

Señor Superintendente
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

E. S. D.
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Evento : 378
Actuación : 412

Referencia : Expediente No. 13.025.488
Asunto : Solicitud de Patente para: "MÉTODO PARA PRODUCIR EL FACTOR VON WILLEBRAND (VWF) DE ALTO PESO MOLECULAR RECOMBINANTE EN CULTIVO CELULAR"
Solicitante : BAXTER HEALTHCARE S.A.; BAXTER INTERNATIONAL INC.
Fecha de solicitud : 7 de Febrero de 2013.

I. RECURSO VÍA GUBERNATIVA

Yo, Luz Clemencia DE PÁEZ, en mi condición de apoderada de las sociedades **BAXTER HEALTHCARE S.A. y BAXTER INTERNATIONAL INC.** solicitantes de la patente de la referencia, por el presente escrito atentamente manifiesto a usted que debidamente notificada de la Resolución No. **605** del 15 de Enero de 2015, interpongo en su contra el recurso de **REPOSICIÓN**, para que sea revocada y en consecuencia se conceda el privilegio de patente de invención para "MÉTODO PARA PRODUCIR EL FACTOR VON WILLEBRAND (VWF) DE ALTO PESO MOLECULAR RECOMBINANTE EN CULTIVO CELULAR" a favor de **BAXTER HEALTHCARE S.A. y BAXTER INTERNATIONAL INC..**

II. OPORTUNIDAD DE LA IMPUGNACIÓN

La Resolución No. **605** del 15 de Enero de 2015, tiene fecha de publicación en listado 20 de Enero de 2015, fecha de vencimiento 20 de Febrero de 2015, y fecha del término legal 6 de Marzo de 2015.

"EXCELENCIA LEGAL EN UN MUNDO SIN FRONTERAS"

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III. LA DECISIÓN RECURRIDA

3.1. Mediante la Resolución objeto de la presente impugnación, el Superintendente de Industria y Comercio decidió:

3.1.1. Denegar la patente de invención a la creación titulada: **“MÉTODO PARA PRODUCIR EL FACTOR VON WILLEBRAND (VWF) DE ALTO PESO MOLECULAR RECOMBINANTE EN CULTIVO CELULAR”**.

3.2. En la parte motiva de la providencia, se mencionaron los siguientes fundamentos:

3.2.1. Las reivindicaciones 1 a 42 carecen de novedad sobre el documento D1: WO2009086309.

3.2.2. Lo anterior debido a que se considera que controlar las concentraciones de amonio en el sobrenadante ya había sido divulgado en el documento D1.

IV. OBJETO DEL RECURSO

De acuerdo con los argumentos que expongo a continuación, solicito atentamente a su Despacho lo siguiente:

4.1. Revocar la Resolución No. **605** del 15 de Enero de 2015.

4.2. Conceder el privilegio de patente de invención para **“MÉTODO PARA PRODUCIR EL FACTOR VON WILLEBRAND (VWF) DE ALTO PESO MOLECULAR RECOMBINANTE EN CULTIVO CELULAR”** a favor de **BAXTER HEALTHCARE S.A.** y **BAXTER INTERNATIONAL INC..**

V. FUNDAMENTOS DEL RECURSO

Respetuosamente me permito disentir de lo expuesto por ese Despacho en la Resolución objeto del presente recurso, toda vez que la presente solicitud sí cumple con los requerimientos de novedad y nivel inventivo exigidos en la Decisión 486, como se demostrará a continuación:

5.1. Presentación de un nuevo pliego reivindicatorio

De acuerdo con el artículo 34 de la Decisión 486 de la Comisión de la Comunidad Andina, donde se establece que la modificación de una solicitud es posible en cualquier momento del trámite mientras no implique una ampliación de la solicitud inicial, me permito presentar un nuevo pliego reivindicatorio conformado por 50 reivindicaciones.

El nuevo pliego reivindicatorio es esencialmente igual al anterior, pero se ha incluido la materia de la reivindicación 15 en la nueva reivindicación 1 y se ha especificado, en la misma reivindicación, que la concentración de NH_4^+ es mantenida durante al menos 7 días de cultivación continua.

Adicionalmente, se han añadido las nuevas reivindicaciones dependientes 42 a 50, las cuales se encuentran soportadas en los párrafos [0169] a [0176], [0187], [0196] y [0205]. Las reivindicaciones 42 a 46 restringen aún más el rango de concentraciones de amonio durante el proceso, mientras que las reivindicaciones 47 a 50 especifican la actividad del cofactor de risticetina específico del rVWF después de al menos 7, 14, 21, 28 días y 5 semanas de cultivación continua respectivamente.

5.2. Argumentos a favor de la novedad de la presente solicitud

En la tabla 1 de la resolución 605, que compara la presente invención con el documento D1, el Señor Examinador afirma que D1 enseña amonio (NH_4^+), al igual que la reivindicación 1. Sin embargo, D1 en realidad enseña amoniaco (NH_3). Como lo sabe cualquier persona versada en la materia, estos dos compuestos son diferentes entre sí.

Estas diferencias son de gran importancia para los aspectos técnicos de las reivindicaciones ya que, como se enseña en el artículo "The importance of ammonia in mammalian cell culture" (anexo), "las características físicas y químicas del amoníaco y el amonio son importantes, con el primero siendo capaz de difundirse a través de las membranas celulares y el segundo compitiendo con otros cationes del transporte activo por medio de una proteína portadora" (abstract).

El amonio y el amoníaco son dos entidades químicas diferentes con propiedades físicas y químicas diferentes, por lo que no se puede considerar que la divulgación de amoníaco en el documento D1 anticipe el ión de amonio requerido en la reivindicación 1.

El artículo "Mechanisms of ammonia and ammonium ion toxicity in animal cells: Transport across cell membranes" presenta evidencia adicional entre los efectos diferenciales entre el amonio y el amoníaco: "los iones de amoníaco y amonio son tóxicos para las células animales – diferentes concentraciones inhibitorias han sido reportadas para diferentes líneas celulares" (p. 340). Las diferencias entre el amonio y el amoníaco no son superficiales, sino que tienen consecuencias diferentes con respecto al crecimiento celular.

Adicionalmente, el documento D1 no enseña ni sugiere un método en el que el contenido de amonio sea mantenido en una concentración por debajo de 10 mM por un periodo específico de tiempo, como si lo requiere la reivindicación 1, que especifica que dicha concentración es mantenida por al menos 7 días.

Es preciso mencionar que mantener la concentración de amonio durante al menos 7 días es parte esencial del paso de "cultivar" del método reivindicado, que contribuye a la solución del problema técnico. Anexos se presentan dos referencias ejemplares que muestran que el paso de remover amonio del sobrenadante de cultivos celulares es un paso funcional que puede tener un efecto técnico en el resultado final. El artículo "IN SITU REMOVAL OF AMMONIUM IONS FROM HYBRIDOMA CELL CULTURE MEDIA: SELECTION OF ADSORBENT" describe el uso de adsorbentes para la remoción *in situ* de amonio del medio del cultivo celular (abstract y figuras 3 a 5), mientras que el artículo

"Improvement of CHO Cell Culture Medium Formulation: Simultaneous Substitution of Glucose and Glutamine" describe experimentos para reducir la generación de amonio mediante el cambio de las fuentes de nitrógeno en el medio de cultivo celular (Conclusión y páginas 72 a 74, especialmente figuras 1F y 3). Estas referencias muestran que mantener las concentraciones de amonio en un nivel especificado es un paso activo en métodos de cultivo celular. Por lo tanto, la característica de la reivindicación 1, dirigida al mantenimiento de la concentración de amonio del sobrenadante del cultivo celular por debajo de 10 mM es un paso activo que representa una característica esencial del método reclamado. D1 no enseña ni sugiere este paso activo, por lo que no puede afectar la novedad de las reivindicaciones de la presente solicitud.

Adicionalmente, D1 no enseña ni sugiere alguna concentración específica de amonio, sino que únicamente menciona el amoniaco en la página 12. De esta forma, D1 no enseña todas las características esenciales de la presente invención.

Consecuentemente, D1 tampoco comprende enseñanza alguna acerca del tiempo durante el cual debe ser mantenida la concentración de amonio. La nueva reivindicación 1 requiere específicamente que el contenido de amonio debe ser mantenido por debajo de la concentración especificada durante al menos 7 días con el fin de obtener el rVWF con la actividad específica deseada. Así, el tiempo especificado en la nueva reivindicación 1 corresponde a una diferencia adicional con el arte previo que le otorga novedad y nivel inventivo.

Queremos llamar la atención del Despacho, que el Tribunal de Justicia de la Comunidad Andina se ha pronunciado en diversas oportunidades en relación a la forma de evaluar la novedad. En la interpretación 36-IP-2010 el Tribunal ha dicho que *"El Tribunal ha tomado como referencia criterios de las Cámaras de Recursos de la Oficina Europea de Patentes (OEP), mediante los cuales "la falta de novedad de una invención se configurará si su objeto deriva clara, precisa y directamente del estado de la técnica, y si en éste son conocidas todas las características de aquélla"*. En el presente caso se observa que el objeto reivindicado no se deriva clara, precisa y directamente del estado de la técnica dado que:

- Del estado de la técnica no puede desprenderse el paso de mantener la concentración de amonio del sobrenadante de cultivo celular por debajo de 10 mM.
- Del estado de la técnica no puede desprenderse que la concentración de amonio sea mantenida durante al menos 7 días.

Dado que no es posible derivar clara, precisa y directamente de la anterioridad la presente invención con todas sus características técnicas, entonces la presente solicitud goza de novedad.

5.3. Concesiones internacionales

En adición a los argumentos presentados, es relevante mencionar que el mérito investigativo de la presente solicitud ha sido reconocido por otras oficinas de patentes del mundo, las cuales han otorgado patentes para invenciones equivalentes. Tal es el caso de Nueva Zelanda (605404) y Estados Unidos de América (8,852,888).

A este respecto, deseamos poner de presente que si bien cada país es autónomo de conceder o negar una solicitud luego de la realización del estudio de fondo, es claro que el hecho de que la novedad y el nivel inventivo de una invención hayan sido reconocidos por varias oficinas a nivel mundial, es un indicio de la patentabilidad de la misma toda vez que (i) el estudio de novedad y nivel inventivo en todas las Oficinas de Patentes del mundo es realizado por personas con conocimientos técnicos en el área de interés que son consideradas personas idóneas para emitir conceptos sobre la patentabilidad de una invención y que (ii) los resultados de los exámenes de novedad o de patentabilidad efectuados respecto a solicitudes extranjeras pueden ser reconocidos como suficientes para acreditar el cumplimiento de las condiciones de patentabilidad de la invención, tal como se señala en el artículo 46 de la Decisión 486:

"Artículo 46.- La oficina nacional competente podrá requerir el informe de expertos o de organismos científicos o tecnológicos que se consideren idóneos, para que

emitan opinión sobre la patentabilidad de la invención. Asimismo, cuando lo estime conveniente, podrá requerir informes de otras oficinas de propiedad industrial.

De ser necesario, a efectos del examen de patentabilidad y a requerimiento de la oficina nacional competente, el solicitante proporcionará, en un plazo que no excederá de 3 meses, uno o más de los siguientes documentos relativos a una o más de las solicitudes extranjeras referidas total o parcialmente a la misma invención que se examina:

a) copia de la solicitud extranjera;

b) copia de los resultados de exámenes de novedad o de patentabilidad efectuados respecto a esa solicitud extranjera;

(...)

*La oficina nacional competente podrá **reconocer los resultados de los exámenes referidos en el literal b) como suficientes para acreditar el cumplimiento de las condiciones de patentabilidad de la invención*** (Negrilla fuera del texto).

5.4. Conclusión

El documento D1 no resulta anterioridad válida capaz de afectar la novedad y el nivel inventivo del método de la presente solicitud.

Por todo lo anterior, se considera que la presente solicitud cumple a cabalidad con los requerimientos de patentabilidad exigidos por la legislación actual, por lo que atentamente solicitamos en consideración lo siguiente:

VI. PETICIÓN

Por todo lo anterior, solicito a ese Despacho lo siguiente:

6.1. Que al considerar las razones precedentes se revoque la decisión contenida en la Resolución No. 605 del 15 de Enero de 2015, en cuanto resuelve negar el privilegio de patente de invención.

6.2. Que se otorgue el privilegio de patente de invención para **“MÉTODO PARA PRODUCIR EL FACTOR VON WILLEBRAND (VWF) DE ALTO PESO MOLECULAR RECOMBINANTE EN CULTIVO CELULAR”** a favor de **BAXTER HEALTHCARE S.A.** y **BAXTER INTERNATIONAL INC..**

VII. NOMBRE Y DIRECCIÓN

El nombre y la dirección del solicitante recurrente son los siguientes:

BAXTER HEALTHCARE S.A.

Thurgauerstrasse 130,
Glattpark (Opfikon) CH-8152
Suiza

BAXTER INTERNATIONAL INC.

One Baxter Parkway,
Deerfield 60015
Illinois
Estados Unidos de América

VIII. ANEXOS

- Formulario de modificaciones en solicitud pendiente
- Recibo por concepto de presentación de modificaciones a las reivindicaciones por un valor de \$132.000
- Nuevo pliego reivindicatorio conformado por 50 reivindicaciones
- Comprobante de pago por 5 reivindicaciones adicionales después de las 45 previamente pagadas
- Portada y reivindicaciones de la patente concedida en los Estados Unidos de América
- Reivindicaciones concedidas en Nueva Zelanda
- Artículo "The importance of ammonia in mammalian cell culture"

- Artículo "Mechanisms of ammonia and ammonium ion toxicity in animal cells: Transport across cell membranes"
- Artículo "IN SITU REMOVAL OF AMMONIUM IONS FROM HYBRIDOMA CELL CULTURE MEDIA: SELECTION OF ADSORBENT"
- Artículo "Improvement of CHO Cell Culture Medium Formulation: Simultaneous Substitution of Glucose and Glutamine"

IX. NOTIFICACIONES

Atentamente manifiesto a usted que recibiré notificaciones en la Secretaria de su Despacho o en mi oficina de abogada situada en la Carrera 4ª. No. 72-35, piso 4º, de esta ciudad de Bogotá, D.C.

Señor Superintendente,


LUZ CLEMENCIA DE PÁEZ

C.C. 35.456.344 de Usaquén

T.P.A. No. 23.555

COLO-13936-0841-051-6
SML\SML\ID-278661\WPC13936
No. M-2015-278661\SML

 Industria y Comercio SUPERINTENDENCIA	Espacio reservado para el adhesivo de radicación
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DIRECCIÓN DE NUEVAS CREACIONES
MODIFICACIONES Y/O CORRECCIONES

1. **Identificación del Trámite**

☐ Error material
 ☒ Modificación a una Solicitud en trámite

☒ Patente de Invención

☐ Patente de Modelo de Utilidad

☐ Registro de Diseño Industrial

☐ Esquema de Trazado de Circuitos Integrados

2. **Datos del solicitante**

Persona Natural ☐
 Persona Jurídica ☒

BAXTER HEALTHCARE S.A

Y

BAXTER INTERNATIONAL INC.

Nombre o denominación / Nombre o razón social completa

Nombre representante legal

Tipo de empresa Micro ☐ Pequeña ☐ Mediana ☐ Otra ☐

Documento de identificación: ☐ C.C. ☐ C.E. ☐ NIT ☐ Otro Número _____

Nacionalidad/País de constitución	Dirección y domicilio del titular	Ciudad
Estados Unidos de América	One Baxter Parkway Deerfield 60015 Illinois Estados Unidos de América	Deerfield
Y	Y	Y
Suiza	Thurgauerstrasse 130, CH-8152 Glatttpark (opfikon) Suiza	Opfikon
Respectivamente	Respectivamente	Respectivamente
Dirección electrónica	No. Fax	Número telefónico
clientes@cavelier.com	2 11 86 50	3 47 36 11

☐ Autorizo expresamente a la Superintendencia de Industria y Comercio para que todas las comunicaciones sean enviadas a través de la dirección de correo electrónico

3. Datos del apoderado:**Apellidos y Nombre**
De Páez Luz Clemencia**Documento de identificación**
C.C. 35.456.344**Tarjeta profesional**
T.P.A. 23.555**Dirección y domicilio****Ciudad**

Carrera 4ª No. 72 -35

Bogotá

Dirección electrónica**No. Fax****Número telefónico**

cavelier@cavelier.com

2 11 86 50

3 47 36 11

En caso de haber presentado poder general para asuntos que se adelanten ante la Delegatura de Propiedad Industrial, sirvase indicar el número de su radicación o protocolo de poder general

4. Datos del trámite**Corrección:****Expediente No** 13.025.488**Aparece****Debe ser****Modificación:****Modificación al capítulo reivindicatorio.**

Presento nuevo capítulo reivindicatorio compuesto por 50 reivindicaciones y pido respetuosamente que el examen de patentabilidad sea realizado sobre estas nuevas reivindicaciones presentadas. Respetuosamente manifiesto que a pesar de eliminar materia del pliego reivindicatorio originalmente presentado, mi representada no renuncia a la protección que pueda obtener sobre dicha materia y además se reserva el derecho de presentar una solicitud divisional que la contenga durante el trámite de la presente solicitud, beneficiándose de la fecha de prioridad inicialmente reivindicada.

**Modificación al capítulo descriptivo.****5. Anexos**

Comprobante de pago de la tasa para la presentación de la corrección y/o modificación por \$132.000.



Poder, si fuere el caso.



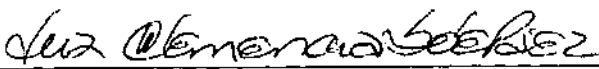
Documento que contiene las modificaciones o correcciones.



Copia de la solicitud y sus anexos en formato magnético.



Comprobante de pago por 5 reivindicaciones adicionales después de la décima reivindicación por \$160.000 teniendo en cuenta que ya fueron pagas 35 reivindicaciones.

6. Firma	
Nombre y apellidos	Firma
LUZ CLEMENCIA DE PAEZ	
C.C. 35.456.344 DE Usaquén	Tarjeta Profesional 23.555

SML\SML\NCA\ID-AF278666\WPC13936

REIVINDICACIONES

1. Un método para producir una composición del Factor Von Willebrand recombinante (rVWF), caracterizado porque el método comprende las etapas de:
 - (a) proporcionar un medio de cultivo de células basales;
 - (b) suplementar el medio de cultivo de células basales con cobre para proporcionar una concentración de cobre final de al menos 2.4 µg/L;
 - (c) proporcionar una o más células que comprenden un ácido nucleico que codifica una proteína del rVWF;
 - (d) cultivar continuamente una o más células en el medio de cultivo de células suplementadas con cobre de manera que el rVWF se expresa y excreta de las células en un sobrenadante del cultivo de en donde el contenido de NH_4^+ del sobrenadante de cultivo celular se mantiene a una concentración por debajo de 10 mM, en donde la concentración de NH_4^+ es mantenida por al menos 7 días de cultivación continua de la una o más células; y
 - (e) recuperar al menos una porción del sobrenadante del cultivo, en donde el sobrenadante recuperado tiene una actividad del cofactor de ristocetina específico del rVWF de al menos 30 mU/µg del rVWF.

2. El método de conformidad con la reivindicación 1, caracterizado porque además comprende la etapa de suplementar el medio de cultivo de células basales con un hidrolizado previo a cultivar una o más células.

3. El método de conformidad con la reivindicación 2, caracterizado porque el hidrolizado es un hidrolizado de planta.

4. El método de conformidad con la reivindicación 3, caracterizado porque el hidrolizado es un hidrolizado de soya.

5. El método de conformidad con cualquiera de las reivindicaciones 1 a 4, caracterizado porque el medio de cultivo de células basales es un medio de cultivo libre de proteína animal.

6. El método de conformidad con cualquiera de las reivindicaciones 1 a 4, caracterizado porque el medio de cultivo de células basales es un medio de cultivo libre de proteína.

7. El método de conformidad con cualquiera de las reivindicaciones 1 a 6, caracterizado porque el medio de cultivo de células basales es un medio de cultivo libre

químicamente definido.

8. El método de conformidad con cualquiera de las reivindicaciones 1 a 7, caracterizado porque la concentración final de cobre del medio de cultivo de células basales suplementado con cobre es al menos 4 µg/L de cobre.
9. El método de conformidad con cualquiera de las reivindicaciones 1 a 8, caracterizado porque la concentración final de cobre del medio de cultivo de células basales suplementado con cobre es entre 2.4 µg/L y 20 µg/L de cobre.
10. El método de conformidad con cualquiera de las reivindicaciones 1 a 9, caracterizado porque el medio de cultivo de células basales suplementado con cobre se proporciona como una sal de cobre, un quelato de cobre, o una combinación de los mismos.
11. El método de conformidad con la reivindicación 10, caracterizado porque la sal de cobre se selecciona del grupo que consiste de sulfato de cobre, acetato de cobre, carbonato de cobre, cloruro de cobre, hidróxido de cobre, nitrato de cobre y óxido de cobre.
12. El método de conformidad con cualquiera de las reivindicaciones 1 a 11, caracterizado porque una o más células son células de mamífero.
13. El método de conformidad con la reivindicación 12, caracterizado porque las células de mamífero son células CHO.
14. El método de conformidad con cualquiera de las reivindicaciones 1 a 13, caracterizado porque cultivar una o más células comprende cultivación por lotes de las células.
15. El método de conformidad con cualquiera de las reivindicaciones 1 a 13, caracterizado porque la cultivación continua de células se realiza en modo quimiostático.
16. El método de conformidad con cualquiera de las reivindicaciones 1 a 13, caracterizado porque la cultivación continua de células se realiza en modo de perfusión.
17. El método de conformidad con cualquiera de las reivindicaciones 1 a 16, caracterizado porque una o más células se cultivan en al menos 100 L del medio de cultivo suplementado a base de células basales.

18. El método de conformidad con cualquiera de las reivindicaciones 1 a 17, caracterizado porque la densidad celular se mantiene a menos de 2.5×10^6 células por mL durante la etapa de cultivar una o más células.
19. El método de conformidad con la reivindicación 18, caracterizado porque la densidad celular se mantiene a menos de 2.0×10^6 células por mL durante la etapa de cultivar una o más células.
20. El método de conformidad con la reivindicación 18, caracterizado porque la densidad celular se mantiene a menos de 1.5×10^6 células por mL durante la etapa de cultivar una o más células.
21. El método de conformidad con cualquiera de las reivindicaciones 1 a 20, caracterizado porque la etapa de recuperar al menos una porción del sobrenadante del cultivo comprende filtración o centrifugación para remover las células de la porción del sobrenadante del cultivo.
22. El método de conformidad con cualquiera de las reivindicaciones 1 a 21, caracterizado porque el sobrenadante recuperado tiene una actividad del cofactor de ristocetina específico del rVWF de al menos 40 mU/ μ g del rVWF.
23. El método de conformidad con la reivindicación 22, caracterizado porque el sobrenadante recuperado tiene una actividad del cofactor de ristocetina específico del rVWF de al menos 50 mU/ μ g del rVWF.
24. El método de conformidad con la reivindicación 22, caracterizado porque el sobrenadante recuperado tiene una actividad del cofactor de ristocetina específico del rVWF de al menos 60 mU/ μ g del rVWF.
25. El método de conformidad con la reivindicación 22, caracterizado porque el sobrenadante recuperado tiene una actividad del cofactor de ristocetina específico del rVWF de al menos 70 mU/ μ g del rVWF.
26. El método de conformidad con la reivindicación 22, caracterizado porque el sobrenadante recuperado tiene una actividad del cofactor de ristocetina específico del rVWF de al menos 80 mU/ μ g del rVWF.
27. El método de conformidad con cualquiera de las reivindicaciones 1 a 26,

caracterizado porque al menos 10% del rVWF en el sobrenadante está presente en un multímero del VWF de alto peso molecular de más de 10 dímeros.

28. El método de conformidad con cualquiera de las reivindicaciones 1 a 26, caracterizado porque al menos 20% del rVWF en el sobrenadante está presente en un multímero del VWF de alto peso molecular de más de 10 dímeros.

29. El método de conformidad con cualquiera de las reivindicaciones 1 a 26, caracterizado porque al menos 30% del rVWF en el sobrenadante está presente en un multímero del VWF de alto peso molecular de más de 10 dímeros.

30. El método de conformidad con cualquiera de las reivindicaciones 1 a 29, caracterizado porque el sobrenadante contiene multímero del VWF de alto peso molecular de 14 a 22 dímeros.

31. El método de conformidad con la reivindicación 1, caracterizado porque el contenido de NH_4^+ del sobrenadante de cultivo celular se mantiene a una concentración por debajo de 4 mM

32. El método de conformidad con cualquiera de las reivindicaciones 1 a 31, caracterizado porque el rVWF es co-expresado con el Factor VIII recombinante (rFVIII).

33. El método de conformidad con la reivindicación 32, caracterizado porque además comprende una etapa de purificación del rVWF lejos de al menos 50% del rFVIII presente en el sobrenadante recuperado.

34. El método de conformidad con la reivindicación 33, caracterizado porque la relación del rVWF a rFVIII después de la etapa de purificación es al menos 10:1.

35. El método de conformidad con cualquiera de las reivindicaciones 1 a 34, caracterizado porque el método además comprende una etapa de enriquecimiento del rVWF.

36. Un sobrenadante de cultivo celular que comprende el Factor Von Willebrand recombinante (rVWF), caracterizado porque el sobrenadante se prepara por un método de conformidad con cualquiera de las reivindicaciones precedentes.

37. Un sobrenadante de cultivo celular que comprende el Factor Von Willebrand recombinante (rVWF), caracterizado porque al menos 20% del rVWF en el sobrenadante

está presente en un multímero del VWF de alto peso molecular de más de 10 dímeros.

38. Un sobrenadante de cultivo celular que comprende el Factor Von Willebrand recombinante (rVWF), caracterizado porque el sobrenadante contiene al menos 0.5 IU de actividad del cofactor de ristocetina por mL.

39. Una composición del Factor Von Willebrand recombinante (rVWF) caracterizada porque se prepara por un método de conformidad con cualquiera de las reivindicaciones 1 a 35.

40. La composición de conformidad con la reivindicación 39, caracterizada porque la composición además comprende el Factor VIII recombinante (rFVIII).

41. La composición de conformidad con la reivindicación 40, caracterizada porque la relación del rVWF a rFVIII es al menos 10:1.

42. El método de acuerdo con cualquiera de las reivindicaciones 1 a 35, en donde la concentración de NH_4^+ es mantenida por al menos 14 días de cultivación continua de la una o más células.

43. El método de acuerdo con cualquiera de las reivindicaciones 1 a 35, en donde la concentración de NH_4^+ es mantenida por al menos 21 días de cultivación continua de la una o más células.

44. El método de acuerdo con cualquiera de las reivindicaciones 1 a 35, en donde la concentración de NH_4^+ es mantenida por al menos 28 días de cultivación continua de la una o más células.

45. El método de acuerdo con cualquiera de las reivindicaciones 1 a 35, en donde la concentración de NH_4^+ es mantenida por 5 semanas de cultivación continua de la una o más células.

46. El método de acuerdo con cualquiera de las reivindicaciones 42 a 45, en donde el contenido de NH_4^+ del sobrenadante del cultivo celular es mantenido a una concentración menor a 4 mM.

47. El método de acuerdo con cualquiera de las reivindicaciones 1 a 35 o 46, en donde el sobrenadante recuperado tiene una actividad del cofactor de ristocetina específico del rVWF de al menos 30 mU/ μg del rVWF después de al menos 7 días de cultivación continua de la una o más células.

48. El método de acuerdo con cualquiera de las reivindicaciones 1 a 35 o 46, en donde

el sobrenadante recuperado tiene una actividad del cofactor de ristocetina específico del rVWF de al menos 30 mU/μg del rVWF después de al menos 14 días de cultivación continua de la una o más células.

49. El método de acuerdo con cualquiera de las reivindicaciones 1 a 35 o 46, en donde el sobrenadante recuperado tiene una actividad del cofactor de ristocetina específico del rVWF de al menos 30 mU/μg del rVWF después de al menos 28 días de cultivación continua de la una o más células.

50. El método de acuerdo con cualquiera de las reivindicaciones 1 a 35 o 46, en donde el sobrenadante recuperado tiene una actividad del cofactor de ristocetina específico del rVWF de al menos 30 mU/μg del rVWF después de al menos 5 semanas de cultivación continua de la una o más células.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800.176.089-2

-/-



RECIBO DE CAJA

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RECIBIDO DE : CAVELIER ABOGADOS

NI 860.041.367

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TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR PAGO
RECIBO DE CA	999999999	23496	26/02/2015		530.000.00
RECIBO DE CA	999999999	23497	26/02/2015		530.000.00

*** Conceptos Pagados ***

CANT.	RENTISTICO	CONCEPTO	Vr.UNDITARIO	Vr.CONCEPTO
1	50005-01-03	CAMBIO DE MODALIDAD Y MODIFICACIONES EN CREACION CORRECCION A SOLICITUD EN TRAMITE / MODIFICA	132.000.00	132.000.00
				\$132.000.00

SON: **CIENTO TREINTA Y DOS MIL PESOS MONEDA CORRIENTE***

Responsable: _____

Recibo de Caja Aplicado al Expediente No. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 13-025488- -00016-0000

Fecha: 2015-03-05 16:29:29 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYI Eve: 378 FASENACIONALI
Act. 412 REPOSPRESRECU Folios: 77

COLO-13936-0841-051-6

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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RECIBO DE CAJA

No. 15 - 0025517

Bogotá D.C., Marzo 03 de 2015 - 13:52:45

RECIBIDO DE : CAVELIER ABOGADOS

NI 860.041.367

***** Soporte del Pago *****

TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR. PAGO
CONSIGNACION	BANCO DE BOGOTA	062754387	669320794	03/03/2015	9.494.000.00

***** Conceptos Pagados *****

CANT. RENTISTICO	CONCEPTO	Vr. UNIDITARIO	Vr. CONCEPTO
5 50005-01-01 SOLICITUDES	1607 REIVINDICACION PATENTE UNITARIA ADICIONAL A LAS 10 INIC	32.000.00	160.000.00
			\$160.000.00

SON: **CIENTO SESENTA MIL PESOS MONEDA CORRIENTE**

Responsable: _____

Recibo de Caja Aplicado al Expediente No. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 13-025488- -00016-0000

Fecha: 2015-03-05 16:29:29 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 412 REPOSPRESRECU Folios: 77

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(12) **United States Patent**
Grillberger et al.

(10) **Patent No.:** **US 8,852,888 B2**

(45) **Date of Patent:** **Oct. 7, 2014**

(54) **METHOD OF PRODUCING RECOMBINANT HIGH MOLECULAR WEIGHT VWF IN CELL CULTURE**

2011/0086813 A1 4/2011 Glaser et al.
2011/0151512 A1 6/2011 Grillberger et al.
2012/0035110 A1 2/2012 Grillberger et al.

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 253 days.

(21) Appl. No.: **13/179,386**

(22) Filed: **Jul. 8, 2011**

(65) **Prior Publication Data**

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(51) Int. Cl.
C12P 21/04 (2006.01)
C12P 21/06 (2006.01)

(52) U.S. Cl.
USPC **435/69.6; 435/69.1**

(58) **Field of Classification Search**
USPC **435/69.6; 69.1**
See application file for complete search history.

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Primary Examiner — Marcia S Noble

(74) *Attorney, Agent, or Firm* — Morgan, Lewis & Bockius LLP

(57) **ABSTRACT**

Among other aspects, the present invention relates to cell culture conditions for producing high molecular weight vWF, in particular, highly multimeric vWF with a high specific activity and ADAMTS13 with a high specific activity. The cell culture conditions of the present invention can include, for example, a cell culture medium with an increased copper concentration and/or cell culture supernatant with a low ammonium (NH₄⁺) concentration. The present invention also provides methods for cultivating cells in the cell culture conditions to express high molecular weight vWF and rA13 having high specific activities.

35 Claims, 11 Drawing Sheets

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TABLE 14-continued

Composition of ADAMTS-13 medium.	
	mg/L
miscellaneous	
D-Glucose	5000.0000
Linoleic Acid	0.0420
Lipoic Acid	1.0050
Putrescine 2HCl	3.6810
Thymidine	0.3650
Hypoxanthine Na	2.3900
Sodium Pyruvate	55.0000
DMEM/F12 BESP845: Total	12738.3
ADAMTS-13 Medium Supplements	
L-Glutamin	1300.0000
Pluronic F68	1000.0000
Ethanolamin	1.5300
ZnSe4*7 H2O	1.0000
Na-Hydrogencarbonat	1500.0000
Supplements Total	3802.5
Total amount of Ingredients	16540.8

To determine if increased copper concentrations affected the integrity of the expressed rA13, supernatant harvested from rA13 cell cultures grown in media containing 0.66 µg/L, 1 µg/L, and 4 µg/L copper was examined by SDS-PAGE analysis. As seen in FIG. 3A (silver stain) and FIG. 3B (anti-A13 western blot), no obvious change in product quality by gel electrophoresis was observed. Specifically, no increase in the level of truncated 170 kD or other low MW rA13 variants and no other additional or increased HCP bands resulted from rA13 expression in the presence of increased copper concentrations.

To estimate an optimal concentration of copper on the activity of rA13, an extrapolation of data from Table 15 of P Frets versus copper concentration (FIG. 4) shows that optimum effect is likely reached at about 2 µg/L, with a conservative estimation of a negative effect above about 4 µg/L.

TABLE 15

Expression of rA13 in mammalian cell culture performed in chemostatic continuous culture mode using media containing variable copper concentrations.				
10 L Scale R&D Experiment Mean of 4 CST weeks	ZZ - NC [10E6 cells/mL]	P Frets [U/(L*d)]	q Frets [U/(10E9 cells*d)]	Spec. Activity U/mg
0.66 µg/L Cu ²⁺	1.35	1322	1028	1759
1 µg/L Cu ²⁺	1.64	1962	1247	1690
4 µg/L Cu ²⁺	2.26	2960	1338	1768

Based on the results obtained above, an additional experiment was performed, which compared the expression of rA13 in cell culture media containing 0.66 µg/L copper with 2.0 µg/L copper. As shown in Table 16, supplementation of the basal cell media with copper, to a final concentration of 2.0 µg/L copper, resulted in a significant increase in volumetric (P) and specific (q) productivity expressed as the total rA13 activity produced per liter culture and day and the total rA13 activity produced per cell and day, respectively, over a period of 8 weeks. The specific data for each week of rA13 production in the two cultures is shown in FIG. 5. From these data, it is clear that copper supplementation has a measurable beneficial effect on cell metabolism, specific growth rate and rA13 productivity.

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TABLE 16

Expression of rA13 in mammalian cell culture performed in chemostatic continuous culture mode using media containing variable copper concentrations.				
10 L Scale Mean of 8 CST weeks	ZZ - NC [10E6 cells/mL]	P Frets [U/(L*d)]	q Frets [U/(10E9 cells*d)]	Spec. Activity U/mg
0.66 µg/L Cu ²⁺	1.29	1049	821	1862
2 µg/L Cu ²⁺	2.17	2470	1146	1740

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

What is claimed is:

1. A method for producing a recombinant Von Willebrand Factor (rVWF) composition, the method comprising the steps of:

- (a) providing a basal cell culture media;
 - (b) supplementing the basal cell culture media with copper to provide a final copper concentration of at least 2.4 µg/L;
 - (c) providing one or more cells comprising a nucleic acid encoding a rVWF protein;
 - (d) culturing the one or more cells in the copper supplemented cell culture media such that rVWF is expressed and excreted from the cells into a culture supernatant and the NH₄⁺ content of the cell culture supernatant is maintained at a concentration below 4 mM or below 10 mM; and
 - (e) recovering at least a portion of the culture supernatant, wherein the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 30 mU/µg rVWF.
2. The method of claim 1, further comprising the step of supplementing the basal cell culture media with a hydrolysate prior to culturing the one or more cells.
3. The method of claim 2, wherein the hydrolysate is a plant hydrolysate.
4. The method of claim 3, wherein the hydrolysate is a soy hydrolysate.
5. The method of claim 1, wherein the basal cell culture media is an animal protein free culture media.
6. The method of claim 1, wherein the basal cell culture media is a protein free culture media.
7. The method of claim 1, wherein the basal cell culture media is a chemically defined culture media.
8. The method of claim 1, wherein the final copper concentration of the copper supplemented basal cell culture media is at least 4 µg/L copper.
9. The method of claim 1, wherein the final copper concentration of the copper supplemented basal cell culture media is between 2.4 µg/L and 20 µg/L copper.
10. The method of claim 1, wherein the copper supplementing the basal cell culture media is provided as a copper salt, a copper chelate, or a combination thereof.
11. The method of claim 10, wherein the copper salt is selected from the group consisting of copper sulfate, copper acetate, copper carbonate, copper chloride, copper hydroxide, copper nitrate, and copper oxide.
12. The method of claim 1, wherein the one or more cells are mammalian cells.

13. The method of claim 12, wherein the mammalian cells are CHO cells.

14. The method of claim 1, wherein culturing the one or more cells comprises batch cultivation of the cells.

15. The method of claim 1, wherein culturing the one or more cells comprises continuous cultivation of the cells.

16. The method of claim 15, wherein the continuous cultivation of cells is performed in chemostatic mode.

17. The method of claim 15, wherein the continuous cultivation of cells is performed in perfusion mode.

18. The method of claim 1, wherein the one or more cells is cultured in at least 100 L of the supplemented basal cell culture media.

19. The method of claim 1, wherein the cell density is maintained at less than 2.5×10^6 cells per mL during the step of culturing the one or more cells.

20. The method of claim 19, wherein the cell density is maintained at less than 2.0×10^6 cells per mL during the step of culturing the one or more cells.

21. The method of claim 19, wherein the cell density is maintained at less than 1.5×10^6 cells per mL during the step of culturing the one or more cells.

22. The method of claim 1, wherein the step of recovering at least a portion of the culture supernatant comprises filtration or centrifugation to remove cells from the portion of culture supernatant.

23. The method of claim 1, wherein the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 40 mU/ μ g rVWF.

24. The method of claim 23, wherein the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/ μ g rVWF.

25. The method of claim 23, wherein the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 60 mU/ μ g rVWF.

26. The method of claim 23, wherein the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/ μ g rVWF.

27. The method of claim 23, wherein the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 80 mU/ μ g rVWF.

28. The method of claim 1, wherein at least 10% of the rVWF in the supernatant is present in a high molecular weight VWF multimer of more than 10 dimers.

29. The method of claim 1, wherein at least 20% of the rVWF in the supernatant is present in a high molecular weight VWF multimer of more than 10 dimers.

30. The method of claim 1, wherein at least 30% of the rVWF in the supernatant is present in a high molecular weight VWF multimer of more than 10 dimers.

31. The method of claim 1, wherein the supernatant contains high molecular weight VWF multimers of 14 to 22 dimers.

32. The method of claim 1, wherein rVWF is co-expressed with recombinant Factor VIII (rFVIII).

33. The method of claim 32, further comprising a step of purifying rVWF away from at least 50% of the rFVIII present in the recovered supernatant.

34. The method of claim 33, wherein the ratio of rVWF to rFVIII after the purification step is at least 10:1.

35. The method of claim 1, wherein the method further comprises a rVWF enrichment step.

* * * * *

What we claim is:

1. A method of producing a recombinant Von Willebrand Factor (rVWF) composition, the method comprising:
 - (a) providing a basal cell culture media;
 - (b) supplementing the basal cell culture media with copper to provide a final copper concentration of at least 2.4 $\mu\text{g/L}$;
 - (c) providing one or more cells comprising a nucleic acid encoding a rVWF protein;
 - (d) culturing the one or more cells in the cell culture media supplemented with copper such that rVWF is expressed and excreted from the cells into a culture supernatant and the NH_4^+ content of the culture supernatant is maintained at a concentration below 4 mM or 10 mM; and
 - (e) recovering at least a portion of the culture supernatant, wherein the supernatant recovered has a rVWF specific ristocetin cofactor activity of at least 30 mU/ μg rVWF.
2. The method of claim 1, further comprising a step of supplementing the basal cell culture media with a hydrolysate prior to culturing the one or more cells.
3. The method of claim 2, wherein the hydrolysate is a plant hydrolysate.
4. The method of claim 3, wherein the hydrolysate is a soy hydrolysate.
5. The method of any one of claims 1 to 4, wherein the basal cell culture media is an animal protein free culture media.
6. The method of any one of claims 1 to 4, wherein the basal cell culture media is a protein free culture media.
7. The method of any one of claims 1 to 6, wherein the basal cell culture media is a chemically defined culture media.
8. The method of any one of claims 1 to 7, wherein a final copper concentration of the cell culture media supplemented with copper is at least 4 $\mu\text{g/L}$ copper.
9. The method of any one of claims 1 to 7, wherein a final copper concentration of the cell culture media supplemented with copper is between 2.4 $\mu\text{g/L}$ and 20 $\mu\text{g/L}$ copper.

10. The method of any one of claims 1 to 9, wherein the copper for supplementing the basal cell culture media is provided as a copper salt, a copper chelate or a combination thereof.
11. The method of claim 10, wherein the copper salt is selected from the group consisting of copper sulfate, copper acetate, copper carbonate, copper chloride, copper hydroxide, copper nitrate and copper oxide.
12. The method of any one of claims 1 to 11, wherein the one or more cells are mammalian cells.
13. The method of claim 12, wherein the mammalian cells are CHO cells.
14. The method of any one of claims 1 to 13, wherein culturing the one or more cells comprises batch cultivation of the cells.
15. The method of any one of claims 1 to 13, wherein culturing the one or more cells comprises continuous cultivation of the cells.
16. The method of claim 15, wherein the continuous cultivation of cells is performed in chemostatic mode.
17. The method of claim 15, wherein the continuous cultivation of cells is performed in perfusion mode.
18. The method of any one of claims 1 to 17, wherein the one or more cells are cultured in at least 100 L of cell culture media supplemented with copper.
19. The method of any one of claims 1 to 18, wherein the cell density is maintained at less than 2.5×10^6 cells per mL during the step of culturing the one or more cells.
20. The method of claim 19, wherein the cell density is maintained at less than 2.0×10^6 cells per mL during the step of culturing the one or more cells.
21. The method of claim 19 or claim 20, wherein the cell density is maintained at less than 1.5×10^6 cells per mL during the step of culturing the one or more cells.
22. The method of any one of claims 1 to 21, wherein the step of recovering at least a portion of the culture supernatant comprises filtration or centrifugation to remove cells from a portion of the culture supernatant.

- 23. The method of any one of claims 1 to 22, wherein the supernatant recovered has a rVWF specific ristocetin cofactor activity of at least 40 mU/ μ g rVWF.
- 24. The method of claim 23, wherein the supernatant recovered has a rVWF specific ristocetin cofactor activity of at least 50 mU/ μ g rVWF.
- 25. The method of claim 23 or claim 24, wherein the supernatant recovered has a rVWF specific ristocetin cofactor activity of at least 60 mU/ μ g rVWF.
- 26. The method of any one of claims 23 to 25, wherein the supernatant recovered has a rVWF specific ristocetin cofactor activity of at least 70 mU/ μ g rVWF.
- 27. The method of any one of claims 23 to 26, wherein the supernatant recovered has a rVWF specific ristocetin cofactor activity of at least 80 mU/ μ g rVWF.
- 28. The method of any one of claims 1 to 27, wherein at least 10% of the rVWF in the supernatant is present in a high molecular weight VWF multimer of more than 10 dimers.
- 29. The method of any one of claims 1 to 28, wherein at least 20% of the rVWF in the supernatant is present in a high molecular weight VWF multimer of more than 10 dimers.
- 30. The method of any one of claims 1 to 29, wherein at least 30% of the rVWF in the supernatant is present in a high molecular weight VWF multimer of more than 10 dimers.
- 31. The method of any one of claims 1 to 30, wherein the supernatant contains high molecular weight VWF multimers of 14 to 22 dimers.
- 32. The method of any one of claims 1 to 31, wherein rVWF is co-expressed with recombinant Factor VIII (rFVIII).
- 33. The method of claim 32, further comprising a step of purifying rVWF away from at least 50% of the rFVIII present in the supernatant recovered.
- 34. The method of claim 33, wherein the ratio of rVWF to rFVIII after the step of purifying is at least 10:1.
- 35. The method of any one of claims 1 to 34, wherein the method further comprises a rVWF enrichment step.

36. A cell culture supernatant comprising recombinant Von Willebrand Factor (rVWF), wherein the supernatant is prepared by the method of any one of claims 1 to 35.
37. The cell culture supernatant of claim 36, wherein at least 20% of the rVWF in the supernatant is present in a high molecular weight VWF multimer of more than 10 dimers.
38. The cell culture supernatant of claim 36 or claim 37, wherein the supernatant contains at least 0.5 IU ristocetin cofactor activity per mL.
39. A recombinant Von Willebrand Factor (rVWF) composition prepared by the method of any one of claims 1 to 35.
40. The composition of claim 39, wherein the composition further comprises recombinant Factor VIII (rFVIII).
41. The composition of claim 40, wherein the ratio of rVWF to rFVIII is at least 10:1.
42. The composition of claim 40 or claim 41, wherein the composition is formulated for pharmaceutical administration.
43. The composition of any one of claims 40 to 42, wherein the composition is formulated for intravenous administration.
44. A method as defined in claim 1 and as substantially described herein with reference to one or more of the accompanying examples.
45. A cell culture supernatant comprising recombinant Von Willebrand Factor (rVWF), wherein the supernatant is prepared by the method of claim 44.
46. A recombinant Von Willebrand Factor (rVWF) composition prepared by the method of claim 44.

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CULLENS

IN SITU REMOVAL OF AMMONIUM IONS FROM HYBRIDOMA CELL CULTURE MEDIA: SELECTION OF ADSORBENT

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ABSTRACT

Various ion-exchange resins and zeolite Phillipsite-Gismondine were tested for their potential application as adsorbents for the *in situ* removal of ammonium ions from animal cell culture media in attempting to increase hybridoma cell density and MAb productivity. A zeolite, Phillipsite-Gismondine demonstrated the best performance in terms of NH_4^+ adsorption capacity, selectivity and autoclavability among the various adsorbents tested. The biocompatibility of Phillipsite-Gismondine was also tested and the result showed improvement in cell density, viability, and antibody productivity.

INTRODUCTION

One of the most challenging problems in animal cell culture is the accumulation of waste metabolites because these metabolites such as NH_4^+ and lactate have been shown to inhibit both cell growth and product formation (Glacken *et al.*, 1988; Glacken, 1988; Miller *et al.*, 1988). The reactor productivity can be dramatically increased by reducing the concentration of those inhibitory waste metabolites as they are formed during cultivation of cells.

Mammalian cells produce NH_4^+ and lactate as waste metabolites of glutamine and glucose catabolism. In a culture with good pH control, ammonium ions are the most detrimental to cell growth (Jeong and Wang, 1990; Miller *et al.*, 1988).

The critical inhibitory concentrations of NH_4^+ for strain L mouse cells, chorioallantoic tissue, mouse ascites tumor cells, canine kidney cells (MDCK), and hybridoma cells have been reported to be 0.5 mM, 10 mM, 40 mM, 5-8 mM, and 3-5 mM respectively (Glacken, 1987). Furthermore, NH_4^+ also inhibits product formation. For example, interferon production by human fibroblast cells is inhibited by 50 % at 2 mM of NH_4^+ (Ito and McLimans, 1981). Vaccine production can also be inhibited by NH_4^+ . The growth of influenza (Jensen and Liu, 1961), Newcastle Disease (Eaton and Scala, 1961), and Columbia SK (Furusawa and Cutting, 1962) viruses were also reported to be inhibited by NH_4^+ .

It is important, therefore, to develop a method for removing NH_4^+ from the media in order to increase the productivities of bioreactors (Glacken, 1988). The simplest and the most promising method for the *in situ* removal of NH_4^+ from the cell culture media is by adsorption (Ito *et al.*, 1985; Jeong and Wang, 1990). To do so, it is most critical to select an adsorbent which is autoclavable, non-toxic and which has high adsorption capacity and high selectivity for NH_4^+ . Several tests were performed to select a good adsorbent based on the requirements mentioned above. *In situ* removal of NH_4^+ from the cell culture media with the selected adsorbent was performed, and the system performances are discussed in this report in terms of cell density, cell viability, and MAb productivity.

MATERIALS AND METHODS

CELL LINE

The mouse-mouse hybridoma cell line VIII H-8 used in this study was kindly given by New Brunswick Sci. Co.. These cells produce mouse immunoglobulin, IgG_{2a} which is specific for the whole cell of bacterium *Rhizobium japonicum* NR-7.

CULTURE MEDIA AND CELL PROPAGATION

The cells were maintained in DMEM (Dulbecco's Modification of Eagle's Medium) containing 4.5 g/l of glucose, supplemented with 0.94 g/l of NCTC 135 (National Cancer Tissue Culture), oxaloacetate (150 mg/l), insulin (75.5 µg/l), sodium bicarbonate (40 mM), streptomycin (100 µg/ml), penicillin (100 U/ml), and 5 % (v/v) calf bovine serum. The medium was filtered with 0.22 µm membrane.

Cells were cultivated in 75 cm² T-flask as a stationary culture and in 250 ml spinner flasks (Corning) of which working volume was 150 ml. T-flasks and spinner flasks were incubated at 37⁰ C in 7 % CO₂ atmosphere.

VIABLE CELL COUNTS

Cells were counted under microscopic examination after small amounts of sample was injected into a haemocytometer. Cell viability was determined by Trypan Blue exclusion method.

ANTIBODY ASSAY

The mouse immunoglobulin, IgG_{2a} was analyzed by using RID (Radial Immuno Diffusion) methods. Anti-IgG was immobilized in the agar on the RID plates. A centrifuged sample of 5 µl was injected into each sample hole on RID plates and incubated at 37⁰ C for 36 h. After incubation, ring was visualized by first staining with a buffaloblack stain solution and then rinsed with RID wash solution (5% v/v glacial acetic acid, 0.5 % v/v glycerol).

AMMONIUM ION

Ammonium ion concentration was measured using an Orion 9512 ammonium ion electrode (Cambridge MA, U.S.A.).

ADSORPTION EXPERIMENTS

Ammonium ion was adsorbed by 2.5 g each of Amberlite CG-120, Amberlite CG-50, Phillipsite-Gismondine, and Sordolite CW-18 in 50 ml of NH₄⁺ solution (5 mM initial concentration) with 200 rpm rotary shaking for 12 hours at 37⁰ C. Another sets of adsorption tests were performed in water or culture media with adsorbents which were autoclaved at 121⁰ C for 15 minutes before testing. Equilibrium was assured by measuring several NH₄⁺ concentrations until the adsorption kinetic curve leveled off.

RESULTS AND DISCUSSIONS

AMMONIUM ION TOXICITY

Ammonium chloride was added into culture medium to vary initial NH₄⁺ concentration. The cell growth and the concentration of monoclonal antibody produced in the T-flask were analyzed at different intervals. The effect of initial NH₄⁺ level on the specific cell growth rate is shown on Fig.1 and that on the monoclonal antibody production kinetics on Fig.2. Both specific growth rate and the highest accumulated monoclonal antibody concentration decrease as initial NH₄⁺ concentration increases, demonstrating NH₄⁺ toxicity to the hybridoma cell line tested. This NH₄⁺ toxicity could be caused by cytoplasmic acidification resulted from the uptake of the weak acid, NH₄⁺, into cytoplasm (McQueen and Bailey, 1990).

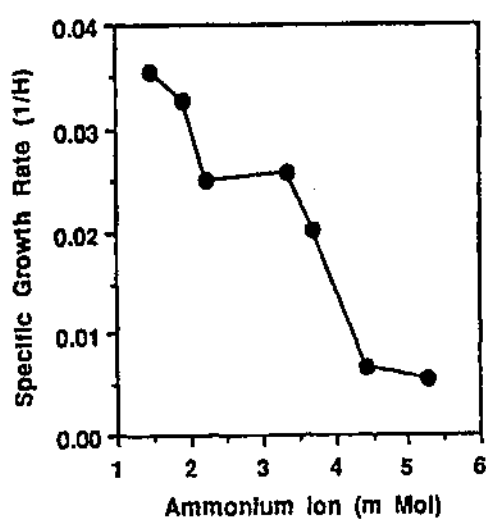


Fig.1 Effect of Ammonium Ion on Specific Growth Rate

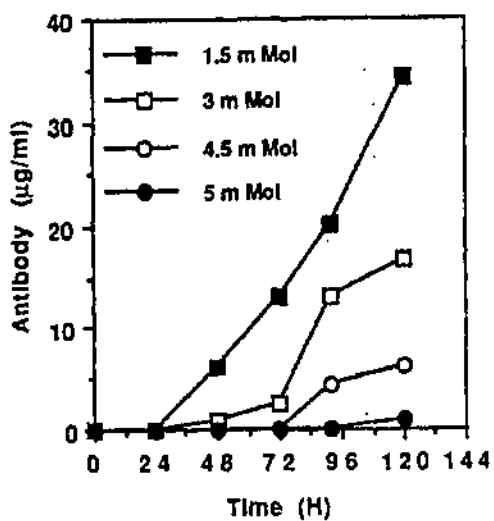


Fig.2 Effect of Initial Ammonium Ion on Antibody Production

SELECTION OF ADSORBENT

Not only high capacity and selectivity for NH_4^+ adsorption but also autoclavability and non-toxicity to hybridoma cells are required for the adsorbents to be used in the *in situ* removal of NH_4^+ in the hybridoma cell culture system. Four adsorbents were tested. Amberlite CG-120 is a strongly acidic cation exchanger, a styrene-divinylbenzene copolymer with a sulfonic group. Amberlite CG-50 is a styrene-divinylbenzene copolymer with a weak acidic carboxyl group. Sordolite CW-18 is a weak acidic cation exchanger with carboxylic group on the basis of polyacrylic acid. Phillipsite-Gismondine is a synthetic zeolite which has an identical structure to natural zeolite 'Merlinolite' (Passaglia *et al.*, 1977).

Fig.3 represents the studies of adsorption capacity and autoclavability of various adsorbents in NH_4^+ solution (initial 5 mM in water). In order to keep the culture system aseptic, adsorbents need to be autoclaved before applying to the media. It is desirable for an adsorbent not to lose its adsorptive capacity after autoclaving. However CG-120, CG-50, and Sordolite exhibit slight decrease in adsorption capacity after autoclaving, implying that their pore structures or functional groups are probably modified by heat. For example, thermal stress will cause the decomposition of sulfonic acid group of CG-120. On the other hand, Phillipsite-Gismondine demonstrates invariant capacity, indicating that the structure of Phillipsite-Gismondine is thermally stable because of its crystalline structure (Passaglia *et al.*, 1977). Therefore, Phillipsite-Gismondine exhibits best performance in autoclavability. In this figure, Phillipsite-Gismondine also shows the highest NH_4^+ adsorption capacity among the adsorbents tested. Fig.4 shows the final NH_4^+ concentration reached after treating 5 mM NH_4^+ solution with various adsorbents. It demonstrates that Phillipsite-Gismondine can easily maintain very low NH_4^+ concentration because it can remove about 98 % of NH_4^+ initially presented under the experimental conditions.

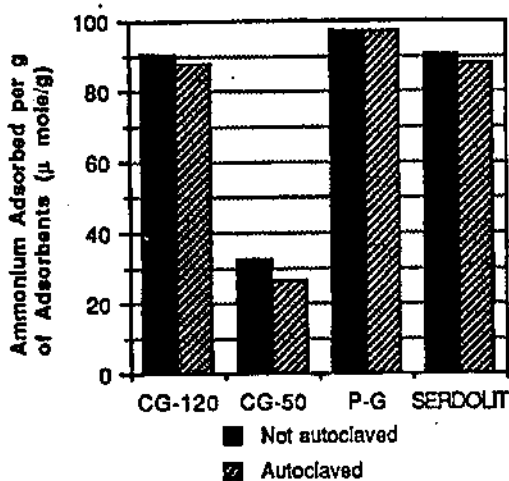


Fig.3 Ammonium Ion Adsorption Capacity Change after Autoclaving

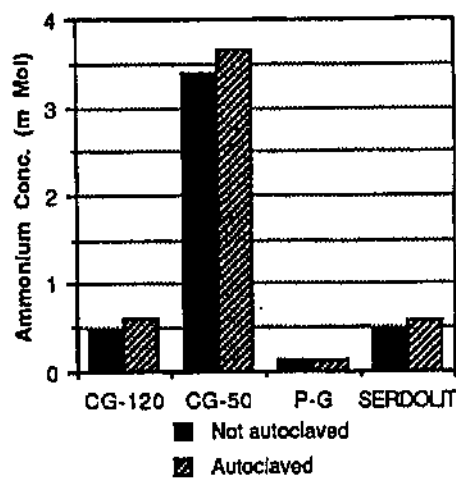


Fig.4 Final Ammonium Ion concentration

Fig.5 shows the studies of NH_4^+ selectivity of various adsorbents in typical animal cell culture media, DMEM and Joklik's medium. There are many kinds of NH_4^+ competitive ions such as Ca^{++} , Na^+ , Mg^{++} , etc., in most animal cell culture. Those competitors bind to the active site of adsorbent, so the available active sites for NH_4^+ adsorption can be easily reduced, consequently low selectivity for NH_4^+ would be expected. Eventhough CG-120 and Serdolite demonstrated relatively high NH_4^+ adsorption capacity in water which is comparable with that of Phillipsite-Gismondine as shown on Fig.3, they show respectively 58 % and 35 % decreases in NH_4^+ adsorption capacity in media. Whereas, Phillipsite-Gismondine shows only 9 % decrease in adsorption capacity. It may be because ion exchangers are usually very sensitive to bivalent ions such as Ca^{++} and Mg^{++} which are subject to strong electrostatic attraction, while zeolite can exclude those ions by size differentials in addition to electrostatic selectivity. Therefore Phillipsite-Gismondine shows the best NH_4^+ selectivity from the culture media of complex ions as well as the best NH_4^+ adsorbing capacity among the adsorbents tested.

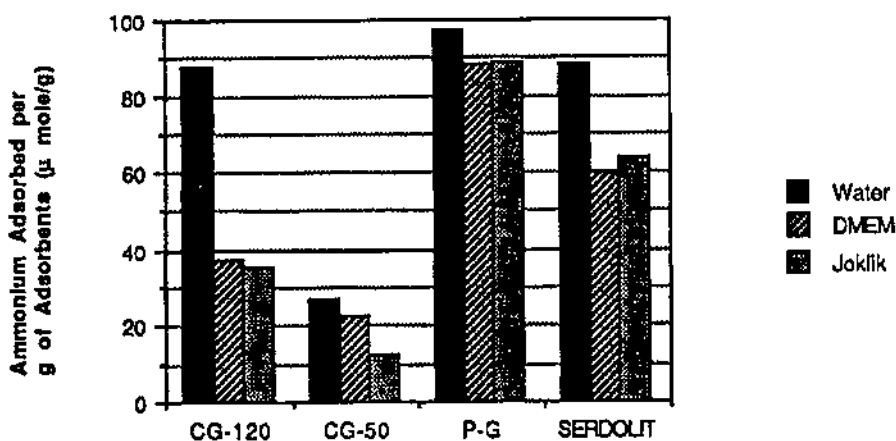


Fig.5 Ammonium Ion Selectivity of Various Adsorbents In Culture Media

BIOCOMPATIBILITY AND IN-SITU REMOVAL OF AMMONIUM ION

Fig.6 shows the result of live cell growth kinetics of hybridoma cells with and without adding zeolite powder in the media. Hybridoma cells were cultured with 0.75 g of Phillipsite-Gismondine powder in a spinner flask of 150 ml working volume at 60 rpm. Fig.7 shows cell viability change and Fig.8 shows NH_4^+ concentration change with respect to time in this run. Applying Phillipsite-Gismondine powder results in 15 % increase in live cell number and 28 % increase in viability after 154 hours. At the same time the difference in NH_4^+ concentration between two spinners becomes significant as shown on Fig.8. So, *in situ* removal of NH_4^+ by Phillipsite-Gismondine can stimulate cell growth and this material is not toxic and it is compatible with hybridoma cells tested. According to Fig.8, the final NH_4^+ concentration of culture with adsorbent is only half of that without adsorbent, demonstrating that Phillipsite-Gismondine can reduce NH_4^+ concentration to below toxic level in later phase of culture. Fig.9 shows the monoclonal antibody (IgG_{2a}) production kinetics in the same run. The enhancement in live cell growth due to maintenance of low NH_4^+ concentration results in 16 % increase in the concentration of monoclonal antibody. Therefore *in situ* removal of NH_4^+ by Phillipsite-Gismondine could stimulate not only cell growth but also monoclonal antibody production.

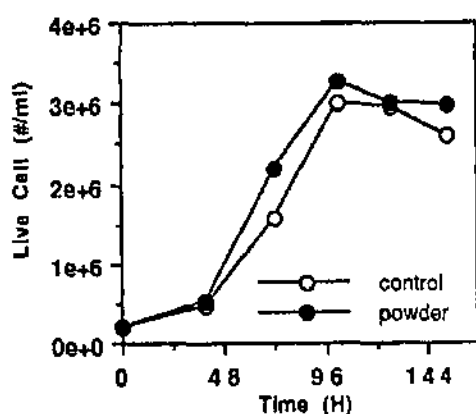


Fig.6 Live Cell Growth Kinetics

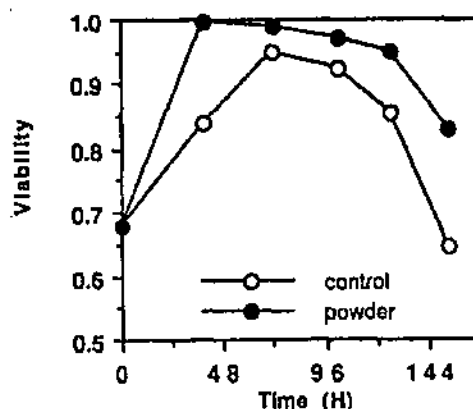


Fig.7 Cell Viability Kinetics

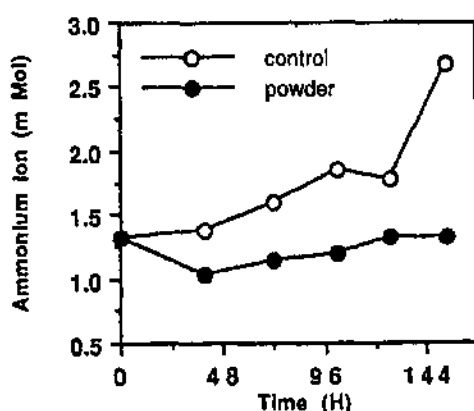


Fig.8 Ammonium Ion Concentration Kinetics

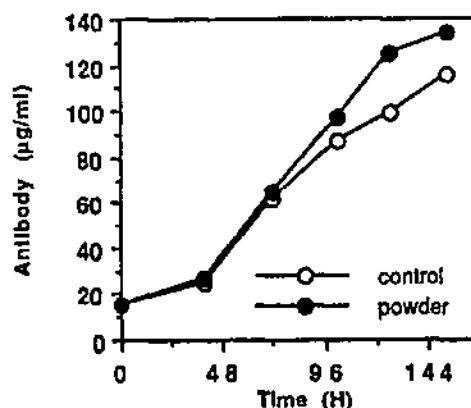


Fig.9 Monoclonal Antibody Kinetics

Although the last few graphs all show positive effects, it is also seen that the effects are rather moderate. This may be caused by following two reasons. The first reason is that the cells in the culture does not produce high enough NH_4^+ to raise up to a seriously toxic level. The final NH_4^+ concentration of culture without adsorbents is only 2.7 mM as shown on Fig.8. According to Fig.1, this level of NH_4^+ is estimated to cause only 30 % decrease in specific growth rate. Another reason is that the nutrient depletion in later phase hampers further increase in cell growth eventhough NH_4^+ accumulation problem has been overcome by adding adsorbents. Nutrient depletion is expected to be more serious in case of culture with adsorbent due to better cell growth and higher antibody production. Therefore, it is suggested that further increase in cell growth and antibody production can be accomplished by combining fed-batch (additional feeding of key nutrients such as glucose and glutamine) and *in situ* removal of NH_4^+ . The combined system which is free of nutrient depletion by fed-batch and free of NH_4^+ accumulation by adsorption showed 60 % increase of final cell density in another experiment. These results will be reported elsewhere very soon.

CONCLUSIONS

NH_4^+ is toxic to the growth of VIII H-8 cells and production of IgG_{2a}. Ammonium ion can be removed *in situ* by adding adsorbent directly to cell culture media. Phillipsite-Gismondine showed the best characteristics in NH_4^+ adsorption capacity, NH_4^+ selectivity, stability in adsorption capacity after autoclaving, and biocompatibility with hybridoma cells. Application of Phillipsite-Gismondine to actual culture system shows better cell growth, higher cell viability, higher antibody productivity and the ability of maintaining low NH_4^+ concentration throughout the culture period. Therefore, Phillipsite-Gismondine can be successfully used as an adsorbent for *in situ* removal of NH_4^+ from animal cell culture media as part of a comprehensive strategy to accomplish high cell density and high monoclonal antibody productivity.

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Minireview

The importance of ammonia in mammalian cell culture

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Abstract

Ammonia has been reported to be toxic and inhibitory for mammalian cell cultures for many years. Reduction of growth rates and maximal cell densities in batch cultures, changes in metabolic rates, perturbation of protein processing and virus replication have been reported. However, cellular mechanisms of ammonia toxicity are still the subject of controversy and are presented here. The physical and chemical characteristics of ammonia and ammonium are important, with the former capable of readily diffusing across cellular membranes and the latter competing with other cations for active transport by means of carrier proteins. The main source of the ammonia which accumulates in cell cultures is glutamine, which plays an important role in the metabolism of rapidly growing cells. Strategies to overcome toxic ammonia accumulation include substitution of glutamine by glutamate or other amino acids, nutrient control, i.e., controlled addition of glutamine at low concentrations, or removal of ammonia or ammonium from the culture medium by means of ion-exchange resins, ion-exchange membranes, gas-permeable membranes or electrodialysis.

Keywords: Mammalian cell culture; Ammonia; Ammonium; Glutamine; Ammonia removal; Ion-exchange membrane; Ion-exchange resin; Gas-permeable membrane; Electrodialysis

1. Introduction

Mammalian cell cultures have become increasingly important and economically interesting in the last decade for the production of high value proteins, including monoclonal antibodies, blood coagulation factors, plasminogen activators or viruses for the use as vaccines (Adamson and Schmidli, 1986; Arathoon and Birch, 1986; Backer et al., 1988; Leist et al., 1990). However, in vitro culture of mammalian cells is still limited in many respects. Thus the cells are much larger and, due to the absence of a rigid cell wall, more fragile and susceptible to shear stresses

than microbial cells. Bioreactors used for mammalian cell cultures have to avoid these high shear stresses, while providing optimal mass transfer characteristics. Furthermore the metabolism of mammalian cells is complex and the understanding of control and regulation is still rather limited. The origin of cells from different organs, in different animal species, considerably complicates this understanding. In addition, many routinely cultured cell lines are derived from cancer cells, with often completely altered metabolic patterns.

Several waste products of cell metabolism have been reported to be inhibitory or toxic to cells. The most important substances mentioned in this context are ammonia and lactate. Lactate excretion is due to incomplete oxidation of glucose by the glycolytic

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Table 1

Cell line	Reported effects	Ref.
3T3 and SV-40 transf. 3T3 fibroblast	60% growth reduction for 3T3 and 15% reduction for SV-40 transformed 3T3 cells upon initial addition of 0.6 mM ammonium (pH 7.6–7.7). 4-fold higher ammonia production by non-transformed cells.	Visek et al. (1972)
BSC-1 monkey epithelial cells	30% growth reduction upon addition of 2 mM ammonium in medium with 0.1% serum, less in medium with 10% serum.	Holley et al. (1978)
MDCK kidney cells	Suspension of 2.3 mM ammonium to be responsible for limiting further cell growth in a microcarrier perfusion culture.	Butler et al. (1983)
BHK 21/C13 kidney Murine myeloma, human hybridoma	75% growth reduction at 3 mM ammonium initially added. Growth inhibition for the myeloma cell line by ammonium. Ammonia removal increased cell density for myeloma and hybridoma (serum free).	Butler and Spier (1984) Iio et al. (1984)
MDCK kidney CRL-1606 murine hybridoma	50% growth reduction at 7–10 mM ammonium. 50% growth reduction at initial ammonium conc. of 6.7 mM, no influence on the specific antibody productivity.	Glacken et al. (1986) Glacken et al. (1986) Glacken (1987)
VIII H-8 hybridoma	Toxic effects on cell growth above 2 mM ammonium, strongly reduced specific antibody productivity above 5 mM ammon.	Reuveny et al. (1986)
HB8178 hybridoma	60% reduction of the initial growth rate with 3 mM ammonium, 90% reduction with 6 mM.	Dodge et al. (1987)
HL-60 human leuk. RPMI 8226 human hematopoiet.	50% growth reduction at 10 and 12 mM ammonium for HL-60 and RPMI 8226, respectively, no influence on respiration rates up to 20 mM ammonium.	Kimura et al. (1987)
AB2-143.2 murine hybridoma	Growth inhibition at 5 mM ammonium, adaptation to 8 mM in continuous culture, lower ammonia release, glutamine and oxygen uptake, and higher alanine production at elevated ammonium conc.	Miller et al. (1988)
TIB 131 murine hybridoma	Growth inhibition at 10 mM ammonium and low extracellular pH, cytoplasmic acidification due to ammonia, specific antibody productivity unchanged.	McQueen and Bailey (1990a,b)
TIB 131 murine hybridoma	Cytoplasmic acidification upon ammonium addition, growth inhibition at 10 mM ammonium, correlation between effects and intracellular pH.	McQueen and Bailey (1991)
PQX B1/2 murine hybridoma	50% growth reduction at 4–7.6 mM ammonium for pH 7.8–6.8, correlation between toxicity and concentration of ammonia (NH ₃).	Doyle and Butler (1990)
CHO C1E3 murine hybridoma	50% growth reduction at 8 mM ammonium. Growth inhibition at ammonium conc. > 3–5 mM, optimal mAb productivity at 2–2.5 mM ammonium, different glutamine metabolism for immobilized and suspended cells.	Kurano et al. (1990) Racher et al. (1990, 1993)
Different	Different amount of growth reduction upon initial addition of 2 mM ammonium in T- flasks.	Hassell et al. (1991)
163.4.G5.3 murine hybridoma	50% growth reduction at initial ammonium conc. of 4 mM, accelerated glutamine and glucose consumption, increased alanine production and reduced ammonia production at elevated ammonia conc., constant specific antibody productivity.	Ozturk et al. (1992)

Table 1 (continued)

VIII H-8 muine hybridoma	40% reduction of the specific growth rate upon addition of 3.5 mM ammonium before inoculation, 80% reduction with 5 mM.	Jeong and Wang (1992)
HL-60 human leukemia	Significant reduction of final cell density in medium with 60 mM lactate and 4 mM ammonium, selection of double-resistant clones.	Schumpp and Schlaeger (1992)
BHK	80% reduction of specific growth rate and 4-fold increase of glucose consumption rate upon addition of 1 mM ammonium chloride.	Wentz and Schügerl (1992)
PQXB1/2 murine hybridoma	Hypothesis that a high rate of ammonia production might coincide with transient increase in intracellular ammonia conc., effecting growth inhibition despite a subtoxic extracellular ammonia conc.	Bushell et al. (1993)
CHO	No inhibition of growth in continuous cultures by ammonium conc. of up to 8 mM, but reduction of recomb. t-PA productivity, reduced spec. ammonia production and increased alanine production at elevated ammonium conc.	Hansen and Emborg (1994)
Murine hybridoma	Correlation between specific growth rate and death rate with concentration of NH_3 , not total ammonia, no adaptation to high ammonia concentration.	Lüdemann et al. (1994)
SP01 murine hybridoma	Significant growth inhibition at initial ammonium conc. > 2 mM, adaptation to 10 mM in batch and continuous c., but with lower cell yield on glucose and glutamine, no influence on specific antibody product.	Newland et al. (1994)
Hybridoma, CHO, BHK, L 929	Increase of intracellular UDP-GNAc concentration upon ammonium addition (15 mM).	Ryll et al. (1994)
NS/0 myeloma (SP2/0) D5 hybridoma	Strong induction of apoptosis by nutrient deprivation, but incubation with ammonium (3 and 5 mM) lead to necrotic, not apoptotic, cell death.	Mercille and Massie (1994)
Hybridoma, plamacyt., CHO, SF9	Moderate induction of apoptosis by ammonium (14 and 18 mM) for hybridoma and plamacytoma, very high conc. (50 mM) lead to necrosis, no apoptosis observed for CHO and SF9 (insect cells).	Singh et al. (1994)
CRL 1606 murine hybridoma	25% higher specific glutamine and glucose consumption rates due to ammonium removal in glutamine fed-batch (final ammonium concentration 7.3 mM reduced to below 2 mM).	Chang et al. (1995a)
Zac3 murine hybridoma	20% growth reduction at initial ammonium conc. of 2.5 mM, complete inhibition at 7.5 mM. 50% reduction of ammonia conc. with ammonia removal, no influence on growth and spec. antibody productivity, reduced lactate production and increased glutamine consumption due to ammonia removal.	Schneider et al. (1995) Schneider (1995)
HeLa S3	20% growth reduction at initial ammonium concentration of 6 mM.	Schneider (1995)
Mouse plasma cells	10 mM ammonium prevented terminal sialic acid transfer to glycosyl side chains of IgM, raise of the pH of Golgi cisternae.	Thorens and Vassalli (1986)
CHO	Inhibition of the N-linked glycosylation of recombinant mouse placental lactogen-I by increasing levels of ammonium (3 to 9 mM), dependance on the extracellular pH.	Borys et al. (1994)
CHO	Significant reduction of terminal sialylation of O-linked glycosylation of recombinant granulocyte colony-stimulating factor by 2–10 mM ammonium.	Andersen and Goochee (1995)

Table 1 (continued)

BG-27 human fibroblast	Inhibition of the ability to establish an antiviral state and reduction of IFN- β production by ammonium.	Ito and McLimans (1981)
BV-2 immortaliz. microglia	Impair of phagocytosis at ammonium conc. > 2mM. increase in pinocytosis, changes in secretion of interferon- γ and interleukins, changes in activity of lysosomal enzymes.	Atanassov et al. (1994)
β TC3 insulinoma AtT20 pituitary	Inhibition of uptake of insulin related peptides to intracellular store vesicles at 6 mM ammonium.	Dyken and Sambanis (1994)
Tumor ascites cells	Significant suppression of growth of Influenza and Newcastle virus under the influence of ammonium, relation between ammonia formation and glutamine.	Eaton and Scala (1961)
Different	Study of the effect of ammonium on 10 viruses in 10 cell lines, inhibition of propagation of influenza virus, independent of the host cell line.	Jensen and Liu (1961)
Mouse ascit. tumor cells	Inhibition of Colombia SK virus propagation by ammonium sulfate.	Furusawa and Cutting (1962)
Mouse L-cells	Strong reduction of Reovirus yield and cytopathic effects on cells upon addition of 10 mM ammonium chloride.	Canning and Fields (1983)
CHSE	90% decrease in virus (pancreatic necrosis) yield upon addition of 30 mM ammonium, inhibition of cellular RNA-synthesis.	Fariás et al. (1988)
Vero	Inhibition of Herpes simplex virus multiplication by ammonium (50 mM).	Koyama and Uchida (1989)

pathway. Pyruvate, the end product of this pathway, is transformed to lactate in order to maintain the oxidation state of the cell, i.e., to recover the oxidized form of the redox cofactor nicotinamide adenine dinucleotide (NAD). Lactate can also be formed from sugars other than glucose, and from glutamine. The toxic action of lactate is probably due to the effect on the pH and osmolarity of the culture medium, only occurring at relatively high concentrations (> 20–30 mM) (Miller et al., 1988; Hassell et al., 1991; Omasa et al., 1992; Ozturk et al., 1992; Wentz and Schügerl, 1992).

Inhibition by ammonia seems to play a much more important role. Thus, total concentrations of ammonia and ammonium as low as 2–3 mM have been reported to reduce cell growth considerably, depending on the cell line and culture conditions. Ammonia release by cells is due to amino acid metabolism, mainly that of glutamine. The latter is routinely added to many culture media since it has been shown to stimulate growth and antibody productivity (Dalili et al., 1990; Ramírez and Mutharasan, 1990; Flickinger et al., 1992; Omasa et al.,

1992). Glutamine is not only a protein constituent, and an amino group donor in some biosynthetic pathways such as purine and pyrimidine synthesis, but also a major energy source. A considerable amount of metabolic energy is derived from glutamine, rather than glucose, oxidation in cultured cells (Donnelly and Scheffler, 1976; Reitzer, 1978; Reitzer et al., 1979, 1980; Moreadith and Lehninger, 1984a; Lanks and Li, 1988; Butler and Jenkins, 1989; Newland et al., 1990; Jenkins et al., 1992; Ljunggren and Häggström, 1992; Fitzpatrick et al., 1993; Sharfstein et al., 1994). Glutamine is chemically labile in a cell culture medium, and decomposition to pyrrolidonecarboxylic acid and ammonia has to be taken into account. Consequently, a part of the ammonia which accumulates in the medium is not due to cellular metabolism but to chemical decomposition of glutamine (Tritsch and Moore, 1962; Ozturk and Palsson, 1990).

Furthermore, another low- to medium-size molecular weight substance, which is neither lactate nor ammonia, has been suspected to be inhibitory or toxic to the cells. This still unknown substance seems

to accumulate in culture media during growth of cells (Holley et al., 1978; Dodge et al., 1987; Rønning et al., 1991; Lee et al., 1995).

In summary, it is a widely held view that the most important inhibitory substance accumulating in cell cultures is ammonia, and that a reduction in ammonia levels has to be overcome in order to achieve the successful economic scale-up of such processes for the production of high value proteins.

This review focusses on the significance of ammonia in mammalian cell culture technology and draws from the considerable volume of literature which has appeared during the last years on this topic, as well as the different strategies used to overcome this problem.

2. Reported effects of ammonia or ammonium in cell cultures

A large number of articles dealing with numerous different effects caused by elevated ammonia and/or ammonium concentrations on cell cultures have been published and are summarized in Table 1.

It can be seen from Table 1 that most authors report a reduction of growth rate and final cell density upon addition of 1 to 5 mM ammonium salts. There are interesting differences in the degree of growth reduction from one cell line to another and for the same cell line from different studies. As an example, Hassell et al. (1991) found a 75% decrease in cell yield, in comparison to control experiments, when 2 mM ammonium chloride was added prior to inoculation of batch cultures of HeLa cells. On the other hand, Schneider (1995) found only a 25% reduction in final cell density, when 6 mM ammonium chloride was added to HeLa batch cultures. Such differences are difficult to explain. Clearly culture conditions, and particularly the previous growth history of the cells, play an important role. This is confirmed by the fact that many authors found an adaptation to higher ammonia concentrations, especially using continuous culture techniques.

Further reported effects of elevated ammonia or ammonium concentrations relate to cellular energy metabolism. Ammonia may reduce metabolic efficiency by forcing excretion of potentially valuable intermediate metabolites, such as alanine, in order to

achieve ammonia detoxification. An increase in alanine production and glutamine consumption rates at elevated ammonia concentrations has been reported by several authors.

The latter group of effects is related to the intracellular compartments involved in the processing of molecules to be secreted. Thus, high ammonia concentrations have been shown to perturb processing and secretion of proteins as well as glycosylation. Virus replication also appears to be inhibited by increased ammonia concentrations. However, in many of these studies, the applied concentrations are much higher than those routinely found in mammalian cell cultures. These effects are most probably due to an increase of pH in the intracellular compartments due to ammonia influx.

3. Energy metabolism as source of ammonia

3.1. Rapid glycolysis and glutaminolysis in cells in culture

The main source of accumulating ammonia in mammalian cell cultures is amino acid metabolism, particularly that of glutamine. Glutamine is present in most cell culture media at concentrations of 2 to 5 mM, a concentration which is much higher than for other amino acids. Glutamine has been shown to be the major nutrient, in addition to glucose, for most fast growing animal cells in culture. Glutamine therefore not only serves as a protein constituent, but as the main energy source (Donnelly and Scheffler, 1976; Reitzer, 1978; Reitzer et al., 1979, 1980; Moreadith and Lehninger, 1984a; Lanks and Li, 1988; Butler and Jenkins, 1989; Newland et al., 1990; Jenkins et al., 1992; Ljunggren and Häggström, 1992; Fitzpatrick et al., 1993; Sharfstein et al., 1994). Glutamine is further required in the first step of de novo purine synthesis. The pivotal role in purine synthesis in leukemic lymphoblasts (Raivio and Andersson, 1982), in lymphoblastic transformation and in plasma cell differentiation (Crawford and Cohen, 1985) has been shown by *in vitro* studies.

Mammalian cell lines derive from different organs with very distinct functions and therefore have very different enzymatic and metabolic patterns. Thus

they cannot be easily compared to one another. Furthermore, many mammalian cell lines cultivated *in vitro* derive from malignantly transformed cancer cells. These cells have metabolic patterns which may be very different from those of normal cells, exhibiting high rates of aerobic glycolysis and glutaminolysis (reviewed by Baggetto, 1992). However, these metabolic characteristics are not limited to malignantly transformed cells. Many normal, fast growing cells in the body such as enterocytes in the small intestine (Watford, 1994) or lymphocytes (Brand et al., 1989), have a similar metabolism. High rates of glutamine catabolism are coupled to an apparently inefficient glucose metabolism, where glucose is predominantly and rapidly metabolized by the glycolytic pathway and subsequently excreted as lactate. Only a very small amount is completely oxidized to carbon dioxide, even in the presence of saturating levels of oxygen (Reitzer et al., 1979, 1980; Morgan and Faik, 1986; Butler and Jenkins, 1989; Newland et al., 1990; Sharfstein et al., 1994). The main requirement for glucose appears to be for the supply of carbohydrate building blocks for anabolic metabolism, such as nucleic acid or polysaccharide synthesis. Thus, a considerable amount is metabolized via the pentose phosphate pathway to provide ribose-5-phosphate. This anabolic role of glucose is supported by the findings of Burton et al. (1981), who reported the continuous growth of HeLa, and other cell lines, in the complete absence of carbohydrates, if the medium contained uridine. The close link between glucose and glutamine metabolism has been shown by the fact that, to a certain extent, they may even replace one another. Thus, for many cell lines a glucose limitation increases glutamine utilization and a glutamine limitation increases glucose uptake (Reitzer, 1978; Zielke et al., 1978; Kuchka et al., 1981; Sumbilla et al., 1981; Miller et al., 1989; Ljunggren and Häggström, 1994; Jeong and Wang, 1995; Zeng and Deckwer, 1995).

The reasons for rapid aerobic glycolysis and glutaminolysis in cancer cells and other fast growing cells is still unclear. Different hypotheses to explain this phenomenon have been raised. The major function of an enhanced aerobic glycolysis in such cells could be to maintain elevated steady state concentrations of glycolytic metabolites for use as precursors in macromolecule synthesis (Hume et al., 1978).

These authors propose, that enhanced aerobic glycolysis might not be unique to malignantly transformed cells, but a requirement for normal rapid cell proliferation. Thus they showed that glycolytic activity rapidly increased in cultured thymocytes, when stimulated with concanavalin A. However, the ability of thymocytes to transform at very low glucose concentrations suggests that enhanced glucose metabolism as such was not essential for the initiation of DNA synthesis. Nevertheless, the continued presence of saturating glucose concentrations was found to be necessary to sustain high rates of DNA synthesis. Concanavalin A stimulated the flow of glucose carbon to the nucleic acid pool, and the increase was proportional to the increase of glucose uptake. High rates of glycolysis could further be necessary, in rapidly dividing cells, to allow for high rates of proliferation when required (Newsholme et al., 1985). Based on regulatory analysis, these authors suggest that the observed high rates of glycolysis and glutaminolysis might be considered as a simple system to ensure sufficient sensitivity in biosynthetic pathways to provide adequately for macromolecular synthesis. It seems to be clear, that mammalian cells are not limited by rates of energy production, but rather by rates of biosynthesis (Newland et al., 1990). This is also supported by the different approach of Savinell and Palsson (1992). These authors tried to interpret hybridoma cell metabolism by means of metabolic network stoichiometric analysis using linear optimization. This analysis showed, that the maintenance demand for ATP did not limit the growth rate under normal growth conditions. The cells used nutrients with a low efficiency, mainly due to a high level of alanine secretion. However, a quantitative description of phenomena such as energy dissipation in futile cycles is very difficult using this approach, since only stoichiometric relationships are taken into account.

In summary, rapid aerobic glycolysis and glutaminolysis with subsequent lactate and ammonia excretion appears to be a characteristic of fast growing mammalian cells. The reason for this is not clear. Growth is definitely not limited by energy availability and neither by biosynthetic capacity. Efficient regulation and control of fast growth and adaptation to rapidly changing conditions might play an important role.

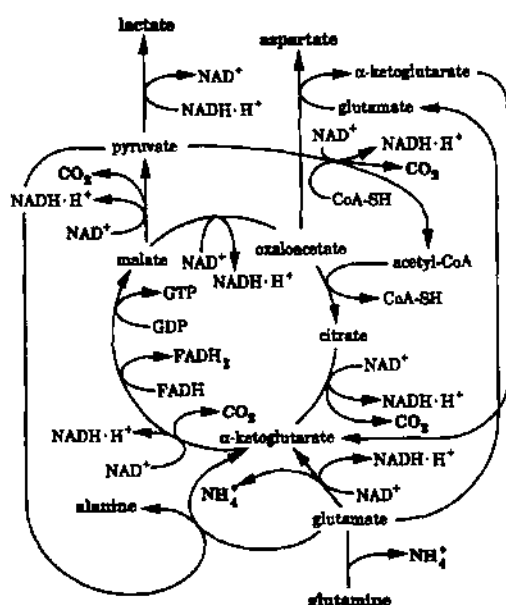


Fig. 1. Schematic representation of the metabolic pathways for glutamine degradation.

3.2. Pathways of glutamine degradation

The cellular degradation of glutamine has been intensively studied by several authors (Ardawi and Newsholme, 1983; Kovacevic and McGivan, 1983; Moreadith and Lehninger, 1984a; Glacken et al., 1986; McKeehan, 1986; Glacken, 1988; Kvamme, 1988; Baggetto, 1992; Watford, 1994).

The metabolic pathways for glutamine degradation are schematically shown in Fig. 1. Glutamine catabolism takes place mainly in the mitochondrial matrix involving the initial removal of the amido group of glutamine to yield glutamate. This reaction is mainly catalyzed by a phosphate-dependent glutaminase (Kvamme, 1988; Swierczynski et al., 1993) and liberates an ammonium molecule. To a much lower extent, the amido group is utilized as an amino group donor by some biosynthetic enzymes, mainly of the purine and pyrimidine biosynthetic pathways, but also for the formation of activated sugars. The second step is the removal of the α -amino group, leading to α -ketoglutarate. This reaction can either be catalyzed by glutamate dehydrogenase, resulting in the liberation of a second ammonia molecule, or an aminotransferase. The latter transfers the amino group to an α -keto acid, mainly pyruvate or oxaloac-

etate, leading to the formation of alanine or aspartate. A further pathway is the transamination of glutamine to form α -ketoglutarate and subsequent deamidation with ω -amidase to yield α -ketoglutarate (glutaminase II in the older literature). However, Nissim et al. (1991) showed that this pathway plays only a minor role for ammonia production in human kidney cells. The α -ketoglutarate subsequently enters the citric acid cycle, and can be completely oxidized to carbon dioxide, or partly oxidized to aspartate via transamination of oxaloacetate. Lactate and alanine formation from glutamine is also possible, through transformation of malate to pyruvate by mitochondrial malic enzyme, a decarboxylating malate dehydrogenase (Sauer et al., 1980; Moreadith and Lehninger, 1984b). This metabolism represents a truncated citric acid cycle, with important contributions from phosphate-dependent glutaminase and mitochondrial malic enzyme, as discussed in detail by Baggetto (1992).

3.3. The energy gain of the different glutamine catabolism pathways

The different metabolic pathways for glutamine degradation yield different amounts of ATP. If 1 mol of NADH is assumed to yield 3 mol ATP in the respiratory chain, 1 mol of $FADH_2$ to yield 2 mol ATP and 1 mol GTP is considered as equivalent to 1 mol ATP, the energetic gain is as follows (see also Fig. 1): Complete oxidation of glutamine to CO_2 yields 27 mol ATP per mol glutamine; incomplete oxidation to aspartate yields 9 mol ATP; reduction of pyruvate to lactate yields 9 mol ATP; and transamination of pyruvate to alanine yields 9 mol ATP. These values are in contradiction to those given by Glacken et al. (1986). These authors did not take into account the NADH produced in the malic enzyme and pyruvate dehydrogenase catalyzed reactions. In the case of aspartate, however, these authors included the NADH resulting from the glutamate dehydrogenase catalyzed reaction, even though glutamate is not oxidatively deaminated in this case. Instead the amino group is transaminated to oxaloacetate, yielding aspartate and α -ketoglutarate. However, in a later publication (Glacken, 1988) similar values to those given here were reported.

3.4. Details of glutamine catabolism

The utilization of the different catabolic pathways depends upon the cell line and on the metabolic state, that is, the concentrations of glucose, glutamine, ammonia, etc. The fate of glutamine nitrogen has been studied by several groups, and includes results of early experiments with labelled metabolites (Eagle, 1959).

Using ^1H - and ^{15}N -nuclear magnetic resonance, Street et al. (1993) could show that most of the ammonia accumulating in cultures of HeLa and CHO cells was derived from the amido group of glutamine. Most of the glutamate was subsequently transaminated, rather than oxidatively deaminated by glutamate dehydrogenase. Between 35 and 50% of alanine in the medium was labelled due to transamination of glutamate, when $[2\text{-}^{15}\text{N}]\text{glutamine}$ was used. This percentage was considerably lower in a medium containing a low glutamine concentration. Incubating the culture with $^{15}\text{NH}_4\text{Cl}$ led to some labelled glutamate and alanine, indicating that the glutamate dehydrogenase catalyzed reaction was close to equilibrium, and was also active in the direction from α -ketoglutarate to glutamate. With CHO cultures, some ^{15}N was also found in the amido group of glutamine, due to glutamine synthetase activity. Direct incorporation of ammonium and bicarbonate into carbamyl phosphate, catalyzed by carbamyl phosphate synthetase, would have led to labelled arginine, which was not determined in this study. However, carbamyl phosphate synthetase, involved in urea synthesis, is active in kidney or liver cells, but can not be expected in all tissues.

The short-term metabolic fate of glutamate-nitrogen was investigated by Filc-DeRicco et al. (1990) in a mouse kidney carcinoma and in normal kidney cells in vivo, using the short-life nitrogen isotope ^{13}N . Aspartate aminotransferase activity was shown to be high in normal kidney and in tumor cells. Aspartate was rapidly labelled in only a few minutes, once glutamate entered the cells, indicating that aspartate aminotransferase was in thermodynamic equilibrium. In contrast, alanine aminotransferase was not in equilibrium, and the activity of this enzyme was much lower. The activity of glutamate dehydrogenase in the tumor was only about 6% of that in normal kidney cells. In the tumor, most glutamate

was converted to α -ketoglutarate via aspartate aminotransferase.

Similar results were found by Moreadith and Lehninger (1984a) and Moreadith and Lehninger (1984b) in studies of mitochondria from five different murine tumor ascites cells. Glutamine was deamidated by phosphate-dependent mitochondrial glutaminase, and glutamate was subsequently transaminated to α -ketoglutarate. No detectable flux through glutamate dehydrogenase could be measured, in spite of the high activity of this enzyme. However, the complex allosteric regulation of glutamate dehydrogenase may have strongly reduced the activity (Tipton and Couée, 1988). An unexpected finding was the inhibition of aspartate production from glutamate by the addition of malate to tumor mitochondria, since in normal mitochondria addition of malate stimulated aspartate formation. The authors explain this as resulting from differences in enzyme patterns in normal and tumor cell mitochondria. Tumor cell mitochondria exhibit malic enzyme activity, catalyzing the oxidative decarboxylation of malate to pyruvate, whilst normal kidney or liver mitochondria lack this enzyme. Externally added malate was transformed to pyruvate, whilst internally formed malate was oxidized to oxaloacetate, catalyzed by malate dehydrogenase. The end products of simultaneous glutamate and malate oxidation in tumor cell mitochondria were therefore citrate and alanine. Why malic enzyme has this preference for externally added malate could not be explained. Furthermore malate dehydrogenase activity in tumor cell mitochondria seems to be regulated in a different way to that in normal mitochondria, since the maximum activity of this enzyme is considerable greater than that of malic enzyme. However, it was later shown that mitochondrial enzymes undergo specific interactions with one another and with the inner mitochondrial membrane (Fahien et al., 1988, 1993; Teller et al., 1990). These authors studied the specific interactions between pyruvate carboxylase, aspartate aminotransferase, malate dehydrogenase and glutamate dehydrogenase in mitochondria. Ternary and quaternary complexes of these, and other, enzymes were formed under certain conditions. Preference of an enzyme for a substrate generated by another enzyme is therefore the consequence of steric proximity and direct interaction of the two enzymes. High protein concentra-

tions render the mitochondrial matrix very viscous, thus free diffusion of metabolites from one enzyme to another is unlikely to occur.

Kovacevic et al. (1988) raised the hypothesis, that the high level of aspartate production from partial glutamine oxidation in tumor cells might be essential for re-synthesis of AMP in the purine nucleotide cycle. Aspartate formed by mitochondrial aspartate aminotransferase is not in equilibrium with aspartate in the matrix but is directly transported to the cytosol (Teller et al., 1990). Aspartate is utilized together with inosine monophosphate (IMP), in the purine nucleotide cycle, to form adenylo-succinate and subsequently AMP. IMP is provided by means of the purine salvage pathway. The reason for the need to replenish the adenine nucleotide pool is the frequent episodes of anoxia that may occur in rapidly growing malignant cells. Since under anaerobic conditions there is a depletion of a part of the pool, re-oxygenation should ensure not only rapid synthesis of ATP, but also regeneration of the pool of adenine nucleotides. Similar results were published by González-Mateos et al. (1993). These authors found that glutamine and asparagine inhibited glycolytic

activity in tumor ascites cells, while contributing to the maintenance of high levels of adenylates by stimulating de novo synthesis of purines. The significance of the purine nucleotide cycle for ammonia production has been discussed by Lowenstein (1972).

A further aspect is the compartmentalization of amino acid metabolism in the cell, with a considerable part taking place in the mitochondria. Transport characteristics of the different carrier systems in the inner mitochondrial membrane may therefore play a role in regulation. However, in a recent review McGivan (1992) concluded that mitochondrial amino acid metabolism is not limited by transport capacity, and that in general there is no evidence to suggest that mitochondrial amino acid transport systems play any more than a permissive role.

In summary, ammonia accumulation in mammalian cell cultures is mainly due to glutamine catabolism. Tracer experiments showed that ammonia in the medium is derived from the glutamine amido group, the α -amino group is used in transamination reactions to form aspartate or alanine, rather than being oxidatively deaminated. Glutamine is rapidly catabolized in rapidly growing cells and

Table 2
Data for glutamine decomposition kinetics at 37°C

Medium	Serum	pH	Half-life (d)	Ref.
Eagle	containing (10% ?)	7.2	6.5	Tritsch and Moore (1962)
Eagle	serum free	7.2	16.9	Wein and Goetz (1973)
Eagle	10%	7.2	3.3	
DMEM	8%	7.1	6.7	Seaver et al. (1984)
DMEM	serum free	7.1	8.0	
DMEM	10	7.3	6.0	Glacken (1988)
MEM- α	10%	7.6	14.4	Kurano et al. (1990)
DMEM	10%	7.25	21.9	Lin and Agrawal (1988)
DMEM	serum free	7.25	50.7	
DMEM	10%	7.2	25.1	
DMEM	10%	7.6	8.6	
IMDM	0–10%	7.4	9.9	Ozturk and Palsson (1990)
RPMI1640	0–10%	7.4	6.8	
DMEM	0–10%	7.4	13.3	
OPTI-MEM	0–10%	7.4	11.3	
Gibco	serum free	7.3	6.2	Heeneman et al. (1993)
RPMI1640	10%	7.2	10.7	Schneider (1995)
FMX-Turbodoma	serum free	7.3	9.6	
FMX-Chomaster	serum free	7.3	17.0	

serves not only as a protein constituent but as a main energy source.

4. Chemical decomposition of glutamine

The second important source of ammonia accumulating in mammalian cell cultures is through chemical decomposition of glutamine (Bray et al., 1949; Gilbert et al., 1949; Tritsch and Moore, 1962; Lin and Agrawal, 1988; Ozturk and Palsson, 1990). Glutamine is chemically labile in aqueous solutions and cyclizes to form pyrrolidonecarboxylic acid and ammonia. The reaction is an intramolecular S_N2 -reaction, where the free electron pair of the α -amino nitrogen attacks the carbonyl carbon of the final amido group. After formation of a tetraedric transition state, the amido nitrogen is released as ammonia with the formation of pyrrolidonecarboxylic acid. This reaction can be considered as irreversible under cell culture conditions. Pyrrolidonecarboxylic acid can probably not be metabolized, and is not toxic at concentrations which routinely accumulate in cell cultures. Concentrations of up to 20 mM were reported not to affect growth of a murine hybridoma cell line (Stoll et al., 1995). A HPLC method for the quantitative determination of pyrrolidonecarboxylic acid in cell culture media has been reported by Stoll et al. (1994).

Glutamine decomposition follows first-order kinetics, leading to an exponential decrease of the glutamine concentration:

$$[gln]_t = [gln]_{t=0} \cdot e^{-k_{gln} \cdot t}$$

With $[gln]_t$ being the concentration of glutamine at time t and $[gln]_{t=0}$ the initial concentration; k_{gln} is the first-order reaction rate constant. The reported half-life varies between 6 to 20 days in different media (see Table 2).

The reaction kinetics strongly depend on the temperature and the chemical environment. The dependence on temperature can be described by an Arrhenius type equation (Tritsch and Moore, 1962). The reaction rate strongly increases with increasing pH (Bray et al., 1949; Seaver et al., 1984; Lin and Agrawal, 1988; Ozturk and Palsson, 1990), as the amino nitrogen is rendered less nucleophile due to protonation at lower pH. Lin and Agrawal (1988), as

well as Ozturk and Palsson (1990), found a 3-fold increase in reaction rate in certain media, when the pH was raised from 7.2 to 7.6. Both authors propose the following empiric correlation to calculate the reaction rate constant at different pH values:

$$\ln k_{gln} = a + b \cdot pH$$

Numerical values for the constants a and b for different media have been reported by the authors. Elevated concentrations of phosphate have further been shown to increase the reaction rate (Bray et al., 1949; Gilbert et al., 1949), while arsenate had a smaller effect, and chloride, sulfate, nitrate, acetate and pyruvate, in concentrations of up to 20 mM, did not show any measurable effect (Gilbert et al., 1949). Wein and Goetz (1973) and Lin and Agrawal (1988) reported a strong increase of the reaction rate with increasing serum content, while other authors found that the serum concentration had little or no influence (Seaver et al., 1984; Ozturk and Palsson, 1990).

The data given by the different authors is summarized in Table 2. The values are all of the same order of magnitude, except for those of Lin and Agrawal (1988). Why the latter values for the half-life are considerably higher is not clear. The contradictions concerning the influence of serum could depend on how it is pretreated, since serum may possess significant glutaminase and asparaginase activity (Wein and Goetz, 1973).

In conclusion, the chemical decomposition of glutamine strongly depends on the chemical environment in the medium. The reaction kinetics have therefore to be determined in every type of medium under the corresponding operating conditions.

The influence of glutamine decomposition on specific glutamine uptake rates and ammonia production rates has often been underestimated and therefore neglected. A detailed study by Ozturk and Palsson (1990) showed that the difference between apparent and real glutamine uptake rates and ammonia production rate may exceed 200%. This is confirmed by the results of Schneider et al. (1995) and those of Griffiths and Pirt (1967). However, until now, many authors do not take this into account for the calculation of specific rates. This omission easily leads to false conclusions, since the relative contribution of chemical glutamine decomposition becomes more important with higher glutamine concentrations. As

an example, the total amount of glutamine utilized in a batch culture of a hybridoma cell line increased by 25% when the initial glutamine concentration was raised from 4 mM to 10 mM, however, the actual glutamine uptake by the cells decreased by 15% (Schneider et al., 1995). The correction can be made by integration of the glutamine concentration during the culture, assuming that the reaction rate is constant during the whole period. However, considering the strong dependance on pH, it would be advantageous to measure directly the pyrrolidonecarboxylic acid produced (Stoll et al., 1994).

5. Mechanisms of ammonia toxicity

Relatively few articles have been published about the actual mechanisms of the toxic or inhibitory action of ammonia in the cell, and the exact mechanisms remain unclear. Ammonia or ammonium can either perturb the intracellular or intraorganelle pH and electrochemical gradients, or directly interact with enzymes.

5.1. Perturbation of intracellular pH and electrochemical gradients

In aqueous solution ammonia and ammonium are linked in a pH-dependent equilibrium according to the following equation:

$$\text{pH} = \text{pK} + \log \frac{[\text{NH}_3]}{[\text{NH}_4^+]}$$

with the pK having a value of 9.3 at 37°C. The protonation and deprotonation reactions are extremely fast thus ammonia, ammonium and the protons can be considered in equilibrium at any time and at any place. At the physiological pH of 7.1–7.5, which is also the pH at which the majority of mammalian cell cultures are carried out, only about 1% of the total concentration of ammonia and ammonium is present as NH_3 , the rest being NH_4^+ .

NH_3 (unprotonated ammonia) is a small, uncharged, lipophilic molecule, which readily diffuses across cellular membranes (Boron and De Weer, 1976; Knepper et al., 1989). The diffusion will follow the gradient of the chemical potential of NH_3 ,

which can be approximated by the gradient of the partial pressure of NH_3 . The small percentage of NH_3 present in the extra- and intracellular aqueous phases will diffuse across the membranes, thus rapidly equilibrating any transmembrane gradient of NH_3 . As protonation is extremely fast, the pH equilibrium will be reconstituted immediately. In a compartment with low pH, the partial pressure of NH_3 is lower, leading to a constant flow of NH_3 across the membrane into this compartment, until equilibrium is attained, i.e., the pH rises. In this way, ammonia can seriously disturb the functioning of organelles with low internal pH, such as lysosomes.

On the other hand diffusion of the protonated form, NH_4^+ , across cellular membranes is extremely slow. Values for the diffusion rate range from four to five orders of magnitude below those for unprotonated ammonia (Knepper et al., 1989). However, NH_4^+ can be transported actively across the cell membrane by specific transport proteins such as the Na^+K^+ -ATPase (Post and Jolly, 1957; Kikeri et al., 1989; Knepper et al., 1989), by facilitated diffusion by means of the $\text{Na}^+\text{K}^+2\text{Cl}^-$ -cotransporter, and possibly by the Na^+/H^+ -exchanger (Kikeri et al., 1989; Knepper et al., 1989). In the first two proteins, NH_4^+ is interacting with the binding site for K^+ , as hydrated NH_4^+ has practically the same ionic radius as K^+ (Knepper et al., 1989). In this way NH_4^+ is in competition with K^+ and perturbs the transmembrane transport of the latter (Wall and Koger, 1994). This may have important constraints for ion gradients over the cell membrane. Moreover, a coupling between aerobic glycolysis and the activity of Na^+K^+ -ATPase has been found by Lynch and Balaban (1987a,b) in two renal cell lines. These authors found that the maximum capacity for inward K^+ transport was substantially higher when the cells were grown in the presence of 10 mM glucose and 2 mM glutamine, than when the cells were grown on glutamine alone with all metabolic energy being derived from glutamine oxidation. The higher glutamine turnover in the latter case would increase the production of ammonia, and ammonium would compete with K^+ for the binding sites on the Na^+K^+ -ATPase. Furthermore, the Na^+K^+ -ATPase has a very high energy demand. For rabbit renal cells, it was estimated to be 50% of the total cellular energy production (Harris et al., 1981). Thus the mainte-

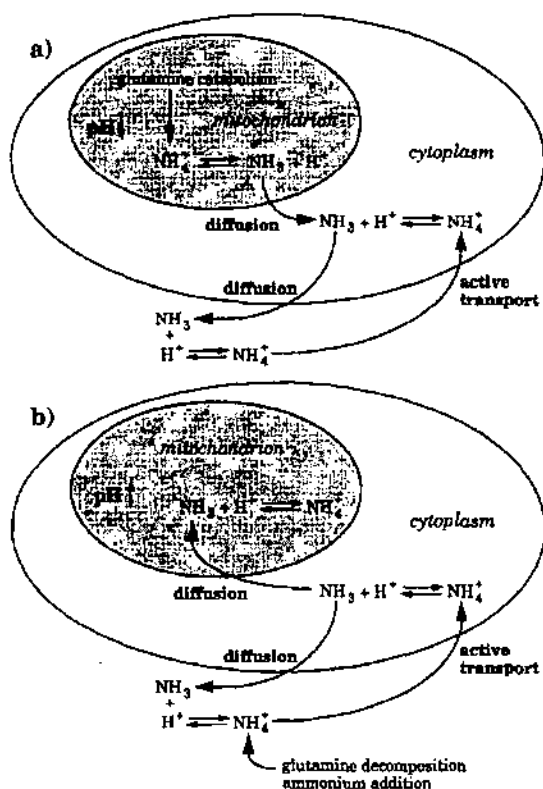


Fig. 2. Perturbation of intracellular pH and electrochemical gradients.

nance energy would be substantially increased and the efficiency of the carrier lowered, due to the competition between K⁺ and NH₄⁺.

Martinelle and Häggström (1993) have discussed the idea that a combination of NH₃-diffusion and NH₄⁺-transport across membranes leads to futile cycles and an increase in maintenance energy. Ammonia is produced, in the form of NH₄⁺, inside mitochondria (Fig. 2a) by the action of glutaminase and glutamate dehydrogenase, with the equilibrium between NH₃ and NH₄⁺ being immediately established according to the pH of the matrix. The inner mitochondrial membrane is extremely impermeable for ions, however, NH₃ readily passes from the mitochondria into the cytoplasm. This NH₃ outflow from the mitochondria leads to a decrease of the pH in the matrix, since a proton is left behind. NH₃ can diffuse out of the cytoplasm into the environment, however it can be transported back as NH₄⁺ by the carrier proteins. The consequence of such a

futile cycle, of NH₃ diffusing out of the cytoplasm and NH₄⁺ transported back, results in an acidification of the cytoplasm, and an alkalisation of the environment. Active transport by means of the Na⁺K⁺-ATPase can occur against the concentration gradient. As already mentioned, competition with K⁺ will increase the energy demand, since the K⁺ gradients have to be maintained. The net result of ammonium production by the aforementioned enzymes is an acidification of the mitochondrial matrix and of the cytoplasm, and an alkalisation of other organelles and the cellular environment.

Externally added ammonia (Fig. 2b) will transiently increase the pH of the cytoplasm due to rapid diffusion of NH₃ into the cell. This alkalisation is followed by an acidification due to transport of NH₄⁺ by carrier proteins. Diffusion of NH₃ into the mitochondria, and other organelles, leads to an increase of the pH inside these compartments. The result is an alkalisation of the cellular environment and of the interior of the organelles, mitochondria included, and an acidification of the cytoplasm. Ammonia resulting from the the decomposition of glutamine in the medium would have the same effect.

Thus, it is very important to realize that the physiological consequences of adding ammonia extracellularly to the medium are very different to those resulting from ammonia produced intracellularly. The first provokes an increase of the pH of the mitochondrial matrix, while the second will decrease this pH. As the main reactions of energy metabolism are located in the mitochondria, externally supplied ammonia could have very different effects on this metabolism compared with ammonia produced within the cells. This has to be taken into account when the ammonia sensitivity of a cell line is determined by the common practice of adding different amounts of ammonia to the medium.

5.2. Ammonia and enzymatic reactions

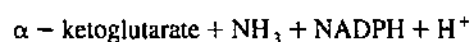
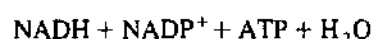
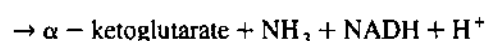
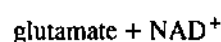
Ammonia or ammonium can participate in enzyme reactions and displace equilibria or interact with regulatory sites of enzymes.

It has been suggested that ammonia might drive a futile cycle of glutaminase and glutamine synthetase (Glacken, 1988). Glutaminase catalyzes the deamida-

tion of glutamine, to yield ammonium and glutamate, while glutamine synthetase catalyzes the ATP-dependent reverse reaction. An intercellular cycle of this kind is found in the intact liver between periportal hepatocytes and perivenous hepatocytes. Energy consuming cycling of glutamine serves as an efficient means for adjusting ammonia flux into urea or glutamine according to the needs of the acid base situation (Häussinger et al., 1992). Elevated ammonium concentrations have been reported to activate glutaminase of the liver type (Kovacevic and McGivan, 1983; McGivan and Bradford, 1983; Verhoeven et al., 1983). Glutamine synthetase activity was shown to be controlled by the glutamine level in the medium in cultures of neuroblastoma cells (Lacoste et al., 1982) and skeletal muscle cells (Feng et al., 1990). The latter showed, that neither glutamine depletion nor glutamine addition caused any change in the level of glutamine synthetase mRNA, and thus concluded that regulation must be post-transcriptional. Street et al. (1993) found elevated glutamine synthetase activity in CHO cells in the absence of glutamine, and increased glutamine synthetase activity in HeLa cells at low glutamine concentrations. In contrast, Miller et al. (1978) found no influence of glutamine concentration on glutaminase activity in cultures of 3T3-L1 cells, a transformed fibroblast cell line. Activation of glutaminase by ammonia, combined with deficient regulation of glutaminase activity, would lead to an ammonia driven futile cycle of these two enzymes.

Glutamate dehydrogenase might participate in a further futile cycle, which was discussed in a study upon ammonia and energy metabolism in isolated mitochondria and liver cells by Tagler et al. (1975). This mitochondrial enzyme catalyzes, on the one hand the oxidative deamination of glutamate and on the other hand the reverse reaction, the reductive amination of α -ketoglutarate. Vertebrate glutamate dehydrogenase can use both NAD and NADP as substrate, in contrast to plants and microorganisms which contain two different isoenzymes (Tipton and Couée, 1988). However, in the latter examples the catalytic properties depend on the coenzyme used. Excess of one component influences the equilibrium of the reaction, with accumulation of ammonia leading to a more oxidized state of the mitochondrial nicotinamide dinucleotides. The authors showed,

from kinetic considerations, that glutamate required NAD⁺, while α -ketoglutarate and ammonia required NADPH. Together with the energy-dependent transhydrogenase reaction, a futile cycle can thus be formed:



The sum of these reactions is an energy dissipation in the form of oxidation of NADPH by NAD⁺, driven by ammonia. The NMR study of Street et al. (1993) showed that if HeLa and CHO cells were incubated with ¹⁵N-ammonium, some labelled glutamate and alanine were found, indicating the formation of glutamate from ammonium and α -ketoglutarate catalyzed by glutamate dehydrogenase. Interestingly, the percentage of labelled glutamate and alanine was considerably higher, when a medium containing a low glutamine concentration was used. This would not support the hypothesis of an ammonium driven futile cycle, since ammonium incorporation into glutamate would be expected to increase at higher ammonium concentrations, such as those resulting from deamination of high concentrations of glutamine.

The transamination reactions, transferring the α -amino group from glutamate to pyruvate and α -ketoglutarate to yield alanine and glutamate, respectively, may play the role of ammonia detoxification reactions. Ammonia release by cells has been reported to be reduced in the presence of high ammonia concentrations in the surrounding medium, with a resulting increase in alanine production (Miller et al., 1988; Ozturk et al., 1992; Street et al., 1993; Hansen and Emborg, 1994). Excessive use of transamination for ammonia detoxification was suspected to lead to depletion of citric acid cycle intermediates. However, since numerous different anaplerotic pathways, such as the pyruvate carboxylase reaction, allow replenishment of this pool, this hypothesis appears to be unfounded.

The key enzyme of the glycolytic pathway, phos-

phosphofructokinase (PFK), has been reported to be activated by ammonia (Uyeda and Racker, 1965; Parmeggiani et al., 1966). Ammonium ions have also been shown to competitively counteract the inhibition of PFK by ATP in yeast (Sols and Salas, 1966). Thus elevated ammonium concentrations could disturb the feedback regulation of carbohydrate metabolism, leading to a high rate of glycolysis and lactate production. However, many different phosphofructokinase isoenzymes with different regulatory characteristics are known, and those of hybridomas, or other routinely cultivated cell lines, have not been investigated to our knowledge, consequently definitive conclusions are not possible.

A completely different mechanism of ammonia toxicity was proposed by Ryll and Wagner (1992) and Ryll et al. (1994). These authors investigated the intracellular pools of various ribonucleotides and showed that these nucleotides reflected the exact physiological state of a culture. UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine (UDP-GNAC) were shown to be the main targets during the inhibitory action of ammonium. These sugar nucleotides, which represent the activated forms of the corresponding sugars for glycosylation reactions, are synthesized in the cytoplasm of the cell. Synthesis starts with the amination of fructose-6-phosphate, from the glycolytic pathway, with glutamine or ammonium to form glucoseamine-6-phosphate catalyzed by glutamine-fructose-6-phosphate transaminase or glucosamine-6-phosphate deaminase, respectively. UDP-*N*-acetylglucosamine is formed by acetylation of glucosamine-6 followed by activation with UTP. The total amount of UDP-GNAC has been shown to increase just before the phase of reduced growth (Ryll et al., 1994). Moreover, effective glycolysis seems to be a pre-requisite for the formation of these activated sugars since the authors could not find a rapid increase of the UDP-GNAC pool in cells grown in glucose deficient media. This finding is related to the observation that the glycolytic activity of a HeLa cell line dropped by a factor of 900 when grown in the presence of 2 mM fructose instead of 10 mM glucose (Reitzer, 1978; Reitzer et al., 1980). In this case the metabolic energy was completely derived from glutamine, with fructose metabolized by the pentose phosphate pathway to provide ribose for nucleic acid synthesis. The level of intracellular

UDP-GNAC formation is cell line dependent and might be correlated to the ammonia sensitivity. The final reason why an increased UDP-GNAC pool is toxic for the cells is not clear. A high UDP-GNAC formation rate might result in a depletion of UTP, and the altered UDP-GNAC pool could have an influence on protein glycosylation.

Inefficient use of energy sources, due to futile cycles or disturbance of ion pumps, will result in a reduction of thermodynamic efficiency. Metabolic energy, which is not utilized for cell growth or cell function, will be dissipated as heat, leading to an increase of the specific heat production. The influence of ammonia on the specific heat production of mammalian cell cultures could therefore shed light on the metabolic affects of ammonia. An attempt to measure the heat production by animal cells was reported by Randolph et al. (1989).

6. Strategies to reduce ammonia formation

Strategies to reduce ammonia formation in mammalian cell cultures have to focus on the chemical decomposition of glutamine in the medium and, more importantly, on the cellular ammonia production due to amino acid, mainly glutamine, metabolism.

6.1. Strategies to overcome glutamine decomposition in the medium

The chemical decomposition of glutamine in the medium can be prevented by replacing glutamine by stable derivatives. If the α -amino group of glutamine is chemically bound and thus rendered less reactive, the decomposition reaction can be prevented. However, cells must be able to take up the glutamine derivatives rapidly and metabolize them. To this end dipeptides such as glycyl-glutamine have been used to substitute for glutamine (Brand et al., 1987; Roth et al., 1988; Holmlund et al., 1992).

6.2. Substitution of glutamine by glutamate, other amino acids or α -ketoglutarate

Most of the ammonia accumulating in mammalian cell cultures is derived from deamidation of glu-

tamine to glutamate catalyzed by glutaminase (Street et al., 1993). Substitution of glutamine by glutamate, or other amino acids, would overcome this problem and dramatically reduce ammonia formation by cellular metabolism and by glutamine decomposition in the medium.

In 1958, Darnell and Eagle first reported the addition of high concentrations of glutamate (20 mM) as a substitute for glutamine in HeLa cell cultures for Poliovirus production (Darnell Jr. and Eagle, 1958). Griffiths and Pirt (1967) investigated the influence of amino acids on the growth of mouse LS cells in batch and continuous culture. In this work glutamate could replace glutamine, however the requirement for valine was increased. These authors further showed that replacement of glutamine by glutamate significantly increased the efficiency of amino acid nitrogen incorporation into cell mass. Unfortunately, the authors did not give any values for ammonia concentrations, however, ammonia production was certainly reduced by the substitution of glutamine by glutamate.

Nagle and Brown (1971) successfully adapted cat kidney cells, HeLa cells and a substrain of mouse L-929 cells to growth in a chemically defined, heat-stable glutamine-free medium. These authors further found that, for mouse L-cells, supplementation with alanine was essential, in the absence of glutamine, and that glutamate could not replace glutamine. For HeLa cells, however, glutamate could be used as a substitute for glutamine. Keay (1977) reported growth of a substrain of mouse L-cells, and Vero cells, on an autoclavable, serum- and glutamine-free medium when supplemented with 0.5% bactopectone.

Kurano et al. (1990) substituted asparagine for glutamine in T-flask cultures of a CHO cell line. However the medium was serum supplemented, resulting in an initial glutamine concentration, due to the serum, of 0.05 mM. Cells were able to grow, after 4 days of cultivation, to the same cell density, however a 40% lower ammonia concentration was observed in the glutamine-free medium in comparison to control cultures.

The ability of various human leukemia cell lines to grow in the absence of glutamine was compared by Kitoh et al. (1990). These authors found that growth of B-lymphoblastoid cell lines, including the promyelocytic cell line HL-60, is highly dependent

on glutamine, whereas T-cell lines are able to proliferate in glutamine-deficient media. The pivotal role of glutamine synthetase was proposed as a biochemical basis for these differences, since glutamine-independent T-cell lines have a significantly higher activity of this enzyme compared with glutamine-dependent B-cell lines. The cell line HL-60 was adapted to growth in glutamine-deficient media by stepwise glutamine deprivation. This was accompanied by a reciprocal increase of glutamine synthetase activity, although the level was not considered significant.

The successful adaptation of three different cell lines to non-ammoniagenic media has been described by Hassell and Butler (1990). In this work glutamine was replaced by either glutamate or α -ketoglutarate. A mole to mole substitution of glutamine by glutamate was successful for a McCoy cell line and led to normal growth rates after approximately 10 days. Cell yield was increased by 17%, ammonia accumulation was reduced by 70%, and glucose consumption and lactate production both decreased by more than 70%. A BHK and a Vero cell line had to be slowly adapted from an initially high concentration of the glutamine analogue. The metabolic changes associated with this adaptation to glutamine-free growth were investigated by McDermott and Butler (1993). These authors compared the McCoy cell line mentioned above, that acquired normal growth rate rapidly, with a MDCK cell line, which could not be adapted to growth on glutamate. Depletion of glutamine led to increased glutamine synthetase activity in both cell lines, however there were significant differences in the ability to adapt for efficient glutamate uptake. The authors proposed, that glutamate uptake, and not glutamine synthetase, is responsible for the ability of a given cell line to grow glutamine free. This is confirmed by the observation that increased glutamine synthetase activity could be detected once glutamine was exhausted from the medium (Bushell et al., 1993).

In contrast, Bell et al. (1992, Bell et al., 1995) investigated a murine hybridoma cell line which could not be adapted to glutamine-free growth even in presence of elevated levels of glutamate. However, successful transformation with a cloned glutamine synthetase gene resulted in a cell line which could grow in the complete absence of glutamine. Ammonia concentrations in the medium of batch

cultures of these cells were below detection levels. The authors concluded that further strategies for manipulating cells to grow in glutamine-free medium should involve both glutamine synthetase and glutamate transport systems.

Bols et al. (1995) reported the successful growth of several fish cell lines in glutamine-free media. Fish cells, in contrast to mammalian cells, do not require glutamine. Thus they could be grown in media deficient of glutamine, glutamate and α -ketoglutarate without any adaptation. The observed differences with mammalian cells must be due to differences in the biochemistry of fish cells.

6.3. Nutrient control – controlled addition of glutamine and glucose

The excessive formation of ammonia due to glutamine catabolism can be reduced by limiting glutamine and possibly glucose availability, as suggested in a short overview by Glacken (1988).

Glacken et al. (1986) reported a substantial reduction of waste product excretion by feeding glucose and glutamine in a controlled manner to microcarrier cultures of human fibroblast and MDCK cells. Concentrations of glucose and glutamine were controlled at concentrations below 1 mM.

Ljunggren and Häggström (1994) studied glucose- and glutamine-limited fed-batch cultures of a hybridoma cell line and found that glucose and glutamine concentrations below 1 mM did not limit growth, although some unknown substrate was growth rate limiting in all cultures. These authors suspect that most of the published values for the saturation constants (K_s) for glucose and glutamine are too high. Glucose limitation alone did not reduce ammonia formation in comparison to a normal batch, however, glutamine limitation reduced ammonia release by about 50%, while dual glucose and glutamine limitation reduced ammonia release by almost 80%. Alanine and lactate production were also reduced. In the dual-limited culture, very little nitrogen was excreted in form of alanine. Thus most of the glutamine was metabolized by the glutamate dehydrogenase pathway which releases more energy per mol glutamine than the transamination pathway. Regulation of glutamate dehydrogenase is probably strongly dependent on the ammonia concentration,

and the reaction might be reversed by elevated ammonia concentrations. In conclusion, the dual substrate-limited fed-batch culture reduced overflow metabolism to lactate, alanine and ammonium formation, and increased energetic efficiency of the metabolism. An analogous study was performed with a murine myeloma cell line by the same authors yielding similar results (Ljunggren and Häggström, 1992).

A further strategy to reduce cellular ammonia production depends on amino acid nutrition, which has been shown to influence the relative contribution of ammonia and alanine for amino acid nitrogen excretion (Hiller et al., 1994). A substantial decrease of the fraction of nitrogen excreted as ammonia occurred upon supplementation of a continuous hybridoma culture with elevated concentrations of branched chain amino acids (leucine, isoleucine, valine) and lysine. This decrease was compensated by an increase of the fraction excreted as alanine. Alanine seems to be less harmful to cell cultures than ammonia, since the exogenous addition of up to 10 mM alanine had no effect on the growth of two hybridoma cell lines (Duval et al., 1991).

6.4. Substitution of glucose by other sugars

In the hypothesis discussed by Ryll et al. (1994), that the intracellular pool of UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine (UDP-GNAc) might be the central target in the inhibitory action of ammonium, an effective glycolysis for the formation of these activated sugars is required. The first step in the formation of UDP-GNAc is the transamination of fructose-6-phosphate, as already described in the section 'Mechanisms of ammonia toxicity'. The formation of UDP-GNAc at elevated ammonium concentrations is therefore dependent on the availability of fructose-6-phosphate. The authors found that practically no accumulation of UDP-GNAc could be observed when the cells were grown on fructose, mannose or galactose in place of glucose. Drastically reduced glycolytic activity due to substitution of 10 mM glucose for 2 mM fructose was also reported by Reitzer et al. (1980) for a HeLa cell line, and by Glacken et al. (1986) for cultures of human fibroblasts, in which glucose was replaced by galactose. The catabolism of fructose or other sugars, mainly

by the pentose phosphate pathway, seems not to provide sufficient amounts of fructose-6-phosphate for UDP-GNAc formation. Further studies will be needed to show whether ammonia susceptibility of a cell line can be overcome by reducing rapid glycolysis which also reduces UDP-GNAc accumulation.

González-Mateos et al. (1993) further reported an inhibitory effect of glutamine and asparagine on rapid glycolysis. Either of these amino acids decreased the glycolytic flux by about 80%.

7. Strategies for ammonia removal

Strategies using different principles for ammonia or ammonium removal have been reported in the literature: (1) the use of gas-permeable, hydrophobic porous membranes; (2) application of nonporous ion-exchange membranes; (3) use of ion-exchange resins; (4) electrodialysis. Care has to be taken to avoid the non-selective removal of other substances, especially essential cations in the case of ion-exchange resins and membranes. Moreover, the biocompatibility of the different materials used for ammonia removal has to be assured. For applications in production processes the system has to be simple in handling and very reliable.

7.1. Ion-exchange resins

Ion-exchange resins allow removal of ammonium ions by adsorption. The non-selective removal of other cations, mainly potassium and sodium, can cause problems. Moreover, the resins have to be reconstituted after being saturated, making the application to continuous processes difficult.

Iio et al. (1984) utilized the ion-exchange powder silica-aluminium ZCP-50 in a dialysis tubing for ammonia adsorption in culture dishes. The cell density in cultures of a murine myeloma cell line could be increased by reducing the ammonia concentration. However, while growth of a human hybridoma in serum supplemented medium did not improve, in serum-free medium the cell density increased.

Clinoptilite, a volcanic rock with natural cation exchange properties, has been used to selectively adsorb ammonium in mammalian cell cultures (Carbonell et al., 1992; Capiaumont et al., 1995). A

culture of immobilized hybridoma cells was connected to a column containing the adsorbent and a detoxification cycle performed every 2 days for 2 h. By this method the ammonia concentration could be reduced by more than 60% by comparison to a control experiment without ammonium removal. However, the antibody concentration in both experiments were similar, and the cell concentration could not be increased substantially.

Jeong and Wang (1992) added powder of Phillipsite-Gismondine, a zeolite, to a spinner flask hybridoma culture. Ammonium ions were selectively adsorbed, however, cell density and antibody production were not significantly improved.

Nayve Jr. et al. (1991, 1994) used a column packed with Zeolithe A-3 to adsorb ammonium. A perfusion culture, with cell retention, of a hybridoma cell line was connected to a cross-flow ceramic filter module to obtain cell free culture broth. The cell-free filtrate was subsequently dialyzed and passed through the Zeolithe column to remove ammonium. The ammonium removal system was shown to be effective and the viable cell density and antibody production could be substantially increased in comparison to control experiments. Unfortunately this system is very complicated from an experimental point of view. However, the results of this work clearly show the inhibitory effect of ammonia on mammalian cells, particularly for high cell density cultures. The majority of publications concerning mammalian cell cultures in combination with ammonia removal found little effect on cell density due the reduced ammonia concentration. However, the viable cell density, in the case of Nayve Jr. et al. (1994), did increase to $2.5 \cdot 10^7$ cells ml^{-1} . These results suggest that, in contrast to a widely held view, ammonia is probably not responsible for the rapid decline in viable cell densities towards the end of batch cultures.

7.2. Non-porous ion-exchange membranes

Non-porous ion-exchange membranes allow the transport of ammonium across the membrane from the culture to the permeate side, from where it can subsequently be removed. Problems can arise from non-selective removal of other cations.

Sikdar and Sawant (1994) investigated the application of a non-porous ion-exchange membrane of

the perfluorinated sulfonic acid type (PFSA). The removal of ammonia on the permeate side was performed using aqueous phosphate buffer, pH 7.2 or aqueous sulfuric acid 0.025 M. Alternatively, pervaporation using a vacuum or pervaporation using an inert gas sweep could be used. Pervaporation, using an inert gas sweep, was found to be superior to the other methods examined. Since the ammonia flux across the membrane was higher when the pH of the source solution was increased, the authors concluded that NH_3 and not NH_4^+ was the diffusing species. Unfortunately the pH of the sink solution was increased in parallel for these experiments. However, since the use of acid as chemical sink did not increase the transfer rate, as would have been expected, this suggests that NH_4^+ and not NH_3 , might have been the diffusing species. No results of mammalian cell cultures performed in combination with this process were presented.

A cation-exchange membrane has been applied to cultures of a suspended growing hybridoma cell line (Thömmes, 1992; Thömmes et al., 1992). The culture was pumped through an external module equipped with a Nafion-membrane, and the transferred NH_4^+ was trapped in an alkaline acceptor stream on the permeate side. The process was very efficient, due to the much larger driving force in comparison to processes using hydrophobic gas-permeable membranes, where NH_3 is transported. Both cell density and antibody production could be increased at the beginning of the culture by means of the membrane process. However, precipitation of carbonates, probably of magnesium and calcium, in the alkaline acceptor stream possibly led to depletion of these essential cations, resulting in a rapid decline of viable cell density after a certain time. This problem might be overcome by removing ammonia by means of pervaporation, as described by Sikdar and Sawant (1994).

7.3. Gas-permeable hydrophobic membranes

Gas-permeable hydrophobic membranes allow the selective transport of volatile ammonia across the membrane and subsequent removal on the permeate side. The driving force for this transport is a difference in partial pressure of ammonia across the membrane. Hydrophobic porous membranes made of

polytetrafluoroethylene (PTFE) or polypropylene (PP) have been used for this purpose. The membrane is in contact with the culture medium and an acid placed on the permeate side. Due to the hydrophobic nature of the membrane, the aqueous liquids on both sides of the membrane do not penetrate into the pores, and are therefore perfectly separated from each other. However, gases can easily pass the membrane through the pores, following the respective partial pressure gradients. Volatile NH_3 diffuses across the membrane and is trapped in the acid on the permeate side as a result of an instantaneous and irreversible. Ionic NH_4^+ is unable to cross the membrane. Since the pH of the culture medium is generally about two units below the pK , only 1% of the total concentration of ammonia and ammonium is present as NH_3 , the rest being NH_4^+ . The NH_3 concentration gradient is therefore relatively small, thus this method requires a large membrane area. The mass transfer characteristics of the process itself have been investigated in detail (Brose and Van Eikeren, 1990; Schneider et al., 1994), and the process has been patented by Bend Research Inc. (Bend Research Inc. (1990), Eur. Patent Application; Bend Research Inc. (1991), US Patent).

Mammalian cell cultures have been performed in combination with the hydrophobic membrane process, with efficient NH_3 removal (Capiumont et al., 1995; Schneider et al., 1995; Schneider, 1995). Capiumont et al. (1995) connected a hollow fibre module containing PP-membranes to a bioreactor with immobilized hybridoma cells. These authors found a 60% increase of the final cell density due to the reduced ammonia concentration compared with control experiments. However, the antibody concentration in the medium did not significantly increase. The apparent increase in cell density may be unreliable due to the method used for counting since the cells were detached after terminating the experiment, moreover each experiment was carried out only once. Schneider et al. (1995) performed batch suspension cultures of a hybridoma cell line in a purpose-built membrane reactor with an integrated flat PTFE-membrane. No increase in cell density or specific antibody productivity, due to the reduced ammonia concentration, could be found compared with control experiments. This was shown to be due to the low final cell densities attained, since growth was limited

by nutrient availability. However, energy metabolism was affected by ammonia removal, resulting in a reduced lactate production and increased glutamine consumption.

Ammonia removal from aqueous liquids using hydrophobic membranes has also been successfully applied to an antibiotic fermentation (Hecht et al., 1989) and has been described in detail for applications in chemical and environmental engineering (Imai et al., 1982; Blet et al., 1989; Semmens et al., 1990). Thus, it would appear that the use of hydrophobic porous membranes for ammonia removal has considerable potential, particularly for high cell density systems such as those involving immobilized cells.

7.4. Electrodialysis

Ammonium ions can be removed from cell culture media by the application of an electrokinetic technique with an electrophoretic mechanism (Chang et al., 1995a,b). This technique involves the continuous application of a direct current electric field to selectively remove ammonium, while simultaneously removing lactate. Externally added ammonium (10 mM) and lactate (45 mM) could be successfully removed under an electric current density of 50 A m^{-2} without changing the chemostatic conditions of the cell-free culture medium. Batch and glutamine fed-batch cultures of a murine hybridoma cell line have been performed in combination with a waste product removal process. Ammonium was almost completely removed and the maximal cell density and antibody concentration were increased by 30% and 50%, respectively, while the specific glutamine consumption rate increased by 20 to 30% compared with control experiments performed in the absence of the electrodialysis process. These findings confirm those obtained by Schneider (1995) and Schneider et al. (1995) in which removal of ammonia or ammonium increased the specific glutamine uptake rate which resulted in an increased ammonia production rate. Thus glutamine catabolism was accelerated by ammonia removal. This situation could be a disadvantage to the application of this approach since Martinelle and Häggström (1993) raised the hypothesis that ammonia produced within mitochondria by catabolic reactions might be more harmful to the

cells than externally added ammonia (see 'Mechanisms of ammonia toxicity').

8. Conclusions

Ammonia accumulation in mammalian cell cultures is due to amino acid catabolism, mainly that of glutamine, and chemical decomposition of glutamine in the medium.

Many different effects of elevated concentrations of ammonium and ammonia (total concentrations from 2 to 10 mM) on mammalian cell cultures have been reported. These include reduction of the specific growth rate and the final cell density in batch cultures, induction of apoptotic and necrotic cell death, perturbation of protein glycosylation and secretion as well as inhibition of virus proliferation in cells. The specific antibody productivity has generally found to be unaffected by ammonia. The critical ammonia concentration appears to be strongly dependent on the cell line and cultivation conditions. However, in contrast to a widely held view, ammonia is probably not responsible for the rapid decline in cell viability towards the end of low density ($< 1 \times 10^6 \text{ cells ml}^{-1}$) batch cultures.

Many cell lines cultivated in vitro are derived from malignantly transformed cancer cells. These cells, but also other normal, fast growing cells such as lymphocytes or enterocytes, have special metabolic characteristics, principally rapid aerobic glycolysis and glutaminolysis. The reason for this apparently very inefficient metabolism with a high level of lactate and ammonia excretion is unknown, since growth seems not to be limited either by energy expenditure nor biosynthetic requirements.

Glutamine is metabolized to glutamate and α -ketoglutarate, which enters the cellular energy metabolism. The carbon of glutamine is mainly recovered as CO_2 , alanine, aspartate and lactate. The amido-nitrogen of glutamine is mainly liberated by glutaminase activity and excreted into the medium in the form of ammonium, whereas the amino-nitrogen is mainly transaminated to α -ketoacids such as pyruvate or oxaloacetate, to yield alanine and aspartate, respectively. The activity of glutamate dehydrogenase, which is possibly reduced in the presence of elevated ammonium concentrations, liberates a second ammonium molecule per glutamine molecule.

Ammonia influences cellular energy metabolism by influencing the relative amount of carbohydrate and glutamine taken up, and by altering the utilization of the different metabolic pathways, including glycolysis, the citric acid cycle, pentose-phosphate pathway and the different mechanisms of glutamine degradation. This influence could be due to interactions with regulatory key enzymes of the different pathways. Increased ammonia concentrations generally reduce the specific ammonia production rate of the cells, with more nitrogen being excreted in the form of amino acids such as alanine, glutamate and aspartate.

The cellular mechanisms of ammonia or ammonium toxicity are still controversial subjects. Ammonia toxicity has been shown to be more pronounced at higher pH, indicating that unprotonated ammonia (NH_3) and not ammonium ions (NH_4^+) is responsible for the toxic action. Several hypotheses to explain ammonia toxicity have been proposed. Some are based on the physico-chemical characteristics of ammonia and ammonium, others suspect ammonia or ammonium to interact directly in enzymatic reactions or regulatory sites of enzymes. It is an important fact that NH_3 readily diffuses across cellular membranes, while diffusion of NH_4^+ is extremely slow, although NH_4^+ can be transported across most membranes by means of carrier proteins. Competition of NH_4^+ with potassium for inward transport with the Na^+/K^+ -ATPase could considerably increase the maintenance energy. Ammonia has further been suspected to trigger different futile cycles in the cell while, recently, activated sugar molecules were suspected to be the main target of ammonium toxicity.

The chemical decomposition of glutamine is considerable and has to be taken into account for the calculation of the specific glutamine uptake rate and specific ammonia production rate. The reaction rate constant has to be determined for each medium under the operating conditions, taking particular account of the rapid increase of the decomposition rate with increasing pH. The amount of glutamine decomposition can be calculated (integrated) from reaction kinetics or, better still, by measurement of pyrrolidonecarboxylic acid produced by means of HPLC techniques.

Ammonia production may be reduced by optimization of the glucose and glutamine feeding strat-

egy. For many cell lines glutamine may be substituted by glutamate, resulting in a strong reduction of the specific ammonia production rate. Substitution of glucose by other sugars, such as fructose, can prevent the high rate of aerobic glycolysis with subsequent lactate formation.

Ammonia removal systems allow a reduction in ammonia inhibition of mammalian cell cultures. Such systems include gas-permeable, hydrophobic porous membranes, ion-exchange membranes or ion-exchange resins and electrodialysis. However, a substantial improvement to viable cell density and antibody concentration are possible only in high density culture systems where very large amounts of ammonia are produced.

While a considerable amount of literature has appeared during the last 30 years, progress in mammalian cell technology appears to be limited. This possibly reflects the fact that, despite all the efforts made, the underlying principles of regulation and utilization of different metabolites by mammalian cells remain poorly understood.

In summary, ammonia and lactate formation are due to an inefficient overflow metabolism. In the presence of high concentrations of glucose and glutamine, glucose is rapidly metabolized by aerobic glycolysis and most metabolic energy is derived from glutamine oxidation. The accumulation of these metabolites to potentially inhibitory levels can be overcome by nutrient control, controlled feeding of glucose and glutamine, or substitution of these substrates by other sugars and amino acids, which are metabolized more efficiently. If these approaches are not successful a number of ammonia removal systems may be applied.

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Improvement of CHO Cell Culture Medium Formulation: Simultaneous Substitution of Glucose and Glutamine

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The formulation of the culture medium for a Chinese hamster ovary (CHO) cell line has been investigated in terms of the simultaneous replacement of glucose and glutamine, the most commonly employed carbon and nitrogen sources, pursuing the objective of achieving a more efficient use of these compounds, simultaneously avoiding the accumulation of lactate and ammonium in the medium. The key factor in this process is the selection of compounds that are slowly metabolized. Among the different compounds studied, galactose and glutamate provide the best results, allowing support of cell growth with an optimal balance between nutrient uptake and cell requirements and the generation of minimal quantities of lactate and ammonium. The attained results also highlight the capacity of the cells to redistribute their metabolism as a response to the changes in medium composition.

Introduction

The formulation of optimal culture media for animal cell growth has received a lot of interest for many years as a result of its critical impact on the development of bioprocesses based on animal cell technology (Sato, 1975; Kovar and Franek, 1986; Castro et al., 1992; Keen and Rapson, 1995; Lao and Shalla, 1996). Undoubtedly, the replacement of fetal calf serum is the most noteworthy advancement, particularly if it can be achieved by media containing low protein concentration or media that are completely protein-free. When this is achieved, the process can be operated with a medium that is well defined, that is, with constant quality and composition, and this has a major impact in terms of process reproducibility, downstream processing, and sometimes in the medium cost. Also, from the point of view of regulation and acceptability of the final product, especially if it has a therapeutical use, it will be an increasingly important requisite to grow the cells in media without any components originated from an animal source, such as fetal calf serum. All of these reasons have generated a lot of interest, and various successful formulations have been developed, leading to a number of protein-free and serum-free media that are commercially available today. In addition to these efforts, the reformulation of media with different types of compounds, such as vitamins, growth factors, hormones, lipids, trace elements, or amino acids, has been addressed. An important criterion in the development of new media is, in addition to the supplementation of all the necessary nutrients, to do it in a balanced way, avoiding an unnecessary increase in the osmolarity of the medium and also in the generation of metabolic waste products (Castro et al., 1992; Xie and Wang, 1994; Lao and Shalla, 1996).

In this effort for medium reformulation, it has been generally accepted that the compounds to use in the medium as main carbon and energy sources are glucose and glutamine. However, the rapid glucose and glutamine metabolism that animal cells exhibit when they are cultured *in vitro* leads to a very inefficient use, causing

their rapid depletion from the culture medium and eventually the accumulation of lactate and ammonium ions in the medium. These products often have inhibiting effects for the cells, specially in the case of ammonium (Hansen and Emborg, 1994; Ozturk et al., 1992; Ryll et al., 1994). It has previously been demonstrated in chemostat cultures (Sanfeliu et al., 1997) that cells cultured in media with high glucose and glutamine concentrations exhibit much higher uptake rates than those strictly necessary for their metabolism. The solution to this problem has been addressed in many different ways. From the macroscopic point of view, that is, the definition of the extracellular conditions in the bioreactor where cell growth takes place, the fed-batch addition of glucose and glutamine to the medium (Ljunggren and Häggström, 1994; Xie and Wang, 1994; Bibila and Robinson, 1995; Zhou et al., 1995; Zhou et al., 1997) or the *in situ* removal of the inhibitory compounds (Matsumura and Nayve, 1995) are two possible strategies that generally require a high degree of instrumentation to be performed in an optimal way. From the microscopic point of view, which is at the cellular level, one of the findings to highlight is the modification of hybridoma and myeloma cells with the glutamine synthetase gene (Bibila and Robinson, 1995; Zhou et al., 1997; Paredes et al., 1999). In this way, cells can grow in medium with glutamine being replaced by glutamate, which is consumed in a more efficient way, producing a lower ammonium accumulation and allowing the extension of fed-batch cultures for a longer time. In this case it is interesting to point out that the glucose consumption and lactate accumulation is also reduced, showing how glutaminolysis and glycolysis pathways are interconnected in the metabolism of these cells. An intermediate possibility to improve cell growth patterns is to find alternative compounds for the cells to use as carbon and energy source, in theory without the need to undertake any genetic modification. The basic requirements for this substitution would be, first, that cells could grow in the new conditions, and second, that the metabolic uptake of the new compounds would be markedly

lower than glucose and glutamine uptake. In this way, it could be envisaged that the cells would use the main compounds of the medium more efficiently, generating a smaller amount of metabolic waste products, particularly lactate and ammonium, and therefore allowing the establishment of fed-batch or high-density perfusion cultures without inhibition limits.

The simultaneous substitution of both glucose and glutamine in the medium formulations has not been addressed previously, and only partial substitutions have been discussed, although in most cases not as a result of an analysis at the process level, linked to the metabolic patterns of the cells. Indeed, for some cells, particularly for hybridomas, it has been shown that this substitution is not possible (Wagner, 1997), at least not without genetic modification of the cells, for example, with the glutamine synthetase approach already discussed. For other cell lines, more studies have been reported. Wagner et al. (Wagner et al., 1991) showed that human tumor kidney cells could be grown on microcarriers, with slower cell death, using a mixture of glucose and galactose. Kurano et al. (Kurano et al., 1990), after discussing the inhibitory levels of ammonium and lactate, replaced glutamine by asparagine for the culture of CHO cells, obtaining a clear reduction in ammonium accumulation and the improvement of cell growth. These results were also confirmed by the work of Hansen and Emborg (Hansen and Emborg, 1994). Reitzer et al. (1979) in their very interesting discussion on the identification of glutamine as the major energy source for HeLa cells showed that cells can grow in a similar way on galactose or fructose as the main sugar in the medium instead of glucose, providing remarkably lower amounts of lactate. Marquis et al. (1996) provided data on the growth of a lymphoblastoid cell line on a medium with glucose replacement by galactose, with lower lactate production.

In addition to the changes in the primary metabolism that the substitution of glucose and glutamine would generate in the cells, it is also very interesting to study other metabolic modifications, for example, at the level of amino acids consumption or excretion, to generate hypotheses about the overall changes introduced in the cell behavior. In the present work, the substitution of glucose and glutamine in a low-protein medium for the culture of a Chinese hamster ovary (CHO) cell line producing t-PA in batch systems is analyzed from the point of view of the physiological changes observed in the cells and their internal metabolic redistribution. This substitution has been addressed by selecting a slowly metabolized sugar compound to replace glucose as carbon source. This compound should be a hexose sugar, as the cells used (a CHO cell line) do not perform gluconeogenesis activity. The entry of the sugar molecule into the glycolytic pathway should therefore be upstream of the metabolic step generating phosphogluconate, which in turn is essential to generate pentose molecules for the nucleic acids synthesis. With regards to glutamine, the objective of this work is to investigate its replacement by glutamate, as CHO cells do have the glutamine synthetase activity expressed constitutively. In addition to the fact that glutamate has only one amino group in its structure, the fact that glutamate is transported into the cell at a lower velocity should provide a more efficient use of this compound with lower generation of ammonium, because spontaneous decomposition would also then be minimized, with respect to the situation where glutamine is used.

Material and Methods

Cell Culture. The cell line CHO TF 70R, kindly provided by Pharmacia & Upjohn (Stockholm, Sweden), has been manipulated genetically to produce t-PA. The cell cultures were carried out in spinner flasks (Techné) with a working volume of 125 mL (or 250 mL) and stirred at 50 rpm in a CO₂ incubator (Forma Scientific CO₂ incubator), at 37.0 °C, with 96% relative humidity in an atmosphere of 5% CO₂ in air.

Culture Medium. The basal medium used in all of the cultures was a proprietary serum-free and low-protein medium denominated BIOPRO1, kindly provided by Bio Whittaker Europe (Verviers, Belgium). BIOPRO1 was supplemented with vitamins (Sigma), lipids and cholesterol (GibcoBRL), proline, serine, and aspartic acid (Sigma). This medium was also supplemented with 20 mM glucose, fructose, mannose, or galactose and 7 mM glutamine or glutamic acid in the different experiments, as indicated in Figures 1 and 3. The culture of the cells in the different media was carried out without previous adaptation. Cells used for inoculum ($0.18\text{--}0.20 \times 10^6$ cells/mL) were centrifuged to remove the culture medium, particularly to ensure that neither remaining glucose nor glutamine from inoculum preparation medium was present. This step was repeated 3–5 times, keeping the cells subcultured in the corresponding carbon and nitrogen source combination under study, before the realization of the experiment. In those passages the growth of the cells was very reproducible.

Analytical Methods. Cell Number. Cells were counted using a hemacytometer (Neubauer improved, Brand). Cell viability was determined by the trypan blue exclusion method (1:1 mixture of 0.2% trypan blue in normal saline and cell sample). After cell counting, the remainder of each sample was centrifuged (5000g, 1 min) to remove the cells, and the supernatant was frozen for further analysis.

Metabolite Determinations. Glucose and lactate concentrations were determined with a YSI 2700 automated glucose and L-lactate analyzer. Ammonium concentration was determined by a flow injection analysis system as previously described (Campmajó, 1994). Amino acids concentrations were measured by HPLC (Hewlett-Packard 1090) using a reversed-phase column (aminoquant 200 mm $\times$ 2.1 mm) after derivatization of the samples with *O*-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) for the analysis of both primary and secondary amino acids. The concentration of t-PA was quantified by enzyme immunoassay (Biopool Imulyse t-PA kit, Diagnostic International, Hannover, Germany).

Specific Rate Calculations. The specific growth rate (μ) in the exponential growth phase was calculated by linear regression of the logarithm of the concentration of viable cells, X_v , as a function of time, t , (eq 1)

$$X_v = X_{v0} \exp(\mu t) \quad (1)$$

In the above expression, X_{v0} is the initial cells number (10^6 cell mL⁻¹). The specific consumption/production rates, q , in nmol h⁻¹ 10⁻⁶ cells, were calculated using the following expressions

$$S = S_0 + \frac{q_s \cdot 10^{-3} X_{v0}}{\mu} [\exp(\mu t) - 1] \quad (2)$$

where S is the concentration of the different compounds

investigated (carbon and nitrogen sources, and products) and S_0 refers to its initial concentration (mM).

In the case of glutamine, this spontaneously decomposes, releasing ammonia following first-order kinetics. Its rate constant, k_d , has been evaluated in the experimental conditions used in this work, obtaining a value of $2.01 \times 10^{-3} \text{ h}^{-1}$. Thus equations for glutamine and ammonium were modified conveniently to take into account this fact:

$$\text{Gln} = \text{Gln}_0 \exp(-k_d t) + \frac{q_{\text{Gln}} 10^{-3} X_{v0}}{k_d + \mu} [\exp(\mu t) - \exp(-k_d t)] \quad (3)$$

$$\text{NH}_4^+ = \text{NH}_4^+ + \frac{X_{v0} 10^{-3}}{\mu} \left[q_{\text{NH}_4^+} + \frac{k_d q_{\text{Gln}}}{\mu + k_d} \right] [\exp(\mu t) - 1] + \left[\text{Gln}_0 - \frac{q_{\text{Gln}} 10^{-3} X_{v0}}{\mu + k_d} \right] [1 - \exp(-k_d t)] \quad (4)$$

Results and Discussion

Substitution of Glucose by a Hexose. In a first series of batch experiments, the glucose substitution in the formulation of the culture medium was investigated, with glutamine maintained in all cases as nitrogen source. Three different compounds were tested: fructose, galactose, and mannose. Glucose was used as the control system. The results are presented in Figure 1. It can be observed (Figure 1a) that the maximal cell concentration achieved for glucose and mannose is similar, reaching a level of 1.2×10^6 cells/mL, whereas for galactose and fructose it is lower, 0.8×10^6 and 0.5×10^6 cells/mL, respectively. The specific growth rate on glucose and mannose was almost the same, as reflected in Table 1, and higher than for galactose and fructose. With respect to the consumption of the carbon source (Figure 1c), two groups of compounds can be distinguished, with marked differences: glucose and mannose are consumed at a high rate, whereas fructose and galactose are consumed much more slowly. The specific consumption rates are provided in Table 1, and it should be highlighted that the values observed for the consumption of fructose and galactose are around 25% of that for glucose. As a consequence of these different consumption rates, the generation of lactate also differs greatly. For glucose and mannose, the high consumption rate leads to high lactate generation, and lactate concentration reaches levels of about 16 mM (Figure 1d), with high values for the specific production rate (see Table 1). On the other hand, when cells are grown on fructose and galactose, the lactate production decreases drastically, and final lactate concentrations are below 1 mM, the specific lactate production rates being about 22% of the values corresponding to glucose. When the glutamine consumption is analyzed (Figure 1e) a similar profile is observed for all of the cultures; however, taking into account the different level of cells generated, it can be calculated that the specific consumption of glutamine is notably higher when galactose and fructose are used, as reflected in Table 1. It can be observed that in the case of the medium formulation with galactose, an abrupt cell concentration decline occurs at the point when glutamine is exhausted. These profiles are linked to those corresponding to the ammonium ion generation in the medium (Figure 1f), which are higher for the compounds providing a higher specific consumption of glutamine (fructose and galactose), reaching levels of 13 mM for ammonium, and lower when glucose or mannose

are used, reaching 9 mM for ammonium. Finally it should be mentioned that the profile of the t-PA concentration follows that of the cell growth (Figure 1b), and therefore a higher t-PA level is obtained when a higher cell concentration is generated.

The analysis of the previous results from the metabolic approach can be done at different levels. The scheme of the most relevant metabolic pathways for the metabolism of sugar compounds and glutamine in animal cells is presented in Figure 2. It can be observed that mannose enters into the glycolytic pathway at the level of fructose-6-P, whereas galactose does so at the level of glucose-6-P. Nevertheless, mannose is consumed at a much faster speed than fructose, with a rate similar to that for glucose.

The observation of the experimental data suggests some hypotheses with respect to the transport of these compounds into the cells. Mannose and glucose seem to be transported at the same velocity into the cell, either through the same transporter or a different one. On the other hand, fructose is probably transported more slowly into the cells, as a result of a lower affinity of the fructose transporter. With respect to galactose, in theory one might expect to find a consumption rate similar to glucose, as both sugar molecules are incorporated into the glycolysis at the level of glucose-6-P. However, the observed rate is considerably slower, and again this fact could be explained by a low transport rate of galactose into the cells. Indeed, it has been previously reported that, for several mammalian cell lines (Plagemann et al., 1981) galactose is taken up into the cells through the same transporter as glucose but with an affinity which is eight times lower. A second possibility that could explain the different consumption rate of the compounds studied is a lower affinity of the enzyme hexokinase for galactose and fructose (Wagner, 1997).

For the quickly metabolized sugars, glucose and mannose, the cell converts most of them into pyruvate through the glycolytic pathway, and probably a small percentage is cycled through the pentose phosphate cycle to obtain the necessary molecules to build compounds essential for the cell viability. It can also be observed from the experimental results that a high percentage of pyruvate is metabolized to lactate. The explanation for this fact may be found in the need for the cell to balance its redox potential (Paredes et al., 1998). It should also be mentioned that it has been previously demonstrated that this pattern has been observed in other cell lines, independently of the oxygen level in the culture system (Sanfeliu et al., 1996), and in consequence it cannot be attributed to a lack of oxygen.

It can be observed that for the sugars presenting a lower glycolytic rate, fructose and galactose, glutamine is consumed at a higher specific rate (see Table 1) and that for similar total consumption values (see Figure 1c), a higher ammonium yield is obtained. (see Figure 1f). The main metabolic intermediate through which glycolysis and glutaminolysis pathways are connected is pyruvate, which also is the key intermediate compound between glycolysis and the tricarboxylic acids cycle. Because of the low specific consumption rate for fructose and galactose, it should be assumed that, in this situation, most of the carbon is diverted to the pentoses synthesis and therefore only a small amount of pyruvate is generated (Reitzer et al., 1979). This fact leads to two consequences: first, the amount of lactate generated in the culture medium is minimal; second, the depletion of pyruvate would stop the TCA cycle, unless the cell could provide more pyruvate from glutaminolysis. One possible

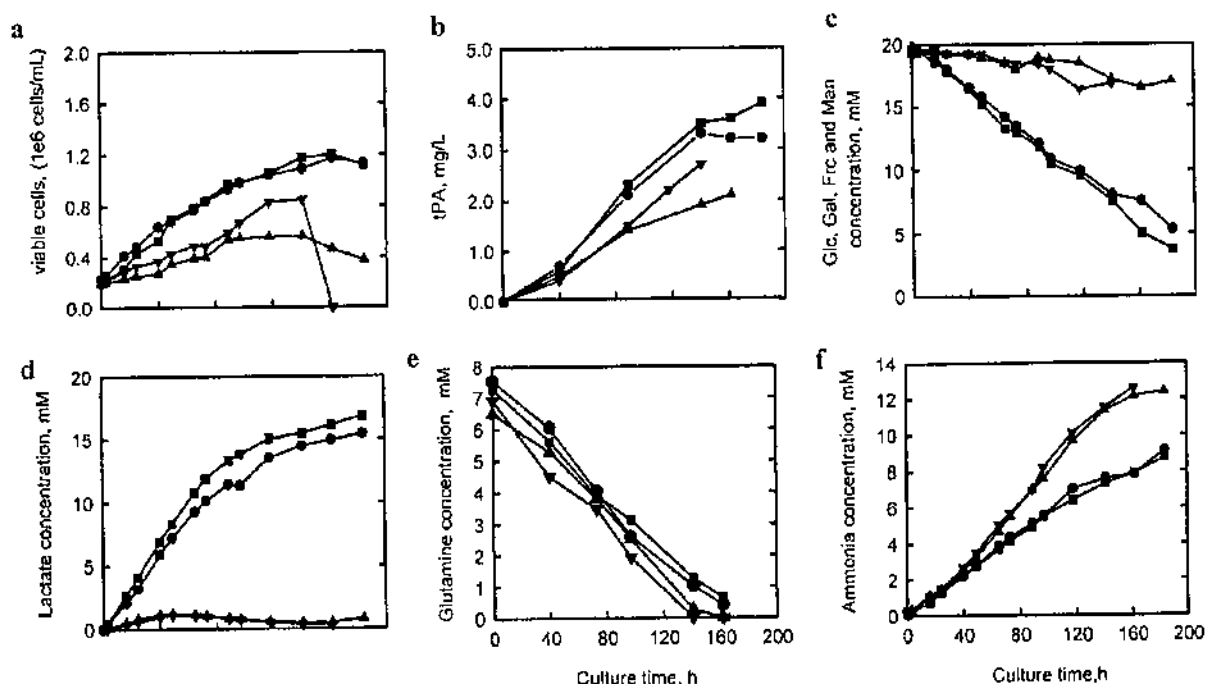


Figure 1. Results of the batch cultures of the CHO cells, using glutamine (7 mM) and four different sugar compounds in the medium formulation: 20 mM fructose (▲), 20 mM galactose (▼), 20 mM mannose (■), and 20 mM glucose (●) (control). (a) viable cells; (b) t-PA concentration; (c) sugar concentration; (d) lactate concentration; (e) glutamine concentration; and (f) ammonium concentration.

Table 1. Values of the Specific Cell Growth Rate and Specific Consumption Rates for the CHO Cell Line Growing on Different Carbon Sources

	glucose	fructose	mannose	galactose
μ (h^{-1})	0.0227	0.0109	0.0220	0.0153
q_c	-176.1	-48.7	-203.7	-48.0
q_{Lac}	336.2	78.8	364.7	61.9
q_{Gln}	-65.9	-103.3	-79.9	-115.0
q_{NH_4}	48.7	85.2	52.8	78.5

reaction that the cell can activate to compensate the low generation of pyruvate is that catalyzed by the malic enzyme (Sauer et al., 1980; Zielke et al., 1980), enabling the transformation of malate (that is then exported from TCA) into pyruvate (see Figure 2).

It is also of interest to discuss the differences encountered with respect to alanine production. Indeed, as already mentioned, although glutamine is consumed in almost the same total quantity for the four sugars studied, ammonium is produced in higher amounts when the sugar is consumed slowly (i.e., fructose and galactose), and at the same time alanine is produced in a much lower quantity than for the culture on glucose or mannose (level of alanine at 180 h of culture, 0.7 mM for galactose and fructose and 3.0 mM for glucose and mannose). Alanine is normally obtained in cell culture as a product of the transamination reaction between pyruvate and glutamate. The reaction also produces α -ketoglutarate, and generally this is the most common way to obtain this compound in cells that are proliferating rapidly, simultaneously reducing the ammonium generation (Matsuno et al., 1994). The low alanine production that is observed for the slowly metabolizable sugars should probably be linked to the low availability of pyruvate in the cell due to the slow glycolysis. In that situation, most of the pyruvate provided by the low glycolytic flux must be incorporated by the cells into the TCA cycle, and the transamination to alanine is then less favored. Alternatively, the generation

of α -ketoglutarate is provided by the action of the enzyme glutamate dehydrogenase.

Finally, it should be pointed out that glycine excretion is also changing with the carbon source used. For sugars rapidly metabolized, a lower glycine production is observed when compared to slowly metabolized sugars (level of glycine at 180 h of culture, 1.2 mM and 2.5 mM, respectively). Glycine is related to pyruvate metabolism.

Replacement of Glutamine by Glutamate. In the second series of experiments of this work, the same approach was followed to substitute glutamine in the culture medium, with the main objective of obtaining a more efficient use of the nitrogen source, with a lower release of ammonium ions. In this case, taking into account the previous results published in this respect (Hansen and Emborg, 1994; Ozturk et al., 1992; Ryll et al., 1994) and considering the glutamine metabolism (see Figure 2), glutamate was selected directly as the compound to be used, for three main advantages: first, glutamate does not suffer spontaneous decomposition as does glutamine (ammonium ions will not be generated as a result); second, glutamine can be obtained directly from glutamate by the CHO cells, as they exhibit glutamine synthetase activity; and third, glutamate has only one amino group. With respect to the carbon source used in this second series of experiments, in view of the results presented in the previous section, it was decided to select galactose, as a slowly metabolizable sugar, and glucose as the control system. The results of the four experiments carried out, using glutamine and glutamate as nitrogen sources and glucose and galactose as carbon sources, are presented in Figure 3.

In Figure 3a it can be observed that the final cell density attained growing on glucose-glutamate is the highest, being more than two times the concentration attained with the combination galactose-glutamate (1.8×10^6 and 0.8×10^6 cells/mL, respectively), and also notably higher than the final cell concentration for the

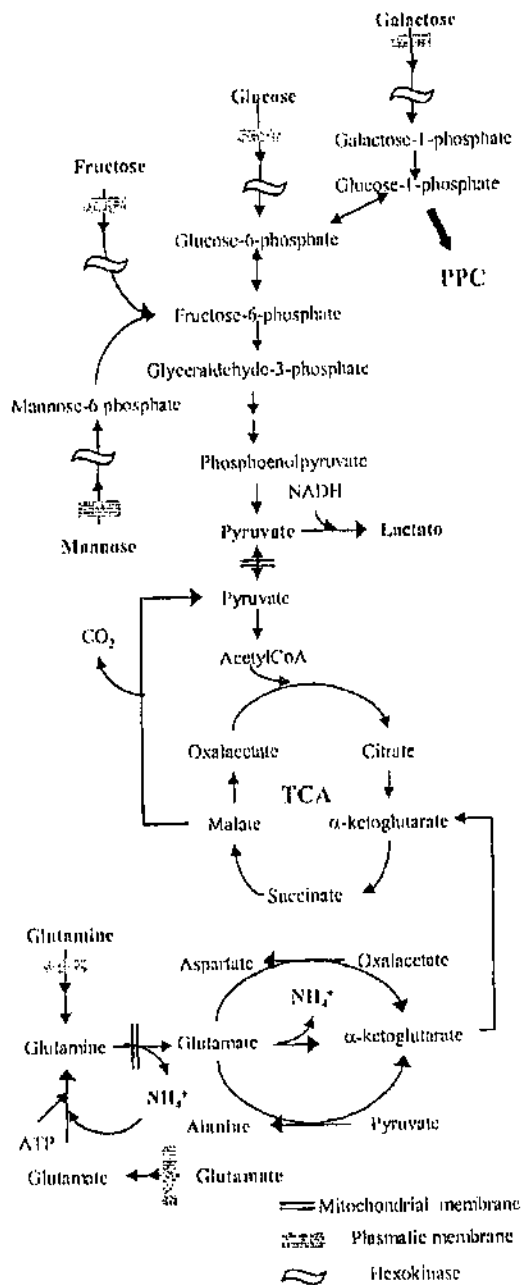


Figure 2. Scheme of the most relevant metabolic pathways for the metabolism of sugar compounds and glutamine in animal cells.

control culture, glucose-glutamine. The cell profiles for the growth on galactose-glutamine and galactose-glutamine are quite similar, with the main difference being that the abrupt drop in the cell concentration observed for galactose-glutamine is not observed for galactose-glutamine, probably because of the very much lower ammonium level generated, as will be discussed below, and also because glutamine is exhausted more rapidly in the first case, that of galactose-glutamine formulation. Regarding t-PA concentration profiles, as in the previous section, there is a clear proportionality between cell growth and product formation.

Figure 3c reflects the profiles for the consumption of the carbon source. It can be observed that galactose is consumed more slowly than glucose, as discussed in the

previous section, and also that in the presence of glutamate, the consumption of the carbon source is always lower than in the presence of glutamine, both for glucose and galactose, as reflected in Table 2. As previously mentioned this fact highlights the relationship existing between glycolysis and glutaminolysis. When the glutaminolysis rate is decreased by the replacement of glutamine by glutamate, the cells possibly have a lower need for pyruvate to be incorporated into the TCA cycle, and therefore the glycolysis rate is also reduced. This behavior is also found in other cell lines; the most evident example is for hybridoma cells modified genetically with the introduction of the glutamine synthetase gene. These transformed cells can then be grown on medium with glutamine being replaced by glutamate, and the pattern observed is a lower glutamate consumption rate, together with a decrease of about 50% in the consumption of glucose (Bibila and Robinson, 1995; Paredes et al., 1999).

With respect to the generation of lactate, Figure 3d shows that it is minimal for the media formulated with galactose, whereas for the media containing glucose, a clear difference can be observed between the control culture, glucose-glutamine (16 mM of lactate) and the experiment with glucose-glutamate (7 mM), clearly as a consequence of the lower consumption rate for glucose when glutamine is replaced by glutamate.

The data for the consumption of the nitrogen source are presented in Figure 1e. The trends for the consumption of glutamine and glutamate can clearly be distinguished, the later being a compound more slowly metabolized by the cells. The specific uptake rate values for the different combinations of media formulation are given in Table 2. The most relevant consequence of this different uptake profile is the ammonium generation. Indeed, it is markedly lower when glutamate is used instead of glutamine. It is also interesting to appreciate that the carbon source also affects, although to a minor extent, the ammonium generation, the general trend being that for any of the two nitrogen sources, ammonium concentration always reaches higher values for galactose than for glucose. Again, this fact also reflects the flexibility of the metabolic network. When the cells are cultured in galactose, the low uptake rate is most probably the limiting factor for cell metabolism, and it is compensated by an increase of the nitrogen source uptake (either glutamate or glutamine), generating a higher amount of ammonium. When glutamate is used in the medium, the alanine production decreases for any of the sugars tested (level of alanine at 180 h, glucose-glutamate culture, 0.7 mM; and galactose-glutamate culture, 0.1 mM) probably as a consequence of a lower availability of the key intermediate, pyruvate, which is transaminated to alanine and α-ketoglutarate in a much lower extent. On the other hand, the glycine production reaches similar levels to those obtained when glutamine is used, independently of the carbon source used (glucose or galactose).

Conclusions

The results presented demonstrate the possibility of regulating the metabolic pattern of CHO cells by substitution of the most commonly used main compounds in the medium formulation, glucose and glutamine, by other compounds that are taken up at significantly lower rates. As most of these compounds are incorporated to the central metabolism, it is most probable that transport rate into the cell is the major step controlling this process; or alternatively it could also be due to the different affinity of the hexokinase enzyme for the different

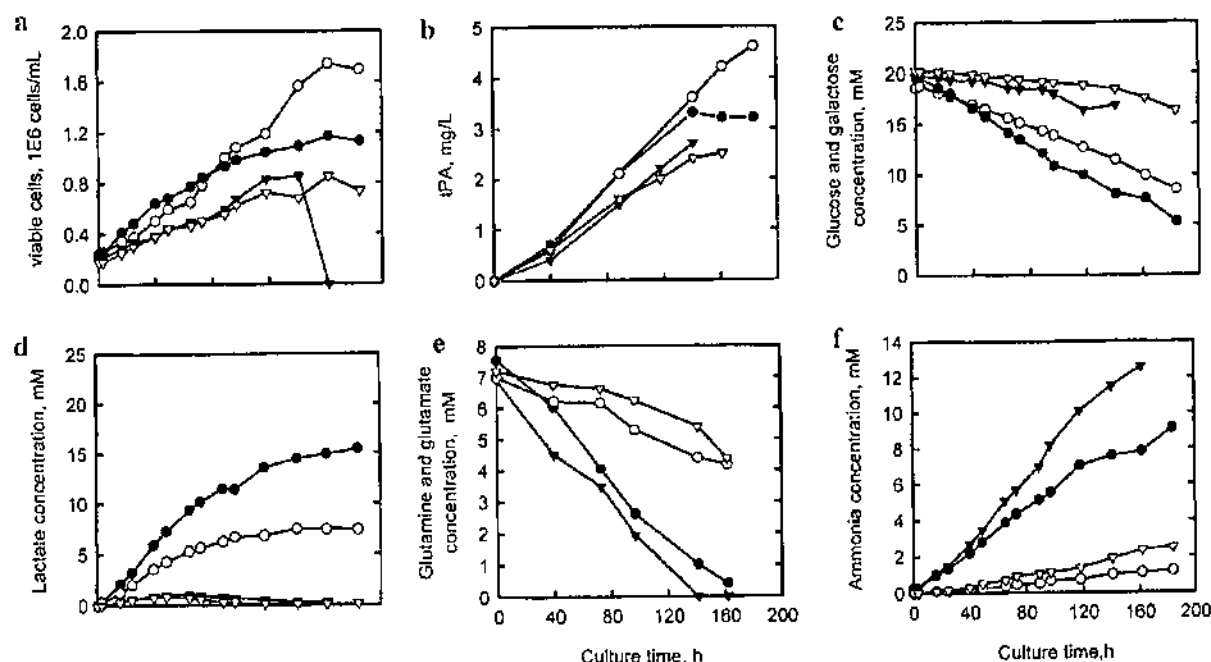


Figure 3. Results of the batch cultures of the CHO cells, using glutamine (7 mM) or glutamate (7 mM) as nitrogen sources and galactose (20 mM) or glucose (20 mM) as carbon sources in the medium formulation: galactose–glutamine (∇), galactose–glutamate (∇), glucose–glutamate (O), and glucose–glutamine ($\bullet$) (control). (a) viable cells; (b) t-PA concentration; (c) sugar concentration; (d) lactate concentration; (e) glutamine concentration; and (f) ammonium concentration.

Table 2. Values of the Specific Cell Growth Rate and Specific Consumption Rates for the CHO Cell Line Growing on Different Nitrogen Sources

	glucose/ glutamine	glucose/ glutamate	galactose/ glutamine	galactose/ glutamate
μ	0.0227	0.0186	0.0160	0.0160
q_C	-176.1	-113.7	-48.0	-37.5
q_{Lac}	326.2	209.3	61.9	47.0
q_{Gln}	-65.0		-115.0	
q_{Glu}	9.7	-32.5	15.3	-37.5
q_{NH_4}	48.7	13.2	78.5	25.0

compounds. From the different possibilities studied, the combination of galactose and glutamate allows the maintenance of cells at an optimal situation with regard to minimal consumption of nutrients and minimal generation of lactate and ammonium, although a lower cell growth is obtained. Alternatively, glucose and glutamate allow the obtaining of maximized cell growth, significantly higher than the control culture with glucose and glutamine, with very low ammonium accumulation and reduced lactate levels. These conditions offer the possibility for optimization of fed-batch cultures, as it will be possible to expand the time span of the cultures because the limiting conditions in terms of osmolarity and toxic compound accumulation will occur later than in the conventional medium formulation with glucose and glutamine.

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Mechanisms of ammonia and ammonium ion toxicity in animal cells: Transport across cell membranes

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Summary

A model for transport of ammonia and ammonium ions across cell membranes is presented. The model suggests that ammonium ions compete with potassium ions for inward transport, over the cytoplasmic membrane, via potassium transport proteins like the Na^+/K^+ -ATPase and the $\text{Na}^+\text{K}^+2\text{Cl}^-$ -cotransporter. It also explains the difference between the ammonia/ammonium that is added to the cells and which is formed by the cells during metabolism of amino acids, especially glutamine and glutamate. The ammonium transport and subsequent events lead to predictable intracellular and extracellular pH (pH_e) changes. Experiments which verified the model and the predicted consequences were performed by measurements of the pH_e in concentrated cell suspensions. Addition of ammonium ions caused a time-dependent pH_e increase which was inhibited by potassium ions. The test system is not per se specific for transport measurements but the effect of potassium ions on the pH_e strongly favors our suggested model. Simple diffusion of ammonium ions would not be counteracted by potassium ions. The results show that ammonium ion transport in the murine myeloma cell line (Sp2/0-Ag14) used is inhibited by an excess of potassium ions. Results from experiments with specific inhibitors of suggested transport proteins were not conclusive. It is postulated that one important toxic effect of ammonia/ammonium is an increased demand for maintenance energy, caused by the need to maintain ion gradients over the

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cytoplasmic membrane. The results also suggest that potassium ions can be used to detoxify ammonia/ammonium in animal cell cultivations.

Myeloma cells; Ammonium transport; Ammonia toxicity; Ammonium toxicity; Potassium ions; Bioreactor control

Introduction

Ammonia and ammonium ions are toxic to animal cells – different growth inhibiting concentrations being reported for different cell lines (Glacken et al., 1986; Miller et al., 1988; Ozturk et al., 1992; Reuveny et al., 1986). The problem with $\text{NH}_3/\text{NH}_4^+$ accumulation in cell cultures has been addressed by many workers (e.g. Glacken, 1988; Glacken et al., 1988; Ljunggren and Häggström, 1990, 1992; Miller et al., 1988) and the following mechanisms of toxicity is proposed: Disturbance of electrochemical gradients (Glacken, 1988), inhibition of enzymatic reactions – e.g. the conversion of glutamate to α -ketoglutarate via glutamate dehydrogenase (Glacken et al., 1988), intracellular pH changes (Glacken, 1988; McQueen and Bailey, 1990a,b, 1991) which may lead to disturbance of proton gradients and inhibition of endocytosis and exocytosis (Docherty and Snider, 1991) and an increased demand for maintenance energy (this article).

Knowledge about the mechanisms of $\text{NH}_3/\text{NH}_4^+$ toxicity may provide means for developing control strategies in animal cell processes. The aim of this work was therefore to further study some of the toxic mechanisms of $\text{NH}_3/\text{NH}_4^+$. The traditional way of measuring the inhibitory characteristics of $\text{NH}_3/\text{NH}_4^+$ is to add an ammonium salt to a cell culture and to follow growth, substrate uptake, product and by-product (e.g. lactic acid and $\text{NH}_3/\text{NH}_4^+$) formation. However, it is important to analyse if $\text{NH}_3/\text{NH}_4^+$ added to the cells or produced by the cells have the same effect. As a basis for a discussion on $\text{NH}_3/\text{NH}_4^+$ toxicity a model describing transport over cell membranes of intracellularly produced and extracellularly added $\text{NH}_3/\text{NH}_4^+$ as well as concomitant pH changes is presented. We further present results from experiments designed to test the model.

Model for transport of ammonia and ammonium ions

Figure 1 presents the model: NH_3 diffuses easily through cell membranes (simple diffusion; Table 1). Hence, NH_3 equilibria across membranes are readily established. The equilibrium between NH_3 and NH_4^+ is pH-dependent [$\text{p}K_a = 8.95$ at 35°C , calculated from $\text{p}K_w = 13.6801$ and $\text{p}K_b = 4.7330$ (Handbook of Chemistry and Physics, 1984–1985)]. The concentration of NH_3 in the extracellular bulk liquid determines the concentration of NH_3 in each intracellular compartment. Thus, the pH of a specific cellular compartment governs the total concentration of $\text{NH}_3 + \text{NH}_4^+$ in that organelle according to $\text{pH} = \text{p}K_a + \log \{(\text{NH}_3)/(\text{NH}_4^+)\}$.

TABLE 1
Simple diffusion of ammonia and ammonium ions

Compound	Diffusion rate (cm s ⁻¹)	Cell type	Reference
NH ₃	0.01	Human erythrocyte	Knepper et al., 1989
	0.007	MDCK cells	Knepper et al., 1989
	0.006	Squid giant axon	Boron and De Weer, 1976
NH ₄ ⁺	4.9–5 × 10 ⁻⁶ ^a	Turtle bladder	Knepper et al., 1989
	3.5 × 10 ⁻⁷ ^b	Mouse hybridoma	McQueen and Bailey, 1990b
	6.8 × 10 ⁻⁸	Squid giant axon	Boron and De Weer, 1976

^a Simulated value.
^b NH₄⁺ permeability supposed to be the same as the K⁺ permeability.

Simple diffusion of NH₄⁺ through cell membranes is extremely slow (Table 1) – due to the charge – as is the diffusion of other ions. However, NH₄⁺ can be transported into the cell by certain transport proteins (Fig. 1). Possible candidates for inward transport of NH₄⁺ are the Na⁺/K⁺-ATPase (Post and Jolly, 1957) (active transport) and the Na⁺K⁺2Cl⁻-cotransporter (Good et al., 1984; Kikeri et al., 1989; Knepper et al., 1989) (facilitated diffusion). NH₄⁺ is transported into the cell on the binding sites for K⁺, the natural substrate for these proteins, because hydrated NH₄⁺ has the same ionic radius as hydrated K⁺ (Kikeri et al., 1989; Knepper et al., 1989; Lund et al., 1970). The Na⁺/H⁺-exchanger has also been suggested as a possible NH₄⁺ transporter (Grinstein and Rothstein, 1986; Knepper et al., 1989) (facilitated diffusion).

Formation of NH₃/NH₄⁺ by the cells occurs mainly in the mitochondria by the mitochondrial enzymes glutaminase and glutamate dehydrogenase (Kovacevic and McGivan 1983; McKeehan, 1986). These reactions liberate NH₄⁺ which only slightly changes (decreases) the mitochondrial pH as the equilibrium with NH₃ is formed (Fig. 1). The mitochondrial pH is, however, decreased as NH₃ diffuses out

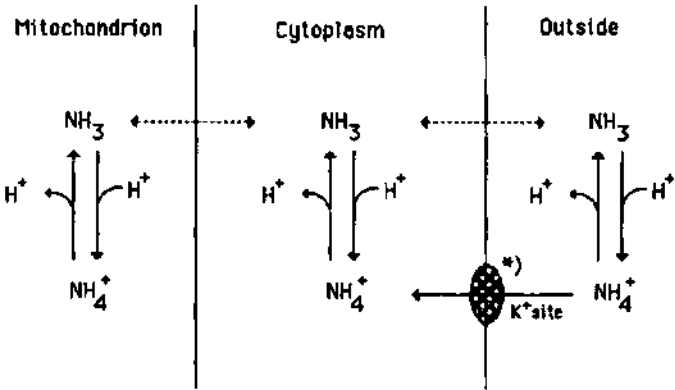


Fig. 1. Model for transport of ammonia and ammonium ions. *) Schematic representation of three possible transport proteins.

to the cytoplasm. The outflow of NH_3 forces formation of new $\text{NH}_3 + \text{H}^+$ from NH_4^+ to reestablish the equilibrium in the mitochondrion. While NH_3 readily diffuses through the extremely ion impermeable inner mitochondrial membrane and transiently increases the cytoplasmic pH the protons are left behind.

Ammonia that diffuses from the cytoplasm to the outside of the cell can be transported back across the cytoplasmic membrane via certain transport proteins (see above) as NH_4^+ , in competition with K^+ (Fig. 1). The transport can occur against a concentration gradient but intracellular NH_4^+ equilibrates immediately with NH_3 which again diffuses out of the cell. The result of such a futile cycle is a time-dependent acidification of the cytoplasm (protons are left behind, as explained above) and an alkalisation of the outside environment. Thus, mitochondrial formation of $\text{NH}_3/\text{NH}_4^+$ decreases both the mitochondrial and the cytoplasmic pH – the cytoplasm becoming progressively more acid by time. Other cellular compartments will turn alkaline as NH_3 diffuses into them.

Addition of NH_4^+ to a cell culture transiently increases the cytoplasmic pH and decreases the extracellular pH due to rapid diffusion of NH_3 into the cells. This is followed by an external alkalisation and a cytoplasmic acidification due to transport of NH_4^+ into the cell via the NH_4^+ transporting proteins and subsequent outward diffusion of NH_3 as explained above (Fig. 1). The mitochondrial pH as well as pH in other compartments will increase due to diffusion of NH_3 . Spontaneous decomposition of glutamine in the cultivation medium will have the same effect as added ammonium.

The difference between addition and cellular formation of NH_4^+ is the effect on the mitochondrial pH. Added NH_4^+ increases it while produced NH_4^+ decreases it. In both cases the cytoplasmic pH decreases (Fig. 1). The proton gradient over the mitochondrial membrane could be strengthened by added NH_4^+ but weakened by intracellularly produced NH_4^+ . This likely influences energy formation as mitochondrial ATP production depends on the proton gradient across the inner mitochondrial membrane. Normally the outside is more acidic than the inside. We speculate that the NH_4^+ formed by the cells may be more toxic than the extracellularly added NH_4^+ .

Materials and Methods

Cell line and medium

A murine myeloma cell line (Sp 2/0-Ag14) cultivated in Eagle's MEM with 5.5 mM of glucose, 4 mM of glutamine, 8.9 mM of sodium bicarbonate, 1 mM of sodium pyruvate, 5% FBS (HyClone) and 20 mM HEPES was used. All medium components except for the FBS were from Nord, Junter and Cell, Sweden. The medium pH was adjusted to 7.1–7.2 with 5 M NaOH.

Preparation of cells

Cells were routinely cultured in T-flasks (75 cm²) with 30 ml of medium, at 37°C. To obtain a concentrated cell suspension ($\sim 10^8 \text{ ml}^{-1}$) for the experiments,

1 l shake flasks with 200 ml of medium were seeded with $1.5-2 \times 10^5$ cells per ml, closed with rubber stoppers and incubated on a shaker (75 rpm) at 37°C for 48 h. The cell suspensions were centrifuged (750 g, 20 min) in a swing-out centrifuge (Wifug Clinic, Wifug) at 4°C and the pellets washed with Dulbecco's PBS (Gibco) without Mg^{2+} and Ca^{2+} and centrifuged (750 g, 15 min) again. The new pellets were suspended in PBS to give a cell density of $\sim 10^8$ viable cells per ml. The cells were counted on a haemocytometer using the trypan blue exclusion method. This cell suspension was used in the experiments below.

pH experiments

The experiments were performed in a 10 ml beaker with a concentrated cell suspension in PBS. One ml cell suspension was added to 9 ml of PBS giving a final cell concentration of $\sim 10^7$ ml⁻¹. Mixing was achieved by a small magnet while the beaker was placed on a magnetic stirrer. The beaker was insulated from the heat generated by the magnetic stirrer to ensure a constant temperature during the experiment. The measurements were performed at room temperature.

The extracellular pH changes following addition of NH_4Cl (Merck), KCl (Merck), ouabain (Boehringer-Mannheim), bumetanide (Sigma) and amiloride (Sigma) were measured each 20 s during 20 min using a combined glass/reference (saturated Ag/AgCl) electrode connected to a three decimal pH meter (PHM84, Radiometer). The final concentration of NH_4Cl was 10 mM. The additional concentration of KCl was 10, 30 or 50 mM – since PBS contains 4.15 mM of KCl this gives a total of 14.15, 34.15 or 54.15 mM. The final concentrations of ouabain, bumetanide and amiloride were 10, 1 and 5 mM, respectively.

For selection of suitable inhibitor concentrations suspensions of cells and inhibitors were incubated in PBS for 20 min at room temperature on a magnetic stirrer. The viability after 20 min (v_{20}) was compared to the starting viability (v_0) to determine the LD₅₀. However, ouabain (0.1–16 mM), 0.1–1 mM of bumetanide or 0.1–1 mM of amiloride did not cause any change in the cell viability during 20 min. At bumetanide concentrations of 5 and 9 mM, v_{20} was 90% and 15% of v_0 , respectively. This decrease in viability could also be due to the decrease in pH caused by bumetanide. At 9 mM of amiloride v_{20} was 95% of v_0 . The applied concentrations of ouabain and amiloride were for practical reasons the maximum obtainable in the complete test system. The applied concentration of bumetanide was restricted by its pH decreasing effect.

The PBS was allowed to equilibrate with air during 20 min on the magnetic stirrer before each experiment. Addition of components to PBS was made in the following order: cells, ouabain/bumetanide/amiloride, KCl and finally NH_4Cl , with 1 min duration between each addition. Appropriate control experiments were performed in parallel to all experiments. No substrate was added to the cells during pH measurements since glucose addition, in early experiments, showed to cause an extensive production of lactic acid which made all other pH changes impossible to measure.

Results

To test the model a series of experiments were designed. According to the model changes in cytoplasmic pH (pH_{cp}), caused by the inward transport of NH_4^+ , should result in opposite extracellular (pH_{e}) changes. Hence, it should be possible to measure the pH_{e} and make conclusions about intracellular events. The following experiments were performed:

- (1) Addition of NH_4Cl to a cell suspension. Transport (or simple diffusion) of NH_4^+ into the cells should be observed as a time-dependent increase of pH_{e} .
- (2) Addition of KCl and NH_4Cl to a cell suspension. If the presence of K^+ could prevent transport of NH_4^+ due to competition for binding to the transport proteins this should be observed as the absence of the pH_{e} increase.
- (3) Addition of the specific inhibitors ouabain (Na^+/K^+ -ATPase), bumetanide ($\text{Na}^+\text{K}^+2\text{Cl}^-$ -cotransporter) or amiloride (Na^+/H^+ -exchanger) and NH_4Cl to a cell suspension. Inhibition of transport of NH_4^+ via the transport proteins should be observed as the absence of the pH_{e} increase after addition of NH_4^+ to the cells.

(1) NH_4Cl

Addition of NH_4Cl to PBS without cells immediately decreased the pH due to proton release as the equilibrium $\text{NH}_4^+ \leftrightarrow \text{NH}_3 + \text{H}^+$ was established (Fig. 2). Addition of cells to PBS also decreased pH_{e} sharply. In both cases pH_{e} remained stable thereafter (Fig. 2). Upon addition of NH_4Cl to cells, already suspended in PBS, again there was a sharp decrease in pH_{e} due to the addition of salt, now

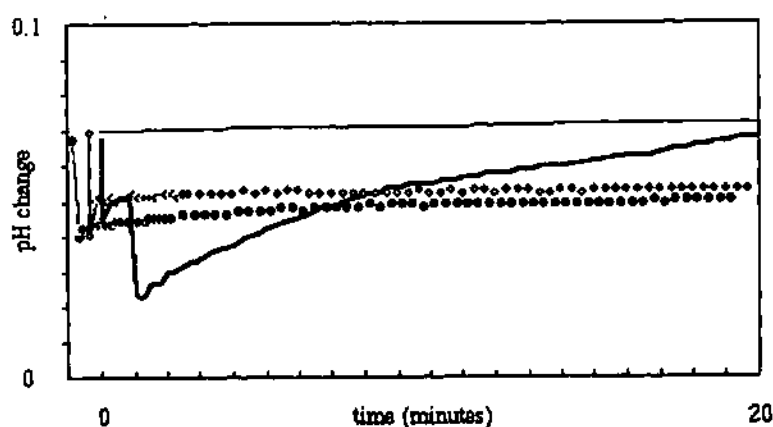


Fig. 2. Addition of NH_4Cl to a cell suspension. The curves are drawn apart at time = 0 minutes for clarity. Symbols: (—) PBS only, ($\diamond$) PBS + cells, ($\bullet$) PBS + NH_4Cl , and ($\blacksquare$) PBS + cells + NH_4Cl .

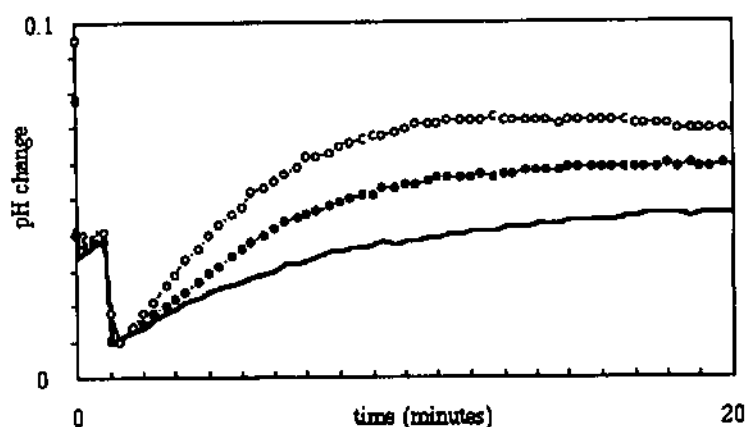


Fig. 3. Influence of cell concentration on the pH_e upon addition of NH_4Cl . Symbols (■) 10^7 ml^{-1} , (●) $2 \times 10^7 \text{ ml}^{-1}$ and (○) $3 \times 10^7 \text{ ml}^{-1}$.

followed by a slow pH_e increase (Fig. 2). The pH_e drop upon addition of NH_4Cl is larger in PBS with cells (0.032 ± 0.0022 pH units, mean value from ten experiments) than in PBS alone (0.024 ± 0.0025 pH units, mean value from ten experiments). This is in agreement with the theory on NH_3 diffusion into cells, leaving protons behind. In samples with double and triple amount of cells the pH_e increase was proportional to the amount of cells (Fig. 3).

(2) NH_4Cl and KCl

If 14.15 mM of KCl was present when NH_4Cl was added the pH_e increase was totally suppressed as compared to a cell suspension with 4.15 mM of KCl (Fig. 4a). No effect could be seen in experiments with 6.65 or 9.15 mM of KCl (data not shown) but 34.15 and 54.15 mM gave the same result as 14.15 mM of KCl (Fig. 4a). All control experiments (PBS alone, PBS + cells and PBS + NH_4^+ in Fig. 2; PBS + K^+ , PBS + cells + K^+ and PBS + K^+ + NH_4^+ in Fig. 4b) showed that pH_e remained stable for the duration of the experiments.

(3) NH_4Cl and ouabain, bumetanide or amiloride

Presence of 10 mM of ouabain or of 5 mM of amiloride in the test system did not influence the pH_e increase after addition of NH_4^+ while the presence of bumetanide or bumetanide + ouabain significantly depressed it as shown in Fig. 5a. Though, the control experiments with bumetanide and bumetanide + ouabain added to a cell suspension caused a fall in the pH_e (Fig. 5b). Because of this pH_e decrease it is not possible to draw any conclusions about the inhibitory effect of bumetanide or ouabain.

Discussion

Figs. 2, 3, 4 and 5 clearly show that a reproducible and time-dependent increase in pH_e occurs after addition of NH_4^+ to a cell suspension. We suggest that this pH_e increase depends on transport of NH_4^+ into the cell and subsequent NH_3 diffusion out of the cell as our model predicts. This conclusion is not immediately evident because our test system is not per se specific for transport measurements. The same result should have been obtained if NH_4^+ entered the cell by simple diffusion. Theoretically it is also possible that other events, not considered here, could give the same result. Though, the inhibition of the pH_e increase by the presence of K^+ strongly favours our suggested model. It appears reasonable to interpret the effect caused by K^+ as competitive inhibition of the transport. Examples of K_m for K^+ transport by the Na^+/K^+ -ATPase are 1.8 mM in crab nerve (Skou, 1960) and 2.1 mM in human erythrocyte (Post et al., 1960). Corresponding K_m for NH_4^+ are 5 mM and 7–16 mM, respectively. Potassium at the applied concentration of 14.15 mM should saturate the binding sites thus preventing transport of NH_4^+ .

Addition and subsequent removal of an ammonium salt solution is an established method of purposely acid loading a cell culture (Aickin and Thomas, 1977; Boron, 1977, 1983). However, the explanations to the mechanism that causes the acidification differ: Aickin and Thomas (1977) suggested active transport of NH_4^+

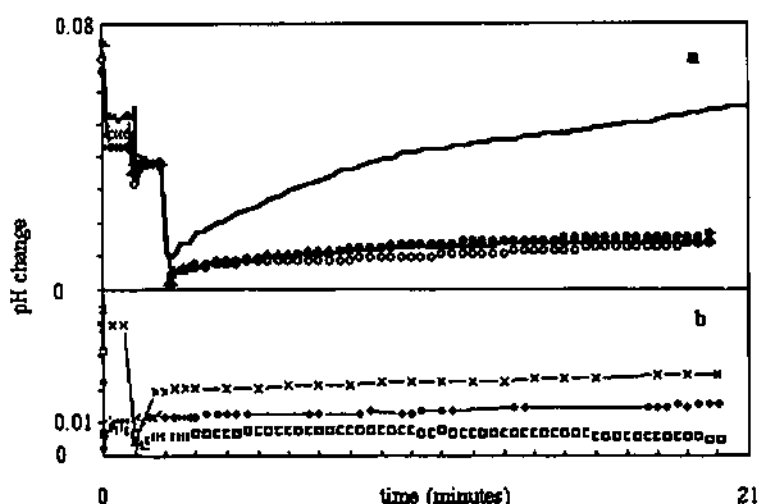


Fig. 4. Addition of KCl and NH_4Cl to a cell suspension. Addition of components to PBS was made in the following order: cells, KCl and NH_4Cl . The sharp pH_e decrease at 2 min (Fig. 4a) represents the addition of NH_4Cl . Symbols in a: (■) PBS + cells + NH_4Cl , (●) PBS + 14.15 mM KCl + NH_4Cl , (○) PBS + cells + 34.15 mM KCl + NH_4Cl and (▲) PBS + cells + 54.15 mM KCl + NH_4Cl . Symbols in b: (□) PBS + cells + 14.15 mM KCl, (◆) PBS + 54.15 mM KCl and (×) PBS + 14.15 mM KCl + NH_4Cl .

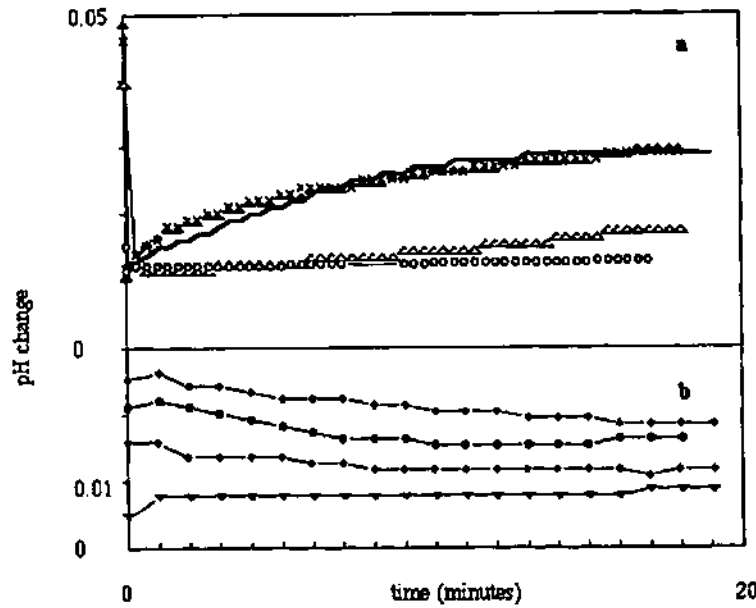


Fig. 5. Addition of specific transport protein inhibitors together with NH_4Cl to a cell suspension. Addition of components to PBS was made in the following order: cells, inhibitor and NH_4Cl . All experiments are shown from the moment when the last addition was made. Symbols in a: (■) PBS+cells+ NH_4Cl , (▲) PBS+cells+ouabain+ NH_4Cl , (×) PBS+cells+amiloride+ NH_4Cl , (△), PBS+cells+bumetanide+ NH_4Cl and (○) PBS+cells+ouabain+bumetanide+ NH_4Cl . Symbols in b: (◆) PBS+cells+ouabain, (◇) PBS+cells+bumetanide, (▼) PBS+cells+amiloride and (●) PBS+cells+ouabain+bumetanide.

via the Na^+/K^+ -ATPase while Boron (1977, 1983) suggested simple diffusion of NH_4^+ driven by concentration and electrochemical gradients. Our experimental results are contradictory to the proposal by McQueen and Bailey (1990b, 1991), Boron and De Weer (1976) and Boron (1977, 1983) which implies that NH_4^+ enters the cell by simple diffusion through the plasma membrane. Although we cannot make any conclusions about which proteins are the most important NH_4^+ transporters (see further below) in the cell line applied, simple diffusion of NH_4^+ would not be counteracted by K^+ . The applied concentrations of NH_4^+ and K^+ in the present investigation should be low enough not to disturb the plasma membrane electrochemical potential.

The results from the experiments with specific inhibitors of the transport proteins were not conclusive. Obviously bumetanide causes a decrease in pH_e which makes it impossible to interpret the inhibition of the pH_e increase as inhibition of NH_4^+ transport. A further explanation could be that the applied concentrations of respective inhibitor were too low. The inhibitor concentrations chosen were based on literature data and our own preliminary experiments (see Materials and Methods). Ouabain concentrations of 0.1–0.2, 0.1 and 5 mM have

been reported to inhibit the Na^+/K^+ -ATPase in 3T3 mouse fibroblasts (Rozen-gurt and Heppel, 1975), mouse soleus muscle (Aickin and Thomas, 1977) and mouse kidney cells (Kikeri et al., 1989), respectively. Bumetanide concentrations of 0.01 and 1 mM is known to inhibit the $\text{Na}^+ \text{K}^+ 2\text{Cl}^-$ -cotransporter in human retinal pigment epithelium (Kennedy, 1990) and mouse kidney (Kikeri et al., 1989), respectively. Amiloride concentrations of 0.1, 0.2 and 2 mM is known to inhibit the Na^+/H^+ -exchanger in mouse soleus muscle (Aickin and Thomas, 1977), Ehrlich ascites tumour cells (Livne and Hoffmann, 1990) and mouse proximal tubule (Roos and Boron, 1981), respectively. It is obvious that the inhibitor sensitivity differs widely between cell lines and cell types, e.g. human cells are much more sensitive than murine cells. Still, the concentrations required for total inhibition of the proteins in the murine myeloma cell line used in the present investigation is not known to us. Furthermore, ouabain (at certain concentrations) is used as a selection agent during cell transformation, killing normal cells and leaving transformed cells intact. An obvious experiment, inhibiting all three of the suggested NH_4^+ transporters, was never performed because it was not possible to obtain the necessary concentrations (see Materials and Methods).

Assuming transport of NH_4^+ via transport proteins – what are then the consequences in terms of toxicity for the cell? Firstly, NH_4^+ is transported into the cell instead of K^+ . This may decrease the intracellular K^+ concentration, an important factor for many enzymes and for the potassium gradient over the cytoplasmic membrane. Secondly, it costs energy – at the direct expense of ATP if NH_4^+ goes in on the Na^+/K^+ -ATPase and indirectly if it goes in on the $\text{Na}^+ \text{K}^+ 2\text{Cl}^-$ -cotransporter. In the latter case the cotransported Na^+ have to be pumped out again to maintain the Na^+ gradient over the cell membrane. This is accomplished by the Na^+/K^+ -ATPase – a process that in turn may accelerate the inward transport of NH_4^+ . Further, in order to counteract the acid load the cell has to pump out protons. This is normally effected by the Na^+/H^+ -exchanger, an enzyme which is activated when cytoplasmic pH falls below its set point (Grinstein and Rothstein, 1986; Gillies and Martinez-Zaguilan, 1991; McQueen and Bailey, 1990b) of approx. pH 7.0 (Livne and Hoffmann, 1990). Again, a requirement for pumping out Na^+ by the Na^+/K^+ -ATPase arises. We therefore suggest that one important toxic mechanism of $\text{NH}_3/\text{NH}_4^+$ exposure is an increased demand for maintenance energy, caused by the need to keep up ion gradients over the cytoplasmic membrane. This effect should be the same whether NH_4^+ is produced by the cells or added to the culture. Though, it is difficult to estimate the magnitude and relative importance of this effect during growth in culture when other metabolic events influence the metabolism and physiology. For example, it should be expected that lactate formation counteracts the acid load in the cytoplasm because lactate exits the cell by simple diffusion as lactic acid, thus simultaneously transporting out a proton. However, in experiments in bioreactors, the cellular yield coefficients for the energy sources glucose and glutamine decreased substantially when NH_4Cl was added to the culture (data not shown). Addition of K^+ to a culture with NH_4Cl restored both growth rate and cellular yield coefficients to normal values (results will be published elsewhere).

Acknowledgements

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