of viability and as an internal control for electrophoretic purposes, respectively, may lead to the misinterpretation of results collected in the laboratory if the differential effects of $pO_2$ on their expression are not considered.

Posttranscriptional Regulation. In addition to the mRNA transcription rate, the decay rate should not be overlooked; it, too, is an important contributing factor in increasing gene expression during hypoxia. A potential advantage associated with this bimodal regulation of gene expression is conservation of energy. Initially, hypoxia increases specific mRNA levels through transcriptional induction, but, during longer exposure times, mechanisms conferring mRNA stability may take over. This phenomenon is illustrated by the increase in TH mRNA stability during long-term hypoxia, which allows maintenance of TH mRNA levels without the energy demand that accompanies synthesis of new mRNA molecules (104). Studying posttranscriptional regulation by hypoxia is complicated by the fact that many inhibitors of transcription nonspecifically stabilize mRNA. For this reason, although hypoxic regulation of EPO mRNA stability is expected, it has yet to be confirmed. In contrast, using nonstabilizing transcription blockers and other techniques, hypoxic regulation at the posttranscriptional level has been demonstrated for VEGF and GLUT-1 mRNAs (152), in addition to TH mRNA (104).

Both stability and instability elements may be involved in the regulation of steady-state EPO, TH, VEGF, and GLUT-1 mRNA levels. These cis-acting sequences within mRNA could direct secondary structure, act as binding sites for trans-acting regulatory proteins, or serve as target sites of nucleases (4). The hypoxia-regulated TH mRNA stability element has been identified as a pyrimidine-rich tract in the 3’ untranslated region (UTR) termed the hypoxia-inducible protein-binding sequence (HIPBS). Oxygen regulation by protein binding to this site was confirmed first by mutations that prevent protein binding and consequently decrease TH mRNA half-life and second by the ability of HIPBS to confer stability on reporter mRNA. The HIPBS closely resembles sequences in the 3’ UTR of both EPO and VEGF mRNAs (153,181). Moreover, similar or the same protein factors as those that bind to HIPBS in TH mRNA also bind to the homologous sequences in EPO and VEGF mRNAs (4). The most common determinants of mRNA stability in mammalian cells are AU-rich elements (182). In addition to three HIPBS motifs, the 3’ UTR region of VEGF mRNA contains multiple AU-rich stabilizing and destabilizing elements that also appear to mediate stability by forming hypoxia-regulated complexes (111,155). Thus, in combination with transcriptional upregulation, the presence of $O_2$-regulated mRNA stability and instability elements could account for enhanced gene expression under hypoxic conditions.

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See also MASS TRANSFER.