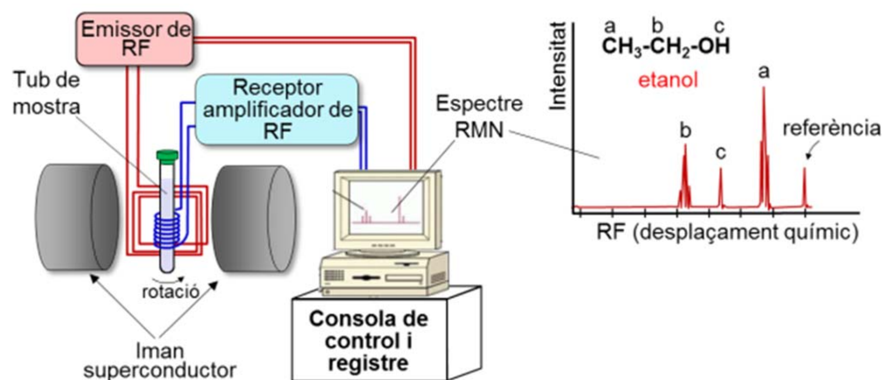
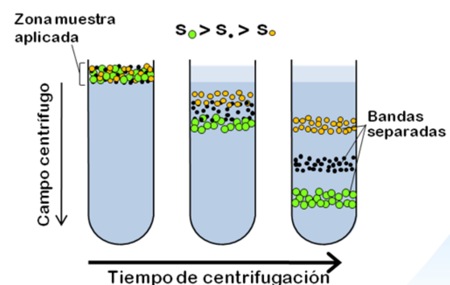
Departament de Bioquímica i Biologia Molecular

PROBLEMES I QÜESTIONS DE MÈTODES EN BIOQUÍMICA i BIOLOGIA MOLECULAR

GRAU EN BIOTECNOLOGIA





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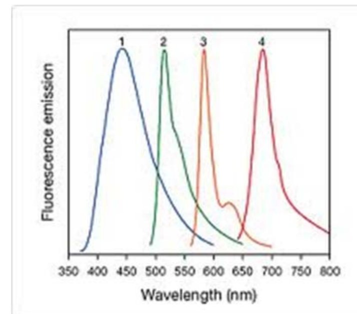
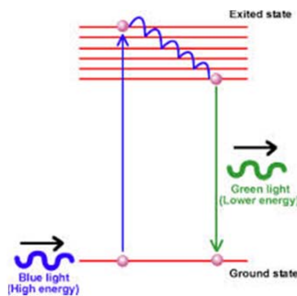
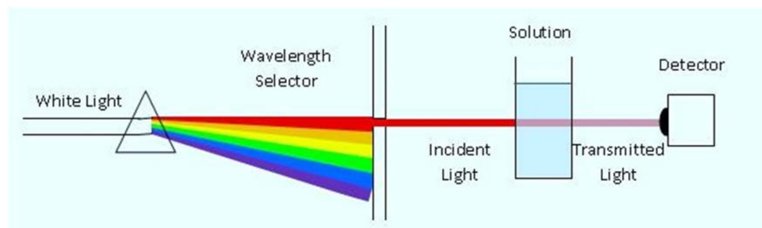
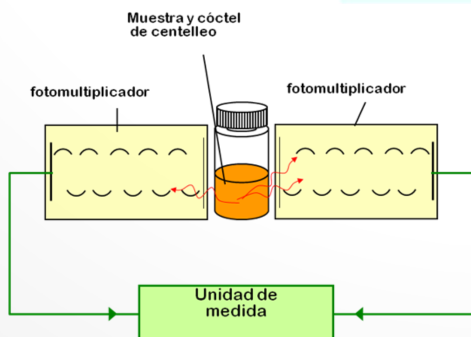
Problemes de Classe

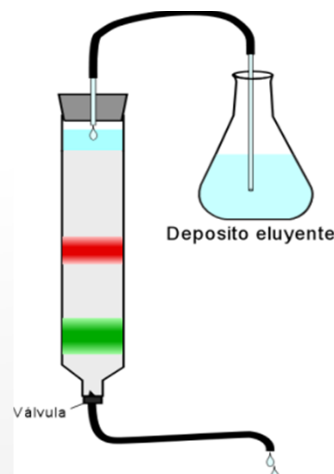
Tema 1: Caracterització de molècules d'interès biològic
Tema 2: Espectroscòpia d'absorció
Tema 3: Espectroscòpia de fluorescència
Tema 4: Espectroscòpia de ressonància magnètica nuclear
Tema 6: Radioisòtops
Tema 7: Electroforesi
Tema 8: Cromatografia
Tema 9: Centrifugació











PROBLEMES T1 (Classe)
CARACTERITZACIÓ DE MOLÈCULES D'INTERÈS BIOLÒGIC

1.1.- Comparar els dos processos de purificació de la triosafosfat-isomerasa indicats en quant a rendiment i grau de puresa aconseguit.

	Conc. Prot. (mg/mL)	**Activitat** (ua/mL)	**Volum** (mL)				
Extracte cru	0.82	120	22				
Procés 1							
Precip. PEG	1.80	475	4				
Crom. Excl. Mol.	0.36	162	10				
Crom. Int. Iònic	0.14	251	6				
Procés 2							
Crom. Afinitat	0.16	338	5				

S: Hi ha que determinar el Rendiment i el Grau de Purificació per tal de fer la comparació. Amb aquestes dades el procés 2 és millor tant pel rendiment com pel grau de purificació.

1.2.- Les següents dades s'han obtingut d'un procés de purificació de la Ribulosa-1,5-Bisfosfat Carboxilasa/Oxigenasa (RuBisCO) de fulles de taronger. Donada l'extraordinària abundància d'aquesta proteïna és possible seguir el procés de purificació paral·lelament per densitometria de electroforogrames tenyits amb blau Coomassie (procediment que permet determinar el percentatge de la proteïna total, en massa, constituït per la RuBisCO). Calculeu el rendiment del procés i el factor de purificació aconseguit tenint amb compte que se sospita que la RuBisCO s'inactiva durant el procés de purificació. És possible confirmar aquesta inactivació de les dades experimentals?

	Prot. total (mg)	**Activitat** (ua)	**RuBisCO** (%)				
Extracte cru	332	88.3	51				
Prec. $(NH_4)_2SO_4$	141	43.3	67				
Centrif.Grad.Sac	94	30.2	84				
Crom. Int. Iònic	54	10.5	98				

S: Amb el Rendiment i el Grau de Purificació deduïts amb l'activitat enzimàtica i amb la quantitat de proteïna es pot concloure que si hi ha inactivació de la RuBisCO.

1.3.- En un procés de purificació d'un àcid nucleic es mesura en cada pas de purificació l'absorbància a 280 nm i 260 nm, i la radioactivitat retinguda per

un filtre en el qual s'ha dipositat una alíquota de la fracció que conté l'àcid nucleic (prèviament desnaturalitzat) i s'ha hibridat amb una sonda radioactiva específica del mateix. Estimar (en la mesura que siga possible) el rendiment i factor de purificació del procés i de cadascun dels passos a partir de les dades obtingudes.

	Volum (ml)	**A_{260}**	**A_{280}**	**Radiact.** (dpm/mL)			
Extracte cru	35.5	1.62	1.80	634			
Fenolització + prec. etanol	5.0	3.06	1.78	3820			
Centrifugació isopícnica CsCl	8.2	0.378	0.210	2146			
Crom. Int. Iònic	10.0	0.060	0.033	1580			

S: La radioactivitat constitueix la mesura de la quantitat de DNA d'interés.

1.4.- Es proposen 2 mètodes colorimètrics diferents per a determinar la quantitat d'un metabòlit en un extracte. Tots dos es basen en dur paral·lelament mostres patrons que contenen el metabòlit en quantitat coneguda i ajustar aquests punts experimentals a una recta patró. Els valors mostrals s'interpolen en la recta patró per a trobar el contingut en metabòlit. Els valors obtinguts per als patrons són:

Quantitat metabòlit (µg)	**Quantitat de color** (Absorbància)	
	Mètode I	**Mètode II**
0	0.001	0.008
10	0.023	0.066
25	0.059	0.160
50	0.117	0.290
80	0.187	0.402
120	0.281	0.546

Discutiu quin dels dos mètodes és més sensible i quin és més exacte. Es pot dir alguna cosa sobre la precisió?

S: El mètode II és més sensible. Mètode I és més exacte. No es pot saber la precisió.

PROBLEMES T2 (Classe)
ESPECTROSCÒPIA D'ABSORCIÓ

2.1.- Una radiació electromagnètica posseeix una longitud d'ona $\lambda = 2\cdot10^{-3}$ cm.

a) Calcular la freqüència, el nombre d'ones i l'energia d'un fotó. A quin tipus d'energia molecular afectarà l'absorció d'un fotó d'aquesta radiació per part de la matèria?

b) Un compost que és capaç d'absorbir a aquesta longitud d'ona es troba en equilibri tèrmic a 51ºC. Calcular la relació de molècules en el primer estat excitat respecte a les del nivell fonamental en absència de radiació.

S: a)Nombre d'ones:500 cm^{-1}, frequencia: $1.5\cdot10^{-13}$ s^{-1}, E=59985 J/mol (0,062 eV). b)11%

2.2.- El triptòfan (Trp) té una massa molecular de 204 Da i presenta un màxim d'absorció en l'UV a 280 nm.

a) Calcular el coeficient d'extinció molar (ε) sabent que una dissolució d'11 μg/mL de Trp en aigua té una transmitància del 50% en una cubeta d'1 cm de pas de llum.

b) Si una dissolució aquosa d'una proteïna de massa molecular 20 kDa a una concentració de 0.3 mg/mL posseeix una densitat òptica (D.O.) de 0.42 a 280 nm, calcular el nombre de residus de Trp que conté la proteïna, suposant que l'absorció a 280 nm es deu exclusivament al Trp.

S: a) ε_{280}=5585 $M^{-1}\cdot cm^{-1}$. b) 5

2.3.- Una substància té un coeficient d'extinció molar ε =348 $M^{-1}\cdot cm^{-1}$ a 482 nm. Una mostra d'aquest compost pur dissolt en aigua té una densitat òptica (D.O.) d'1.60 cm^{-1}. Es realitzen les següents dilucions (afegint volums d'aigua) i s'obtenen els següents valors:

Dilució (vol mostra:vol aigua)	**D.O.** (cm^{-1})			
cap	1.60			
1:1	1.52			
1:2	1.42			
1:3	1.05			
1:4	0.84			
1:5	0.70			
1:6	0.61			

Calculeu la concentració del compost en la mostra original.
S: 0,012 M

2.4.- Una dissolució que conté 2 soluts diferents (A i B) posseeix absorbàncies A_{350}= 0.36 i A_{400}= 0.225. Els valors dels coeficients d'extinció molar (ε) de les dues substàncies són:

Solut	**ε a 350 nm**	**ε a 400 nm**
A	15000	3000
B	7000	6500

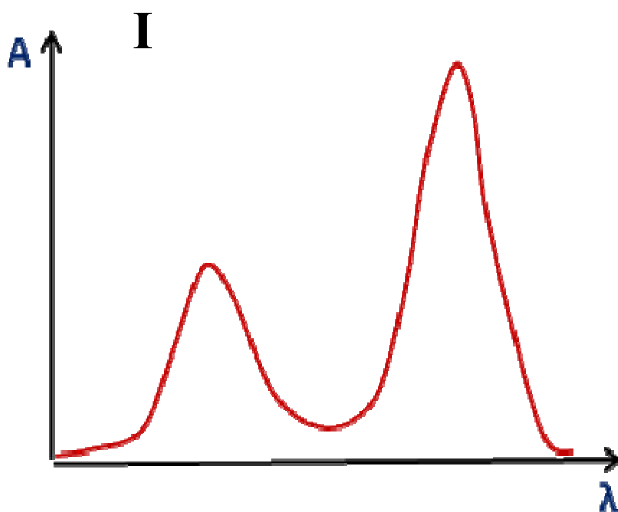
Calculeu les concentracions de A i B en la dissolució.
S: A $1\cdot10^{-5}$ M, B $3\cdot10^{-5}$ M

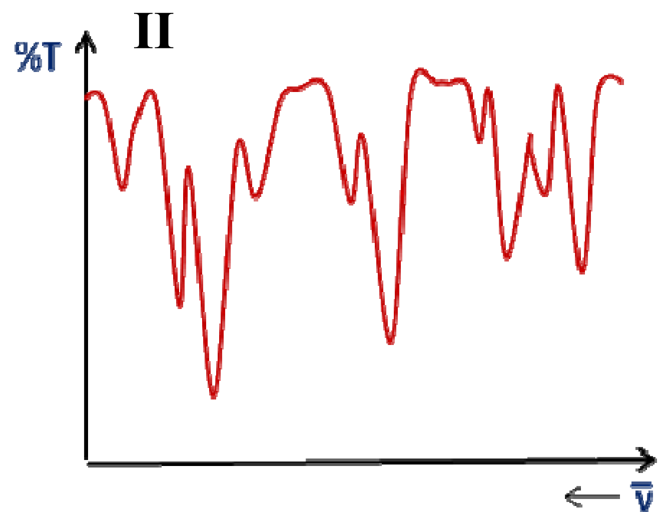
2.5.- La vibració de tensió d'enllaç del grup alcohol (O-H) de la treonina dóna lloc a una banda d'absorció en l'IR a un nombre d'ones de 2850 cm^{-1}.

a) Predir el nombre d'ones al que es desplaçarà aqueixa banda d'absorció en l'IR si es produeix intercanvi isotòpic en aigua deuterada.
b) Si la mostra s'il·lumina amb radiació làser de λ= 810 nm, calcular la longitud d'ona corresponent al pic Raman causat per la vibració d'aquest grup.

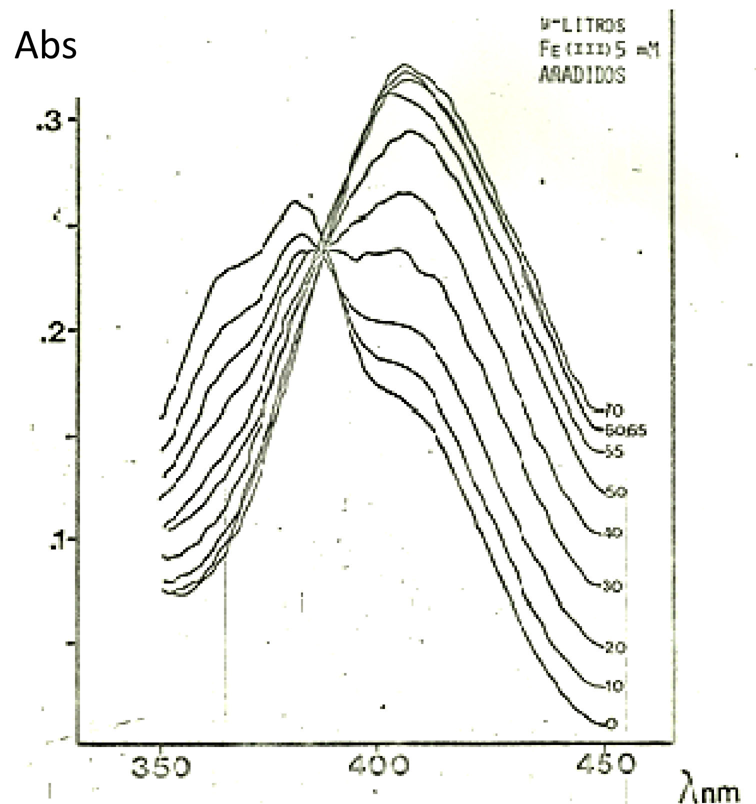
S: a2079 cm^{-1}. b) 1056 nm

2.6.- Els següents espectres d'absorció pertanyen a la mateixa substància. Un d'ells s'ha obtingut en l'IR i un altre en l'UV. Identificar quin és quin i raonar l'elecció.





2.7.-Els sideròfors bacterians són molècules capaces de quelar ions de ferro amb gran afinitat. Els sideròfors són secretats pels bacteris al medi per a capturar el ferro i després són de nou internalitzats mitjançant mecanismes específics de transport. S'ha obtingut un sideròfor de *Pseudomonas syringae* purificat i lliure de ferro en dissolució aquosa i s'ha registrat l'espectre UV-V entre 350 i 450 nm (veure figura adjunta). L'addició de petits volums (menyspreables enfront del volum de dissolució de sideròfor) de clorur fèrric 5 mM produeix modificacions progressives de l'espectre, tal com mostra la figura. Quin pot ser la raó que tots els espectres mostren la mateixa absorbància entorn de 390 nm?



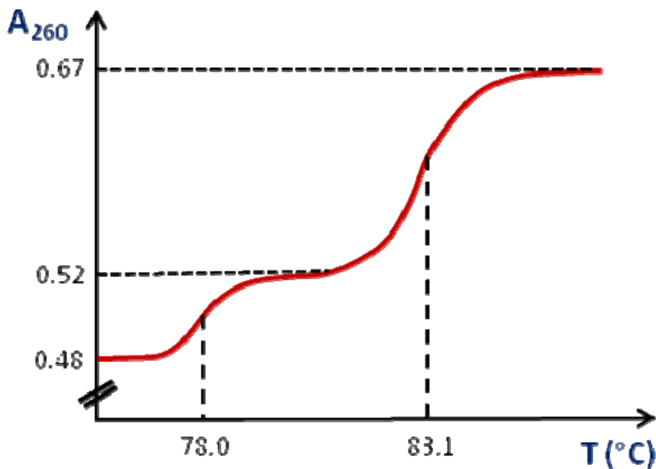
2.8.- Es disposa d'una dissolució aquosa (1.5 mg/mL) d'una proteïna de 15 kDa de massa molecular. Es barregen porcions d'1 mL d'aquesta dissolució amb 1 mL de tampons de diferents pHs. Es determina l'absorbància a 295 nm de les mescles en una cubeta d'1 cm de pas de llum. A aquesta longitud d'ona absorbeix exclusivament la forma desprotonada (ió tirosinat) del cromòfor de la tirosina amb un coeficient d'extinció molar ε= 2600 $M^{-1}\cdot cm^{-1}$. El pKa de la tirosina es troba entorn de 9.5. Les dades obtingudes són:

pH del tampó	A_{295}
7.0	0.010
8.0	0.012
8.5	0.017
9.0	0.035
9.5	0.078
10.0	0.122
10.5	0.140
11.0	0.144
11.5	0.159
12.0	0.187
12.5	0.230
13.0	0.268
13.5	0.270

Determineu quants residus de tirosina posseeix la proteïna i quants d'ells es troben en la superfície de la molècula, accessibles al dissolvent.

S: 2, una interna i una superficial

2.9.- Es té una preparació de DNA d'un cultiu bacterià portador d'un plasmidi. El DNA se sotmet a desnaturalització tèrmica i s'obté la següent variació de l'absorbància a 260 nm amb la temperatura:



Prèviament s'han establert, mitjançant patrons desnaturalitzats en les mateixes condicions, les següents relacions lineals entre percentatge de bases i temperatura de fusió (t) o hipercromicitat (H):

$$t\ (°C) = 0.39 \cdot (\%\ G\text{-}C) + 62.00$$
$$H = 0.004 \cdot (\%A\text{-}T) + 0.200$$

Suposant que el DNA plasmídic és minoritari, calcular el percentatge de bases de cada DNA (cromosòmic i plasmídic) i determinar què fracció de l'absorbància del DNA natiu total es deu al DNA cromosòmic.

S: DNA cromosòmic 81%

2.10.- Calculeu les concentracions de fumarat i malat en una mescla sabent que:

a) afegint 1 mL d'una dissolució que conté NAD^+ en l'excés, semicarbazida (per a eliminar l'oxalacetat), i malat-deshidrogenasa (MDH) a 1 mL de la barreja problema en una cubeta de 3 cm de pas de llum, l'absorbància a 340 nm (A_{340}) passa de 0.020 a 0.247.

b) després de l'addició d'1 mL d'una dissolució de fumarasa (F), amb una A_{340} de 0.030, als 2 mL anteriors, l'A_{340} va augmentar fins a 0.730.

Dada: $\varepsilon_{340\ (NADH)} = 6.22 \cdot 10^3\ M^{-1} \cdot cm^{-1}$

Reaccions implicades:

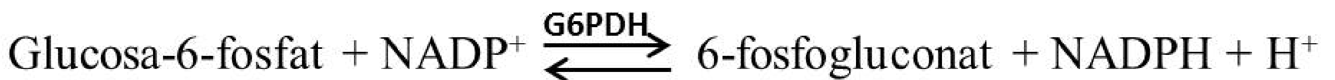
S: Malat, $2{,}43 \cdot 10^{-5}$ M; Fumarat $8{,}9 \cdot 10^{-5}$M

2.11.- Es barregen 0.5 mL d'una dissolució que conté $MgCl_2$, ATP, $NADP^+$ i glucosa-6-fosfat-deshidrogenasa (G6PDH) amb 0.5 mL d'un extracte en el qual se suposa que existeix activitat hexoquinasa (HK). Després d'incubar la mostra un cert temps es mesura l'absorbància a 340 nm en una cubeta d'1 cm de pas de llum i s'observa que s'estabilitza en A_{340}= 0.039.
A la mescla anterior (1 mL) s'afegeixen 2 mL d'una dissolució de glucosa 5 mM (A_{340}= 0.003). Ambdues dissolucions es barregen bé i es mesura l'A_{340} a intervals d'1 min després de l'addició. Els resultats obtinguts són:

Temps (min)	A_{340}		
1	0.106		
2	0.209		
3	0.311		
4	0.411		

a) Quines condicions han de complir-se perquè aquestes mesures siguen representatives de l'activitat HK de l'extracte?
b) Calcular l'activitat HK en mol de glucosa transformats per minut
Dada: ε_{340} (NADPH) = $6.22 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$

Reaccions implicades:

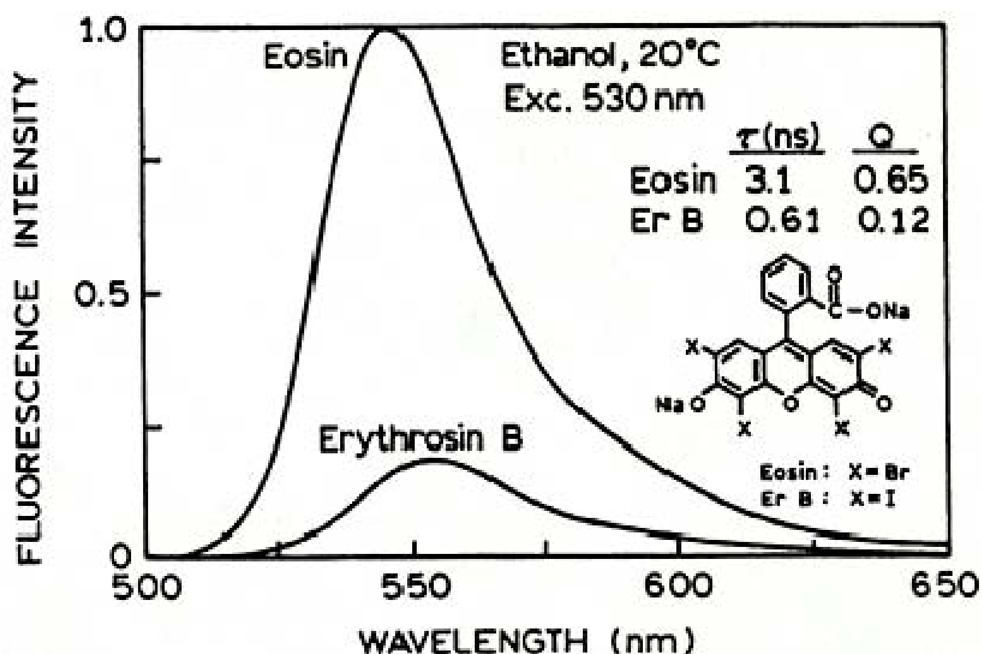


S: b) $4,92 \cdot 10^{-5}$ mol/min

PROBLEMES T3 (Classe)
ESPECTROSCÒPIA DE FLUORESCÈNCIA

3.1.-Una dissolució d'un determinat compost emet fluorescència entorn de λ_{em}= 405 nm quan s'excita a λ_{ex}= 320 nm.
Fer un càlcul aproximat del rendiment quàntic del fluoròfor sabent que el feix incident (cilíndric de secció 0.12 cm^2) posseeix una intensitat I_0= $8.2\cdot10^{-10}$ $J\cdot s^{-1}\cdot cm^{-2}$, el feix transmès té intensitat I_t= $4.2\cdot10^{-10}$ $J\cdot s^{-1}\cdot cm^{-2}$, i la radiació fluorescent captada a 62 mm del centre de la cubeta té una intensitat I_F = $3.3\cdot10^{-14}$ $J\cdot s^{-1}\cdot cm^{-2}$.
S: Q= 0,42

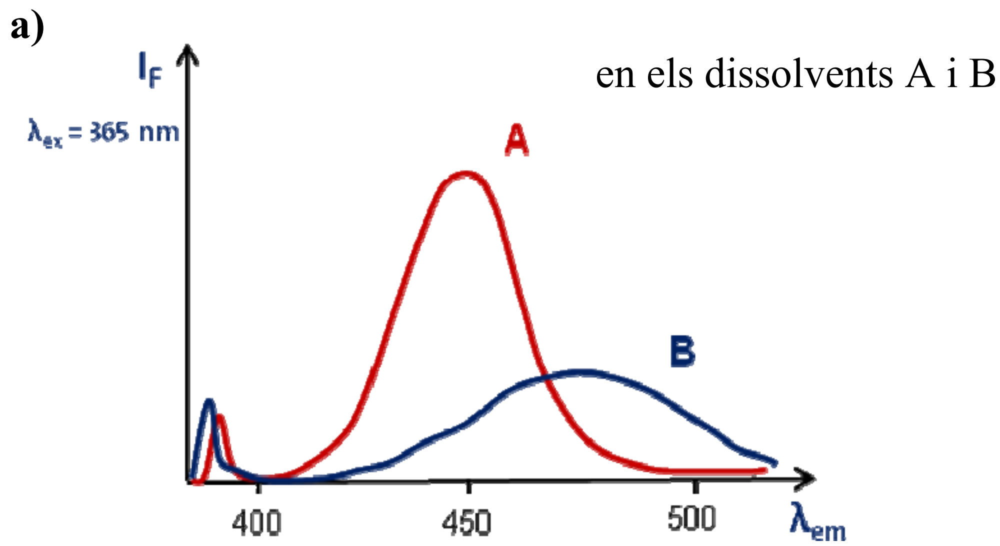
3.2.- La figura adjunta mostra els espectres d'emissió de l'eosina i l'eritrosina B, juntament amb la vida mitjana en l'estat excitat (τ) i el rendiment quàntic (Q) dels dos compostos.
Calcular la vida mitjana intrínseca fluorescent dels dos fluoròfors i la constant cinètica de tornada radiant i no radiant de tots dos. A què es deu el baix rendiment quàntic de l'eritrosina B (relatiu al de l'eosina)?



S: τ_0 (eosina) = 4,77 ns; τ_0 (eritrosina B) = 5,08 ns; K_F(eosina)=0,21 ns^{-1}; K_F(eritrosina B)=0,197 ns^{-1}; K_{NF}(eosina)=0,11 ns^{-1}; K_{NF}(eritrosina B)=1,44 ns^{-1}

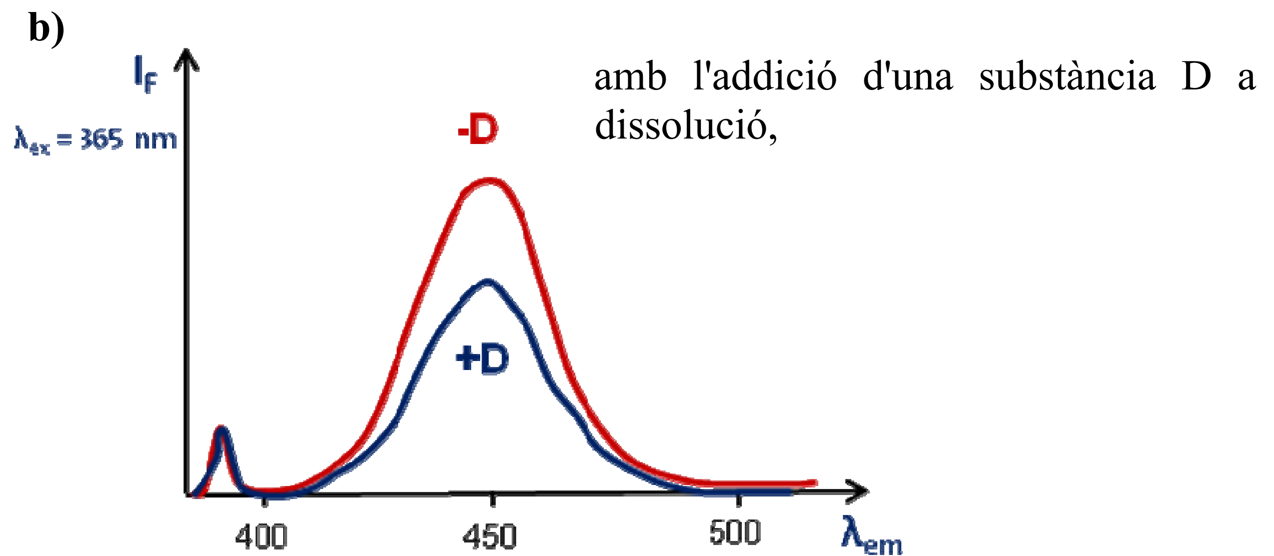
3.3.- Justificar els següents canvis en l'espectre d'emissió d'un fluoròfor:

a)



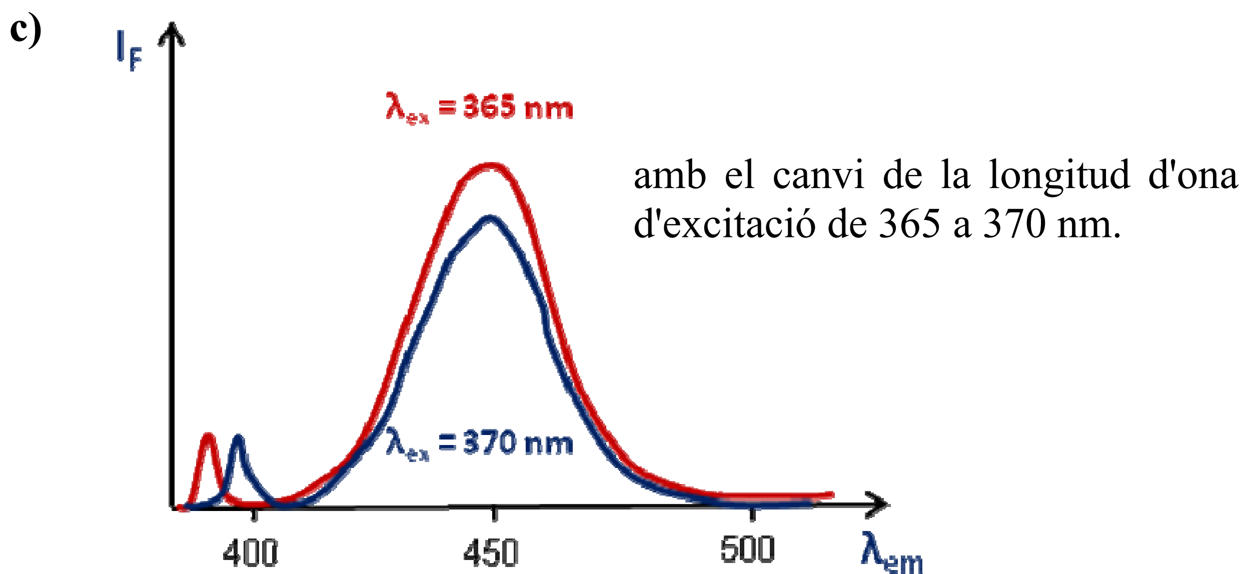
en els dissolvents A i B

b)



amb l'addició d'una substància D a dissolució,

c)



amb el canvi de la longitud d'ona d'excitació de 365 a 370 nm.

3.4.- En l'estudi de l'activitat lactat-deshidrogenasa (LDH) d'un extracte dialitzat de múscul s'ha afegit a 2 mL d'extracte 1 mL d'una dissolució contenint piruvat (en gran excés) i NADH. La mescla es col·loca immediatament en la cubeta del espectrofluorímetre i es mesura l'emissió fluorescent del NADH a 420 nm (excitació a 340 nm) en unitats arbitràries (ua) a intervals de 10 segons. Les dades obtingudes són:

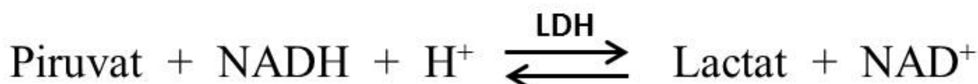
Temp (s)	**I_F (ua)**
10	185.0
20	171.0
30	156.5
45	134.8
60	113.0
90	72.0
120	38.5
150	20.2

D'altra banda, solucions de NADH, a concentracions conegudes, mesurades en les mateixes condicions van donar les següents lectures.

[NADH] (10^4 M)	**I_F (ua)**
0.5	38
1.0	74
1.5	110
2.0	146
3.0	218

Calculeu l'activitat enzimàtica de l'extracte en mol de substrat transformat per minut.

Reacció implicada:



S: $3{,}6\cdot10^{-7}$ mol/min

3.5.- Per a mesurar l'activitat d'una endoproteasa, capaç de tallar un petit fragment de l'extrem N-terminal de la seroalbúmina bovina (BSA), es marca aquesta amb clorur de dansil (un reactiu fluorescent específic de l'extrem amino). Amb açò, l'activitat endoproteolítica pot seguir-se mesurant la variació de la polarització de la fluorescència (p) del grup dansil.
Una dissolució de BSA marcada (que s'ha dialitzat prèviament per a eliminar tot el clorur de dansil lliure sobrant) se sotmet a l'acció de la endoproteasa en un volum de 2 ml (concentració final de BSA marcada= $2\cdot10^{-7}$ M) i es determinen els canvis de polarització del grup dansil amb el temps. S'obtenen els següents resultats:

Temps (min)	**p**
0	0.29
1	0.25
2	0.21
3	0.17
4	0.14
20	0.06

La polarització observada després de 20 minuts (0.06) es manté constant i estable per a temps d'incubació superiors.

a) Expliqueu a què es deu el descens de polarització observat.

b) Calculeu l'activitat enzimàtica (en mol de BSA processada per minut).

S: b) $6{,}96 \cdot 10^{-11}$ mol/min

3.6.- Una deshidrogenasa depenent de NADH posseeix un únic triptòfan (Trp). Se sospita que el Trp està prop del centre actiu perquè en absència de NADH el seu rendiment quàntic és 0.20 i descendeix a 0.16 quan la deshidrogenasa se satura amb NADH. Es creu que la disminució del rendiment quàntic és deguda a la transferència per ressonància entre Trp i NADH. El valor de la distància característica de transferència (R_0) per a aquest parell és de 20 Å.

a) Quins espectres caldria realitzar per a demostrar l'existència de transferència? Dibuixar de forma aproximada els espectres que cabria espera en cas que hi haguera i no hi haguera transferència.

b) Calcular la distància entre Trp i NADH en el cas que hi haja transferència.

Dades: Trpλ_{ex}= 290 nm; NADHλ_{ex}= 340 nm; Trpλ_{em}= 340 nm; NADHλ_{em}= 420 nm

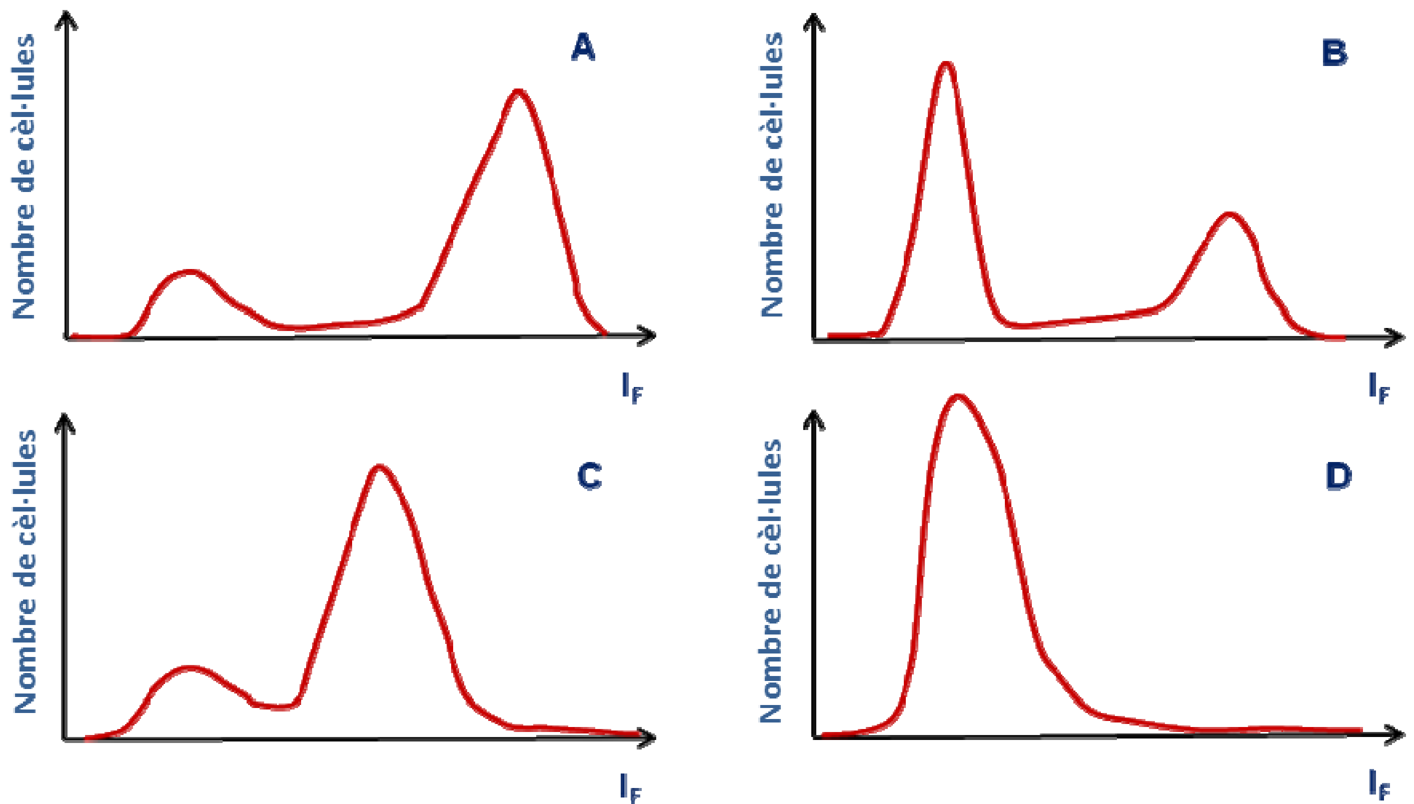
S: b) 25,2 Å

3.7.- Per a determinar la concentració de Ca^{2+} intracel·lular es tracta una suspensió de plaquetes sanguínies amb QUIN2AM (acetoximetil èster). Després de 24 hores d'incubació a 37 ºC les cèl·lules es renten i es resuspenen comprovant-se que la seua fluorescència (a la longitud d'ona d'emissió del QUIN2) s'ha estabilitzat en un valor de 54 unitats arbitràries (ua). A 3 mL d'aquesta suspensió cel·lular se li afegeix 0.5 mL d'una dissolució 50 mM de EGTA (un quelant de Ca^{2+} d'alta afinitat) amb calcimicina (un ionòfor capaç de permeabilitzar les membranes plaquetàries al pas del calci). S'observa que la fluorescència va descendint fins a estabilitzar-se en 24 ua. En afegir a continuació 0.5 mL de $CaCl_2$ 0.2 M es produeix un increment progressiu de la fluorescència fins a 78 ua. Calcular la concentració intracel·lular de Ca^{2+} que existia originalment en les plaquetes (abans de procedir a la seua manipulació).

Dada: K_D (constant d'equilibri de dissociació del QUIN2-Ca^{2+})= $7.8 \cdot 10^{-8}$ M

S: $4{,}06 \cdot 10^{-8}$ M

3.8.- Les gràfiques mostren el perfil obtingut en un citòmetre de flux per a un cultiu cel·lular en creixement continu (no sincronitzat) en el qual s'ha marcat amb fluorescència el DNA mitjançant un agent intercalant.



Les gràfiques corresponen a cultius en condicions diferents:

1) Cultiu control (sense cap tractament)
2) Cultiu tractat amb un inhibidor de la divisió cel·lular (inhibidor de la formació de l'envà intermedi entre les cèl·lules filles)
3) Cultiu en el qual s'ha detingut la replicació del DNA per expressió d'una proteïna que s'uneix en un punt concret (a 800 kb de l'origen de replicació) bloquejant l'avanç de la DNA-polimerasa.
4) Cultiu tractat amb un inhibidor de l'inici de la replicació.

Identifiqueu cadascuna de les quatre situacions (1-4) amb una de les quatre gràfiques (A-D).

3.9.- (Optatiu) Una proteïna receptora d'una hormona esteroide posseeix dos triptòfans (Trp). L'emissió dels Trp en una dissolució aquosa d'aquesta proteïna es pot captar a 350 nm quan s'excita a 290 nm. Si el receptor se satura amb hormona, la fluorescència augmenta lleugerament i se suposa que açò es deu al fet que un (o els dos) Trp han passat a un ambient menys polar (s'han protegit de l'entorn aquós) com a resultat del canvi de conformació causat per la unió de l'hormona. Si s'afegeix acrilamida (un extintor

colisional del Trp) a la dissolució de receptor s'observa la següent variació de la intensitat de fluorescència (IF):

[Acrilamida] (mM)	**I_F (ua)**	
	Receptor sol	Receptor + hormona
0	210	225
1	197	213
5	170	186
20	146	155
50	137	142
200	132	133
1000	130	130

Estan d'acord els resultats amb la interpretació anterior?

S: Hi ha que mostrar, d’alguna forma, que en presència d’hormona l’accessibilitat del Trp al desactivador acrilamida és menor.

PROBLEMES T4 (Classe)
ESPECTROSCÒPIA DE RESSONÀNCIA MAGNÈTICA NUCLEAR

4.1.- L'aminoàcid cisteïna té per fórmula elemental $C_3H_7O_2NS$. Si es col·loca cisteïna pura d'origen natural sota l'acció d'un camp magnètic extern de 16 T, indicar:

a) quins nuclis cal esperar que donen senyal en RMN i al voltant de quina freqüència la donarien.
b) quina seria la relació d'àrees entre els senyals de C i N i quina seria la màxima relació que pot aconseguir-se si la cisteïna es marca amb ^{13}C.

Nucli	I	γ ($T^{-1}\cdot s^{-1}$) x 10^{-6}	Abund. (%)	Sensibilitat	Receptivitat
^{1}H	1/2	267.53	99.98	1.000	1.00
^{2}H	1	41.07	0.0016	0.0096	$1.5\cdot10^{-6}$
^{13}C	1/2	67.28	1.1	0.016	$1.8\cdot10^{-4}$
^{14}N	1	19.34	99.63	0.001	0.001
^{19}F	1/2	251.79	100	0.834	0.834
^{31}P	1/2	108.40	100	0.066	0.066

S: b) $^{13}C/^{14}N$ = 0,54 i amb 13C en totes l'es posicions de la Cys $^{13}C/^{14}N$ = 48

4.2.- Quantes bandes de ressonància de ^{1}H-RMN cal esperar dels següents compostos? (no considerar les multiplicitats de moment):

a) metà
b) età
c) formaldehid
d) acetona
e) metanol
f) etanol
g) formamida

4.3. Dibuixeu de forma aproximada l'espectre de ^{1}H-RMN que cal esperar del etanal (=acetaldehid) i del propanal incloent les multiplicitats.

4.4.- Una mostra dóna un pic de ressonància en ^{1}H-RMN a 78656 G quan es realitza un escombratge de camp magnètic a freqüència constant (ν_0 = 334.90 MHz). En les mateixes condicions el patró de referència (tetrametilsilà) ressona a 78657 G. Trobar el desplaçament químic (δ) i la constant d'apantallament (σ) del pic de la mostra.
Dada: γ (^{1}H) = $267.53 \cdot 10^{6}\ T^{-1} \cdot s^{-1}$
S: δ= 12,72 ppm; (σ= $2{,}1484 \cdot 10^{-5}$

4.5.- Demostrar l'equivalència de les definicions de desplaçament químic (δ) a freqüència (ν) constant i a camp (B) constant.

ν = cte. δ (ppm)= $[(B_{ref} - B_m)/B_{ref}] \cdot 10^6$
B = cte. δ (ppm)= $[(\nu_m - \nu_{ref})/\nu_m] \cdot 10^6$

4.6.- Dos pics (A i B) de ^{1}H-RMN ressonen a camps B_A=75820 G i B_B=75832 G quan s'irradien a una freqüència ν_1= 322.82 MHz. Si existeix acoblament entre ells, a quin segona freqüència (ν_2) caldrà irradiar simultàniament per a eliminar del pic A les multiplicitats induïdes pel pic B? I per a eliminar en B les multiplicitats induïdes per A (ν_3)?
S: ν_2=322,769 MHz; ν_3= 322,871 MHz

4.7.- Es desitja mesurar l'activitat de la polinucleòtid-fosforilasa [(PNF) enzim que produeix un polinucleòtid de seqüència aleatòria a partir de nucleòsids trifosfat alliberant pirofosfat (PPi) amb la incorporació de cada monòmer] mitjançant ^{31}P-RMN. Per a açò s'incuba 1 mL d'extracte enzimàtic amb ATP 1 mM (concentració final) com a únic compost fosforilat en un volum total de 5 mL i s'obté un espectre mitjà (de 200 escombratges) cada 3 minuts. Després de quantificar les àrees dels pics corresponents al pirofosfat i a el β-fosforil de l'ATP en unitats arbitràries (u.a.), les dades obtingudes són:

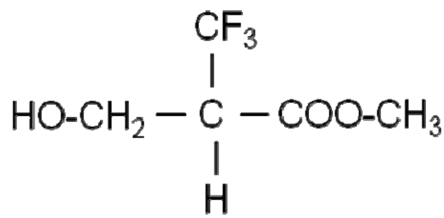
Δ temps (min)	**βP-ATP (ua)**	**PP_i (ua)**
0 - 3	32.2	0.6
3 - 6	31.6	1.8
6 - 9	31.0	3.0
9 - 12	30.4	4.2
12 - 15	29.8	5.3
15 - 18	29.3	6.4

Calcular l'activitat polinucleòtid-fosforilasa en mols d'ATP consumits per min i per mL d'extracte.

Reacció implicada:

S: $3{,}07 \cdot 10^{-8}$ mol/min·ml

4.8.- Es proposa el següent compost artificial (2-hidroximetil 3-trifluoro propanoat de metil) per a mesurar *in vivo* el pH intracel·lular d'un organisme acidòfil per ^{19}F-RMN:



a) Raonar el disseny d'aquesta substància com a indicador de pH.

b) Si el pKa de la forma àcida és 4.5 i els desplaçaments químics observats *in vitro* per al senyal del F en aquest compost són de 4.2 ppm a pH 8 i 8.3 ppm a pH 2, què pH intracel·lular indicaria un senyal a 6.9 ppm observada *in vivo*?

S: b) pH=4,21

PROBLEMES T6 (Classe)
MÈTODES ISOTÒPICS

6.1.- Una mostra que conté ^{35}S ($t_{1/2}$=87,1 dies) exhibeix una activitat de 10 mCi. Quina serà la seua activitat, expressada en dpm, després de 174 dies?
S: $5.55\cdot10^{9}$ dpm.

6.2.- El ^{14}C posseeix un $t_{1/2}$=5570 anys. Calculeu: a) la fracció d'àtoms de ^{14}C que es desintegren anualment i b) l'activitat específica màxima del ^{14}C.
S: a) 0,012%; b) $1,42\cdot10^{14}$ dpm/mol.

6.3.- A quants g de fòsfor equival 1 µCi de ^{32}P? ($t_{1/2}$=14,28 dies).
S: 3,5 pg

6.4.- Calculeu la massa de 1 µCi de ^{14}C ($t_{1/2}$=5570 anys).
S: 0,22 µg

6.5.- Calculeu la màxima radioactivitat específica teòrica que es pot aconseguir per al ^{11}C.
Dada: periode de semidesintegració de l'isòtop= 20,3 min; 1Ci= $3.7\cdot10^{10}$ dps
S: $2,06\cdot10^{22}$ dpm/mol= $9,26\cdot10^{9}$ Ci/mol = $1,86\cdot10^{21}$ dpm/g= $838,6\cdot10^{6}$ Ci/g.

6.6.- Un investigador rep 5 mCi de ^{131}I el dimecres a las 8 de la vesprada. L'experiment està programat per a començar el següent dilluns a las 8 del matí. Indiqueu l'activitat de ^{131}I que disposarà l'investigador per a realitzar l'experiment ($t_{1/2}$=8,04 dies).
S: 3,4 mCi

6.7.- Una mostra d'AMP amb doble marcatge amb ^{14}C i ^{32}P exhibeix una activitat específica de S= 9500 cpm/µmol. El 70% de la radioactivitat inicial procedeix del ^{32}P i la resta del ^{14}C. Calculeu l'activitat específica als 7, 14 i 28 dies.
Dades: λ ^{32}P= $5,65\cdot10^{-7}$ s^{-1}; λ ^{14}C = $1,44\cdot10^{-8}$ $hores^{-1}$
S: 7 dies 7586 cpm; 14 dies 6208 cpm; 28 dies 4546 cpm.

6.8.- Descriu la preparació d'1 mL de solució [γ-^{32}P]ATP 10 mM amb activitat específica $1,5\cdot10^{5}$ dpm/µmol. Per això es disposa de les soluciones estoc d'ATP 0,1 M ("fred") i [γ-^{32}P]ATP amb activitat específica 30 mCi/mmol i concentració 1mCi/mL.
S: 99,77 µL ATP 0,1 M (és a dir: 9,977 µmols) fred + 0,675 µL [γ-^{32}P]ATP (és a dir: $6,75\cdot10^{-7}$ Ci =$1,5\cdot10^{6}$ dpm i 0,0225µmols) completant fins 1 mL amb aigua o solució amortidora.

6.9.- Un estudiant vol determinar la quantitat de fòsfor que absorbeix un cultiu de tomaques en un medi hidropònic. L'experiment es realitza afegint una quantitat coneguda de fosfat marcat amb 1 μCi de ^{32}P a la solució hidropònica on creixeran les plantes. Després d'un període de 7 dies de creixement es determina una activitat de ^{32}P en les plantes de $6{,}78 \cdot 10^4$ dpm. Quin percentatge de fosfat ha assimilat el cultiu de tomaques als 7 dies d'experimentació? ($t_{1/2}$= 14,28 dies).
S: 4,3 %

6.10.- En una experiència de doble marcatge amb ^{3}H i ^{14}C es va introduir 0,2 g de mostra en un comptador discriminador d'altura de pols de dos canals A i B. En les mateixes condiciones es va determinar la radioactivitat de patrons de ^{3}H i ^{14}C en tots dos canals. Una vegada restat el soroll de fons a totes les mesures s'obtingueren les següents dades:

	cpm		
	^{3}H	**^{14}C**	**Mostra**
Canal A	175	80	120
Canal B	75	320	160

Sabent que l'eficiència és del 16 % per al ^{3}H i del 80 % per al ^{14}C, calcula la radioactivitat específica de la mostra para cadascun dels radioisòtops en Ci/g. Dades: 1 Ci= $3{,}7 \cdot 10^{10}$ dps
S: ^{3}H 1,8 nCi/g; ^{14}C 0,43 nCi/g

6.11.- A una sèrie de 6 mostres de patrons estàndard la radioactivitat dels quals és de 1250 dpm se'ls afegeix 5 quantitats creixents d'un extintor i es mesuren en un comptador discriminador d'altura de pols dividit en dos canals A i B, juntament amb un control sense extintor. Una mostra que també conté el radioisòtop i de la qual es sap que té extintors es mesura en el mateix comptador. Els resultats obtinguts són els següents:

	cpm						
	control	**1**	**2**	**3**	**4**	**5**	**mostra**
Canal A	500	478	445	410	379	332	1822
Canal B	500	425	354	290	227	170	1211

Quina és l'eficiència de la mesura de la mostra? Quina és la radioactivitat real de la mostra?
S: i) 53 %; ii) 5723 dpm

6.12.- Un cultiu de fags està marcat amb una mitjana de 42 àtoms de ^{32}P per fag. L'eficiència de detecció de l'isòtop en una emulsió gruixuda és del 90 %. Quant de temps ha d'exposar-se per a tenir una mitjana de 9 braços per estel?
Dades: $t_{½}$ ^{32}P= 14.2 dies S: 5,57 días

6.13.-A fi d'estudiar el recanvi de Na^{+} en la sang, s'injecta $^{24}NaCl$ en el torrent circulatori d'un animal, es prenen mostres de sang periòdicament i es mesura la radioactivitat

Temps (h)	**1**	**2**	**5**	**10**	**16**	**24**
cpm/mL	3604	2928	2376	1412	756	329

Quin és el temps de recanvi de l'ió Na^{+} en la sang? ($t_{½}$ ^{24}Na= 15 h)
S: 17 h

6.14.- Per a estudiar la relació entre iniciació i elongació de cadenes de RNA producte de l'activitat d'una RNA-polimerasa s'incuba aquest enzim amb rNTPs marcats amb ^{14}C en la ribosa (radioactivitat específica 100 cpm/mmol) i amb ^{32}P en el fosfat γ (radioactivitat específica 5000 cpm/mmol) i en presència de motlle i dels cofactors necessaris per a la síntesi. Transcorreguts 3 min, la reacció es deté per precipitació amb àcid tricloroacètic (TCA). La radioactivitat incorporada en el precipitat es mesura amb un comptador analitzador d'altura de pols amb dos canals A i B i s'obtenen els següents resultats:

	cpm		
	^{32}P	**^{14}C**	**Mostra**
Canal A	215	455	407
Canal B	645	245	325

Calcula la longitud mitjana, en nombre de nucleòtids, de les cadenes sintetitzades per la RNA-polimerasa així com el nombre de cadenes.
S: i) 165 N/cadena; ii) $2,09 \cdot 10^{19}$ cadenes.

6.15.- Una mostra de 10 g de proteïna va ser hidrolitzada en els seus aminoàcids. A aquesta mostra se li van afegir 3 mg de treonina marcada amb ^{14}C de radioactivitat específica 1000 cpm/mg. Posteriorment es van aïllar els aminoàcids mitjançant cromatografia d'intercanvi iònic, obtenint-se 60 mg de treonina pura amb una radioactivitat específica 20 cpm/mg. Quin és el percentatge de Thr en la proteïna?
S: 1,47 %

PROBLEMES T7 (Classe)
ELECTROFORESI

7.1.- En unes determinades condicions d'electroforesi, la velocitat de moviment de la Gly a pH 9,5 és de 2 cm/h. Quina serà la velocitat a pH 8,1 i a pH 5,5? Quina seria la velocitat màxima que pot aconseguir la Gly i a quin pH es produiria?
Dades: Gly: $pK_{1(\alpha COOH)}$= 2,34; $pK_{2(\alpha NH3+)}$= 9,6
S: pH= 8,1; 0,136 cm/h,cap el ànode; pH=5,5; 2,75 10^{-3} cm/h, cap el càtode.

7.2.- Una mescla de tres tripéptidos s'intenta separar per electroforesi en paper. Quin pH és el més adequat per a obtenir una bona separació?
A: His-Glu-Ser
B: Lys-Asp-Arg
C: Val-Glu-Arg

7.3.- Els dipèptids α-aspartilhistidina i β-aspartilhistidina posseeixen les següents característiques de dissociació:

	pK_1	pK_2	pK_3	pK_4
α-Asp-His	2,45	3,03	6,82	7,98
β-Asp-His	1,90	2,95	6,90	8,72

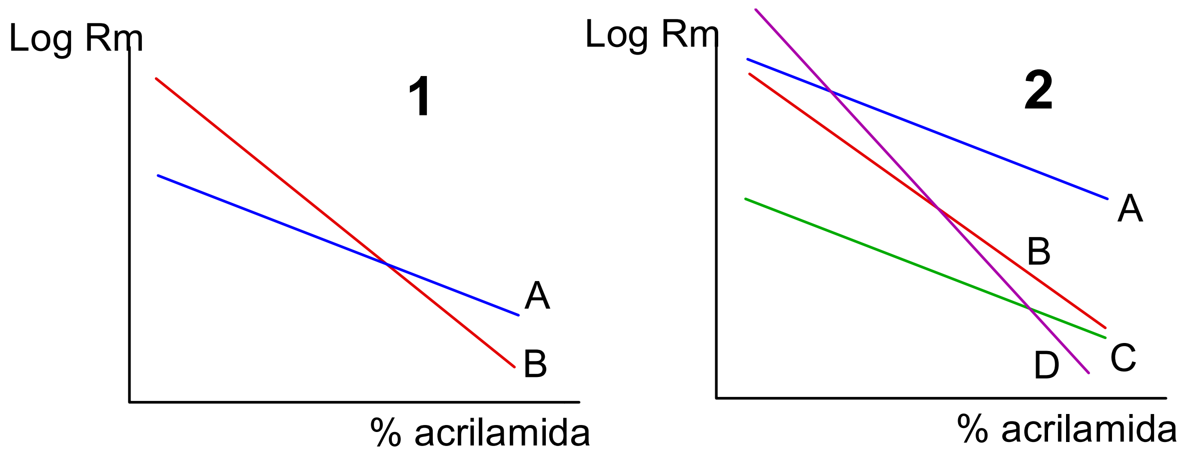
Determineu la relació entre les mobilitats electroforètiques de tots dos pèptids en dissolució a pH 1,90. Quina seria la tècnica més apropiada per a la seua separació: electroforesi de zona o enfocament isoelèctric?
S: μ_α/μ_β = 1,19

7.4.- El gel de poliacrilamida s'empra tant en cromatografia d'exclusió molecular com en electroforesi. En cromatografia les molècules més grans elueixen abans, no obstant açò en electroforesi queden més retardades. A què es deu aquesta diferència de comportament?

7.5.- Fes un esquema del resultat d'una electroforesi en acetat de cel·lulosa (pH 7,0) de les següents proteïnes (el punt d'aplicació de la mostra és el centre de la tira). Comenta-ho breument.

	MM	pI
proteïna A	60000	7,0
proteïna B	20000	8,0
proteïna C	60000	5,0

7.6.- Interpreta les següents representacions de Ferguson. Indica les relacions relatives, quant a càrrega, massa i densitat de càrrega, entre les proteïnes representades en cada gràfica.



7.7.- S'ha determinat la mobilitat electroforètica relativa de l'enzim lactat-deshidrogenasa i de diverses proteïnes patró en electroforesi en gel de poliacrilamida al 7.5 % en presència de SDS, obtenint-se els valors de la Taula:

	Mostra	**Patrons**					
proteïna	Lactat-deshidr.	BSA	Catalasa	Glutamat-deshidr.	Glicerald-3-P-deshidr.	Tripsina	Mioglob
MM (kDa)	¿?	68	60	53	36	23.3	17.2
Rm	0,455	0,18	0,22	0,29	0,40	0,60	0,74

Calcula la massa molecular de la lactat-deshidrogenasa.

S: MM 33,9 kDa.

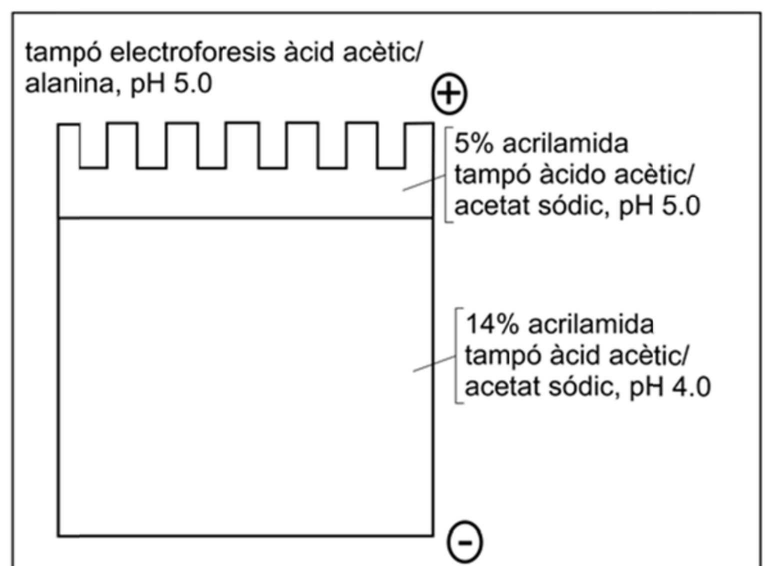
7.8.- Una electroforesi discontinua de proteïnes es duu a terme en les següents condicions:

Tampó d'electroforesi: àcid acètic/alanina pH 5,0

Gel concentrador: 5 % acrilamida; tampó àcid acètic/acetat sòdic pH 5,0

Gel separador: 14 % acrilamida; tampó àcid acètic/ acetat sòdic pH 4,0

Indiqueu quin és el ió guia (*leading ion*), i el ió de rastreig (*trailing ion*). Enumereu les discontinuïtats introduïdes.



7.9.- L'electroforesi d'un enzim, prèviament tractat amb SDS i 2-mercaptoetanol, en gel de poliacrilamida contenint SDS genera dues bandes amb mobilitats relatives de 0,51 i 0,56. La massa molecular de l'enzim actiu, determinada per cromatografia d'exclusió molecular sobre Sephacryl S-200 HR (confirmada per ultracentrifugació) és de 130000.

Indica la possible estructura quaternària de l'enzim així com la massa molecular de les seues subunitats. La mobilitat relativa de proteïnes patró sotmeses a electroforesi en presència de SDS en les mateixes condicions es mostra en la següent Taula:

Proteïna	**Massa molecular**	**Mobilitat relativa**
Mioglobina	17800	0,75
Tripsina	23600	0,60
G3PDH	36000	0,40
Ovoalbúmina	43500	0,16

S: MM= 26000 y 28000.

7.10.- Una proteïna oligomèrica se sotmet a electroforesi en gel de poliacrilamida en presència de SDS sota dues condicions diferents:

1) La proteïna, en estat natiu, es tracta durant un temps limitat amb un agent entrecreuador (açò porta a la unió covalent, parcial, de les cadenes polipeptídiques que estan en contacte en l'estructura nativa). Després de l'electroforesi SDS-PAGE s'obtenen 4 bandes amb Rm 0,10; 0,30; 0,52 i 0,70.

2) La proteïna, sense tractament previ, se sotmet a SDS-PAGE i s'obtenen 2 bandes de Rm 0,52 i 0,70. El densitometrat del gel proporciona uns valors de 33690 i 12578 unitats arbitràries respectivament per a aquestes dues bandes.

D'altra banda se sotmeten a electroforesi en les mateixes condicions 4 proteïnes patró de masses moleculars 15, 40, 60 i 90 kDa les quals generen 4 bandes amb Rm de 0,82; 0,40; 0,22 i 0,04 respectivament.

Quin és la massa molecular de la proteïna en estat natiu? Quines conclusions es poden deduir respecte a l'estructura quaternària de la proteïna?
S: MM natiu 80 kDa

7.11.- La següent Taula mostra el comportament d'una proteïna X, comparada amb patrons de massa molecular coneguda, en SDS-PAGE i en gels en gradient de poliacrilamida (PAGGE).

	SDS-PAGE	**PAGGE**
	Rm (relativa al blau de bromofenol)	**Distància recorreguda (mm)**
Proteïna X	0,47	51
Patró 15 kDa	0,86	81
Patró 28 kDa	0,58	66
Patró 40 kDa	0,43	59
Patró 65 kDa	0,26	52
Patró 89 kDa	0,14	49
Patró 118 kDa	0,04	46

Determina la massa molecular de la proteïna X. S'obté informació addicional per haver realitzat dos tipus diferents d'electroforesis? Es pot determinar Rm, respecte a un marcador (per exemple blau de bromofenol) en PAGGE?

S: MM SDS-PAGE, 38 kDa; MM PAGGE, 76 kDa

7.12.-La migració de RNA en gels de poliacrilamida és inversament depenent de la seua massa molecular. S'ha realitzat l'electroforesi de RNA del fag T5 d'*E. coli*, en un gel de poliacrilamida del 10 %, emprant com a patrons RNAs de massa coneguda, obtenint-se els següents resultats:

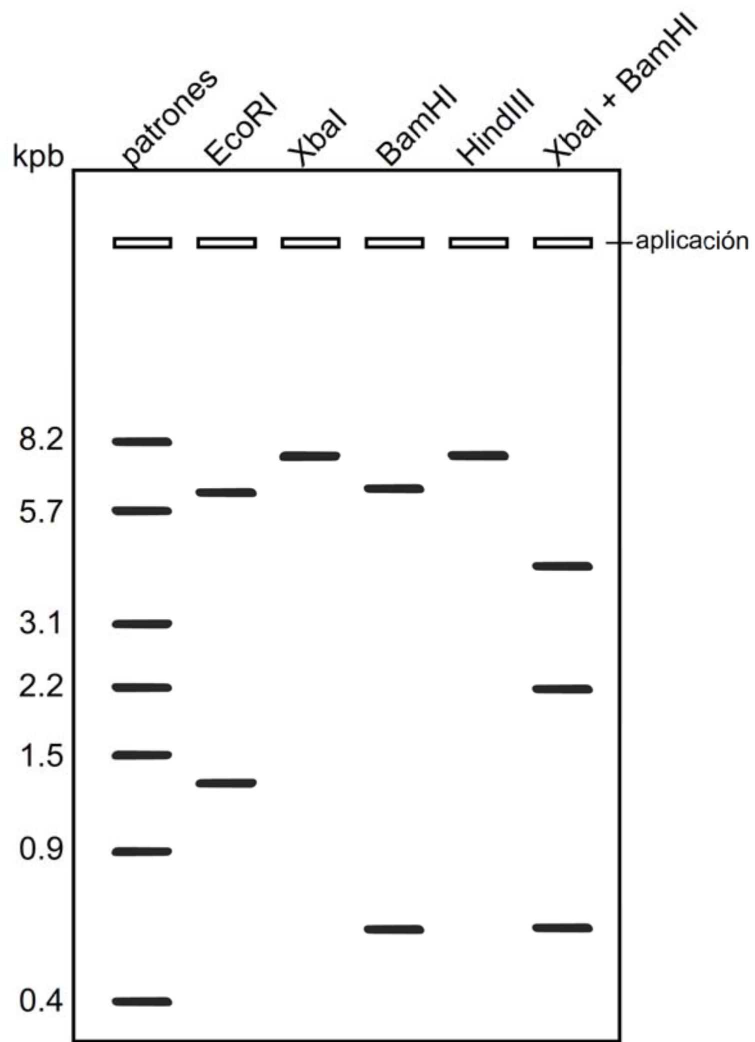
distància (cm)	**8**	**6**	**1,8**
massa molecular	$2{,}6\cdot10^4$	$3{,}8\cdot10^4$	$8{,}5\cdot10^4$

Calcula la massa molecular dels dos tipus de RNA del fag, sabent que migren 4,3 i 2,4 cm en les mateixes condicions que els patrons anteriors

S: $MM_{D4.3}= 5{,}3\cdot10^4$; $MM_{D2.4}= 7{,}6\cdot10^4$

7.13.- Un fragment de DNA amb extrems XbaI-KpnI s'introdueix en el lloc de clonació múltiple (EcoRI-XbaI-PstI-KpnI-BamHI-HindIII) d'un vector pBS (4,2 kpb). El plasmidi circular obtingut se sotmet a digestió per diversos enzims de restricció i els productes es resolen en electroforesis en gel de agarosa (0,8 %) juntament amb patrons de grandària coneguda (fragments de DNA lineals). Un esquema del gel després de l'electroforesi, tenyit amb bromur d'etidi i exposat a llum ultraviolada es mostra en la figura.

Realitza el mapa dels punts de tall dels enzims de restricció utilitzats.


kpb
patrones
EcoRI
XbaI
BamHI
HindIII
XbaI + BamHI
aplicación
8.2
5.7
3.1
2.2
1.5
0.9
0.4

Aminoàcid	**Abreviatura**	**pK_{a_1}**	**pK_{a_2}**	**pK_{a_R}**	**Mr**
Àcid aspàrtic	Asp D	2,09	9,82	3,86	113
Àcid glutàmic	Glu E	2,19	9,67	4,25	147
Alanina	Ala A	2,34	9,69	-	89
Arginina	Arg R	2,17	9,04	12,48	174
Asparagina	Asn N	2,02	8,80	-	132
Cisteïna	Cys C	1,71	10,78	8,33	121
Fenilalanina	Phe F	1,83	9,13	-	165
Glicina	Gly G	2,34	9,60	-	75
Glutamina	Gln Q	2,17	9,13	-	146
Histidina	His H	1,82	9,17	6,00	155
Isoleucina	Ile I	2,36	9,68	-	131
Leucina	Leu L	2,36	9,60	-	131
Lisina	Lys K	2,18	8,95	10,53	149
Metionina	Met M	2,30	9,20	-	131
Prolina	Pro P	1,99	10,60	-	115
Serina	Ser S	2,21	9,15	-	105
Tirosina	Tyr Y	2,20	9,11	10,07	181
Treonina	Thr T	2,63	10,43	-	119
Triptòfan	Trp W	2,38	9,39	-	204
Valina	Val V	2,32	9,62	-	117

PROBLEMES T8 (Classe)
CROMATOGRAFIA

8.1.- Les substàncies A, B, C i D presenten els següents Rfs en cromatografia en paper amb cadascun dels 4 eluents següents (1-4):

	1-Butanol-H_20	**2-**Isopropanol-HCl	**3-**Benzè-à. acètic	**4-**Cloroform-metanol
A	0,23	0,31	0,42	0,09
B	0,24	0,20	0,51	0,62
C	0,38	0,58	0,40	0,64
D	0,41	0,56	0,53	0,10

Quin procediment donaria millors resultats per a separar les quatre substàncies? (assumir desenvolupament de 10 cm i resolució de taques quan els seus centres disten almenys 1 cm).
En una cromatografia en capa fina bidireccional vària la resolució si s'inverteix l'ordre dels eluents emprats?

8.2.- En un sistema cromatogràfic, un solut té un temps de retenció de 407 s i una amplària en la base de 13 s. Un pic veí és eluït a 424 s i té una amplària en la base de 16 s. Calculeu la resolució de la columna per a aquests dos components.
S: Rs =1,17

8.3.- En un laboratori de control de qualitat es vol posar a punt un mètode d'anàlisi per HPLC en fase inversa d'una mescla de dos compostos A i B. Se sap que, en columna de octadecil-sílice de 25 cm de longitud i utilitzant una fase mòbil de metanol-aigua, els temps de retenció són 6,25 i 7,10 min respectivament, el temps mort és 1,4 min i la resolució és 1,05.

a) Quina ha de ser la longitud de la columna per a aconseguir una resolució d'1,5?
b) Calculeu els factors de retenció de A i B. Seran diferents en la nova columna?

S: a) 51 cm, b) K_A= 3,46; K_B= 4,07, iguals

8.4.- En un sistema cromatogràfic, un solut té un temps de retenció ajustat de 407 s i una amplària en la base de 13 s en una columna de 12,2 cm de longitud. Calculeu el nombre de plats teòrics.
S: 15680 plats teòrics

8.5.- En una columna de 10 cm de longitud, es duu a terme la separació cromatogràfica de dues substàncies A i B, obtenint-se factors de capacitat de 0,8 i 1,0 respectivament.

a) Si es considera una resolució d'1 per a tots dos pics, calculeu l'altura equivalent de plat teòric.

b) Si el temps de retenció de la substància no retinguda és d'un minut, calculeu els amples de banda en la base dels analits A i B.

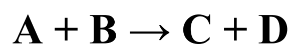
S: a) 0,0692 mm, b) W_A= 0,189 min; W_B= 0,210 min

8.6.- Tres soluts A, B i C són eluïts d'una columna de cromatografia (repartiment, fase reversa) amb volums de elució de 36, 35 i 12 mL respectivament. Se sap que C no pateix cap retard en aquesta cromatografia, ja que no interacciona en absolut amb la fase estacionària. Quin és el nombre mínim de plats teòrics de la columna necessari per a resoldre completament A i B?

S: N= 20164; però N= 8800 si se utilitzen volums d'elució ajustats

8.7.- Quin procediment cromatogràfic podria emprar-se per a separar les histones (proteïnes molt bàsiques, amb un pI entorn de 10) d'un extracte proteic cru?

8.8.- La reacció catalitzada per l'enzim I, el qual requereix Mg^{2+} com a cofactor, és:



A + B → C + D

Es pretén esbrinar si aquesta reacció és ordenada o a l'atzar. Hi ha algun indici previ que l'ordre podria ser: Mg^{2+}, A, B. Com es podria investigar aquesta qüestió mitjançant procediments cromatogràfics? (suggereix més d'una forma).

8.9.- La glutamat-deshidrogenasa (GDH) és un enzim que catalitza la següent reacció:

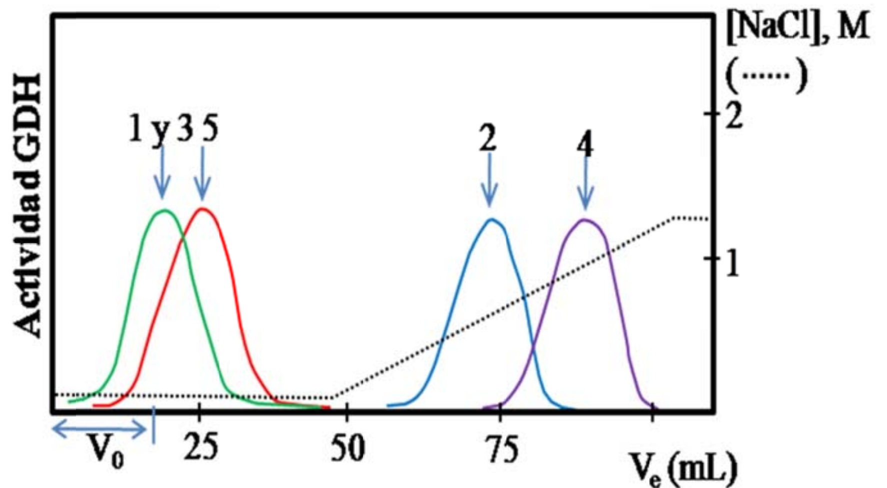
Glutamat + NAD^+ + H_2O → α-oxoglutarat + NH_4^+ + NADH + H^+

L'aspartat actua com a inhibidor competitiu d'aquesta reacció. El GDP és un activador al·lostèric (unió a un lloc diferent al dels substrats) de l'enzim. Per a comprovar aquestes propietats es realitza una cromatografia d'afinitat en columna d'agarosa a la qual s'ha unit l'aspartat (-CO-CHNH$_2$-CH$_2$-COOH) com lligand. S'aplica a la columna una fracció purificada de l'enzim GDH, en forma de diverses alíquotes en diferents mitjans d'elució:

1) Tampó pH=7.0
2) Tampó + NAD^+ 2 mM
3) Tampó + GDP 5 mM
4) Tampó + NAD^+ 2 mM + GDP 5 mM
5) Tampó + NAD^+ 2 mM + Glutamat 0,1 M

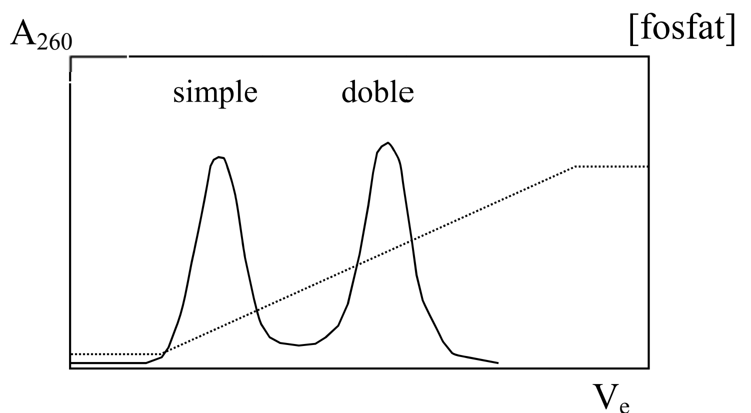
Els perfils d'elució obtinguts es representen a la figura.

A la vista d'aquests resultats raonar si l'aspartat és realment un inhibidor competitiu del glutamat i quin podria ser l'efecte del GDP. Es pot dir alguna cosa sobre el mecanisme (seqüencial o a l'atzar) de la reacció catalitzada per la GDH?



8.10.- Els àcids nucleics de simple i doble cadena poden separar-se en columnes d'hidroxiapatit (Figura). Explica el mecanisme d'interacció en aquest suport cromatogràfic.

Si una molècula amb 50 % de doble cadena i un altre 50 % de simple cadena, on cal esperar la seua elució? Amb el pic corresponent a simple o doble cadena, o al mig?



8.11.- Per una columna (55 cm x 2 cm) de Superdex 75 (rang de fraccionament 3000-80000) se separen 3 proteïnes patró: mioglobina; ovoalbúmina; seroalbúmina, a més de blau dextrà i una proteïna la massa molecular de la qual es desitja conèixer. Els volums d'elució són:

	Massa molecular	V_e (mL)
Mioglobina	20000	150
Ovoalbúmina	40000	123
Seroalbúmina	60000	90
Blau dextrà	1000000	70
Proteïna problema	¿?	128

El volum total de la columna, calculat a partir del radi i l'altura del suport és de 170 mL. Calcula la massa molecular de la proteïna problema.

S: 31 kDa

8.12.- Què ocorre si es representa V_e enfront de log MM en lloc de K_{av} enfront de log MM? Serviria també per a l'obtenció de la massa molecular d'una proteïna problema?

8.13.- Quins són els valors límit teòrics per a K_{av}? Què indicaria si s'obté per a una proteïna una $K_{av} > 1$? Seria correcta la deducció de la massa molecular per a eixa proteïna? Per què? Com podria evitar-se el fenomen que origina valors de $K_{av} > 1$?

8.14.- Es desitja concentrar una dissolució d'una proteïna que es troba dissolta a 0,24 mg/mL en tampó Tris 20 mM pH 8,1 (pK_a= 8,1). La massa molecular de la proteïna és de 24000 i el seu pI=6,0. Descriu almenys dos procediments cromatogràfics que permeten concentrar aquesta proteïna. Descriu els detalls dels procediments.

8.15.- Es va hidrolitzar, per tractament amb HCl 6 M (30 hores), una certa quantitat d'un pèptid i l'hidrolitzat resultant es va passar a través d'un analitzador automàtic d'aminoàcids obtenint els resultats de la Taula.

	μmols	**Mr**
Ala	0,147	89
Arg	0,105	174
Lys	0,126	146
Gly	0,168	75
Met	0,042	149
Ser	0,252	105

El tractament del mateix pèptid amb tripsina va donar lloc a una sèrie de pèptids més curts el nombre dels quals no va poder determinar-se amb exactitud (per solapament de taques en cromatografia en capa fina bidimensional), però es va estimar entorn de 10.
Determina la massa molecular del pèptid.
S: 3825.

PROBLEMES T9 (Classe)
CENTRIFUGACIÓ

9.1.- Calcula la velocitat angular a partir de les següents velocitats de rotació.

a) 2400 rpm

b) 1000 rps

Si una partícula situada a 12,5 cm de l'eix de gir es troba girant a les velocitats anteriors, quin és el camp centrífug relatiu al que es troba sotmesa?

S: a) 251,33 rad/s; b) 6283,2 rad/s; a- 806*g*; b- 503554*g*

9.2.- Un procediment de ultracentrifugació requereix centrifugar durant 10 hores en un rotor del qual el camp centrífug relatiu màxim és de 150000*g*. Si el fons del tub està situat a 9,8 cm de l'eix, quina velocitat ha d'emprar-se? Si a causa d'una deterioració del rotor aquest només pot emprar-se a una velocitat màxima de 30000 rpm, quin pot servir com a procediment alternatiu de ultracentrifugació sota aquesta limitació?

S: 37000 rpm; tenint amb compte que $\omega^2_1 \cdot t_1 = \omega^2_2 \cdot t_2 \rightarrow$ 15,2 hores

9.3.- Una suspensió de material biològic conté quatre classes de partícules subcel·lulars amb les següents característiques:

Partícula	$s_{20,w}$ (Svedberg)	Densitat (g/mL)
P1	$5{,}3 \cdot 10^7$	1,405
P2	$4{,}4 \cdot 10^5$	1,410
P3	$1{,}1 \cdot 10^4$	1,292
P4	$9{,}8 \cdot 10^3$	1,102

Dissenya un esquema de separació que permeta aïllar les partícules amb un grau de contaminació mínim.

S: Per centrifugació diferencial, però no és possible l'aïllament complet donat els valors propers de s. Per centrifugació zonal: P3 i P4 isopícnica (p.e. sacarosa 10%- 1,04 g/mL, 50% 1,23 g/mL). P1 i P2 per centrifugació zonal de velocitat de sedimentació en gradient de densitat.

9.4.-. Demostra que els ribosomes d'eucariotes, formats per les subunitats 40S i 60S, originen una unitat 80S. Assumeix que tant el ribosoma complet com les seues subunitats són partícules esfèriques i que posseeixen idèntic volum específic parcial. Quin seria el s d'una partícula formada per un dímer de ribosomes? (aplica les mateixes suposicions anteriors).

S: Es tracta de demostrar la dependència de s amb M. De Svedberg, $s=m_p(1-\upsilon\rho_d)/f$. Posem m_p en funció de M i nº Avogadro: $m_p=M/N_a$; i $f=6\pi\eta r$ (r= radi d'esfera, de volum $Vol_p=4/3\pi r^3$ y $Vol_p=m_p/\rho_p \rightarrow Vol_p=M/N_a\rho_p$, així $\upsilon=1/\rho_p \rightarrow Vol_p=M\upsilon/N_a$). Substituint $s=M^{2/3}\{[(4/3)^{1/3}(1-\upsilon\rho_d)]/6[N_a^{2/3}\eta\upsilon^{1/3}\pi^{2/3}]\}$. Tot entre les claus són constants per a totes les partícules (enunciat)$\rightarrow s=M^{2/3}K$. Equació aplicable a qualsevol de les partículs. $s_{60}=M_{60}^{2/3}K$; $s_{40}=M_{40}^{2/3}K$ i $s_{rib}=(M_{60}+M_{40})^{2/3}K$. Finalment s'arriba a: $s_{rib}^{3/2}=s_{40}^{3/2}+s_{60}^{3/2} \rightarrow s_{rib}^{3/2}=40^{3/2}+60^{3/2}$. $s_{rib}=80{,}1S$.
$s_{dímer}=[2s_{rib}^{3/2}]^{2/3}$; $s_{dímer}=[2\cdot(80{,}1)^{3/2}]^{2/3}$; $s_{dímer}=127{,}3S$

9.5.- Mitjançant centrifugació diferencial d'un extracte de teixit es van obtenir 4 fraccions en les quals es va determinar l'activitat de 4 enzims marcadors i d'un enzim problema (X) la localització subcel·lular del qual es desitjava conèixer. Les dades d'activitat enzimàtica (u.a./mL), juntament amb els de contingut proteic total en cada fracció, es mostren en la següent Taula:

Fracció	proteïna (mg/mL)	RNA-polimerasa	Citocrom-oxidasa	Glucosa-6-fosfatasa	Lactat-DH	X
I	0,82	9,43	315,4	266,7	169,3	93,7
II	1,23	0,37	2760,4	505,4	539,7	231,4
III	0,65	0,12	312,6	467,4	352,3	179,7
IV	0,32	0,04	30,8	82,2	446,0	38,7

Quina és la localització subcel·lular més probable de X?
S: es divideix l'activitat enzimàtica de cada marcador i de X per la concentració de proteïna (activitat específica).

9.6.- Es centrifuguen a 30000 rpm les subunitats 30S i 50S ribosomals i el tRNA 4S de procariotes en un rotor amb els radis de gir (x) següents: x_{min}= 4 cm i $x_{màx}$=10 cm. Quin és el temps necessari de centrifugació, t_{sed}, per a sedimentar completament la subunitat 50S? Sota aquestes condicions què % de les partícules 30S i tRNA 4S han sedimentat també?
S: amb la forma integrada de l'equació de Svedberg→5hores 9min.
De nou amb la mateixa equació, però ara la incògnita el $x_{mín}$ en el que sedimentarien les altres partícules: 30S→70 %; tRNA 4S→11,83 %.

9.7.- Es pretén separar mitocondris i cloroplasts des d'un homogeneïtzat vegetal. Ambdues partícules presenten una forma similar que pot ser considerada esfèrica, però els cloroplasts són més voluminosos ($8{,}2\ 10^{-3}$ mm^3) que els mitocondris ($5{,}03\cdot10^{-3}$ mm^3). Les densitats també són diferents: 1,22 g/cm^3 per als cloroplasts enfront d'1,28 g/cm^3 per als mitocondris.

a) Si l'homogeneïtzat es centrifuga en un medi uniforme, quines característiques del medi i/o de la centrifugació governen la separació? En quines condicions sedimentarien junts els dos tipus d'orgànuls?

b) En centrifugació zonal en gradient lineal de sacarosa, quins serien els valors extrems de densitat del gradient més adequats per a obtenir una bona separació de cloroplasts i mitocondris? Quin dels dos tipus de partícules sedimentaria més ràpidament en el gradient triat? Tingueu en compte que el valor mínim de densitat del medi ha de ser 1,03 g/cm^3 (resulta isotònica per a les partícules).

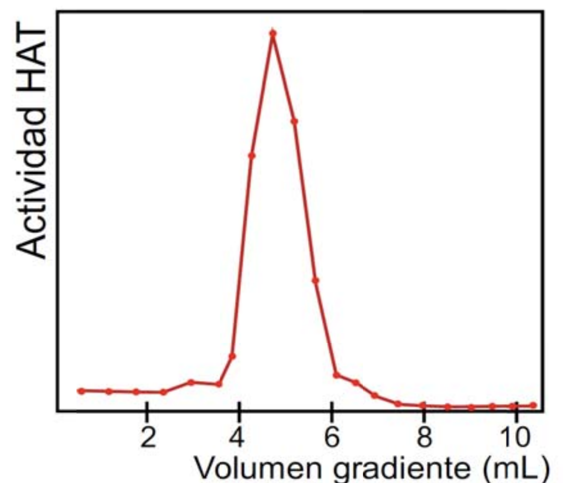
S: a) densitat, viscositat, Tª, velocitat, distància a l'eix (tipus de rotor), temps. En general paràmetres que apareixen en l'equació de Svedberg: $v= s\cdot\omega^2\cdot t$, on $s= m_p(1-\upsilon\rho_d)/6\pi\eta r f_0$ és el coeficient de sedimentació.

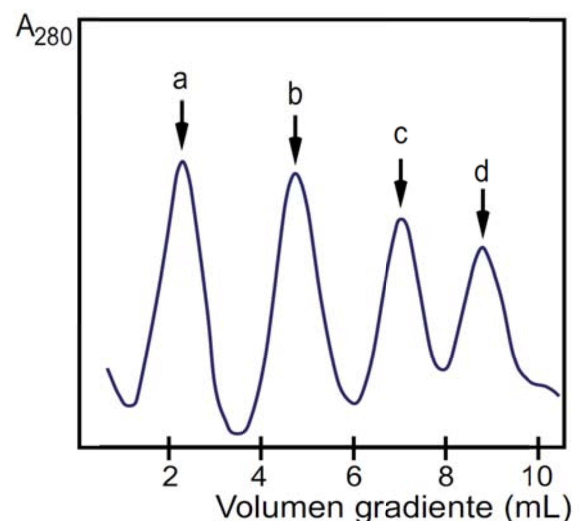
Hi ha que esbrinar la densitat del medi (ρ_d) en el que s de mitocondries (s_m) i cloroplasts (s_c) siguen iguals: $s_m=s_c$. Per a mitocondris, $s_m=m_m(1-\upsilon_m\rho_d)/6\pi\eta r_m$; i per a cloroplasts $s_c=m_c(1-\upsilon_c\rho_d)/6\pi\eta r_c$. Com $m=Vol\cdot\rho$ i $\upsilon=1/\rho$ i també volum d'una esfera: $Vol=4/3\pi r^3 \rightarrow [r=(3Vol/4\pi)^{1/3}]$. Calculem la massa per ambdós orgànuls: $m_m= 6,4\cdot10^{-6}$g i $m_c= 1\cdot10^{-5}$g i també $\upsilon_m= 0,781$ cm^3/g i $\upsilon_c= 0,820$ cm^3/g. Així mateix podem obtenir els radis: $r_m= 0,0106$ cm i $r_c= 0,0125$ cm. Substituint tot açò en s tenim: $s_m= [6,4\cdot10^{-6}\cdot(1-0,781\rho_d)]/6\pi\eta0,0106$ i $s_c= [1\cdot10^{-5}\cdot(1-0,82\rho_d)]/6\pi\eta0,0125$. Igualant arribem a $\rho_d= 1,064$ g/mL.

b) En centrifugació zonal un gradient adequat seria aquell amb densitats entre 1,064 i 1,22. Els mitocondris serien més ràpids.

9.8.- Es pretén l'estimació de la massa molecular d'un isoenzim histona-acetiltransferasa (HAT) mitjançant ultracentrifugació en gradient de densitat. Per a açò es van emprar gradients de sacarosa del 7 al 18 % (11 mL), que després d'aplicar la mostra es va ultracentrifugar 20h a 38000 rpm en rotor basculant SW40Ti (Beckman). Els gradients van ser després fraccionats i les fraccions resultants emprades per a analitzar l'activitat HAT mitjançant un assaig específic. Paral·lelament, en idèntiques condicions, es va ultracentrifugar una mescla de 4 proteïnes de masses moleculars conegudes. En la figura adjunta es mostren els resultats obtinguts.

Determina la massa molecular de l'enzim HAT. Què ha sigut assumit, respecte a l'estructura de les proteïna implicades, per a poder realitzar aquesta determinació?





Las proteïnes patró emprades foren: a, catalasa MM 240.000; b, aldolasa MM 158.000; c, seroalbúmina bovina MM 68000 y d, citocrom *c* MM 12500. El gradient fou fraccionat des del fons cap a la superfície.
S: Aplicant el procediment de Martin i Ames ($M^{2/3}$ *vs* Distància)→ MM=140000. Per el procediment de Redon i Calcagno (log M *vs* log Distància)→MM=141000

9.9.- Quina és la composició de bases aproximada del DNA del fag T1 d'*E. coli* sabent que la seua densitat de flotació (θ) en clorur de cesi és d'1,7057 g/mL?
S: Existeix una relació empírica que permet deduir: % GC fag T1= 47,7 %

9.10.- La centrifugació a 30000 rpm d'una determinada partícula en una ultracentrífuga analítica origina els següents resultats:

Temps (min)	Distància del front a l'eix (cm)
2	6,225
10	6,310
14	6,358
18	6,400
22	6,445
26	6,491

Calcula el coeficient de sedimentació de la partícula.
S: 29,2S

9.11.- Una proteïna de volum específic parcial (υ) 0,72 mL/g se sotmet a ultracentrifugació analítica (60000 rpm) dissolta en un medi de densitat 1,015 g/mL. Repetint la ultracentrifugació amb diferents concentracions de proteïna es van obtenir els següents coeficients de sedimentació:

Concentració (mg/mL)	s, (Svedbergs)
0,02	9,61
0,05	9,09
0,07	8,77
0,10	8,33

a) Coneixent el coeficient de difusió de la proteïna ($D= 3{,}3\cdot10^{-7}$ cm^2/s) i la temperatura de 27ºC, calculeu la massa molecular de la proteïna.

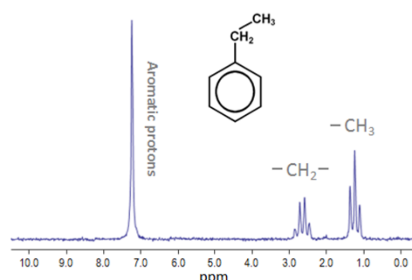
b) Si la viscositat del medi ($\eta_{27,d}$) era de 1,160 centipoises (cP) calculeu el coeficient de sedimentació estàndard de la proteïna ($s^o_{20,w}$).

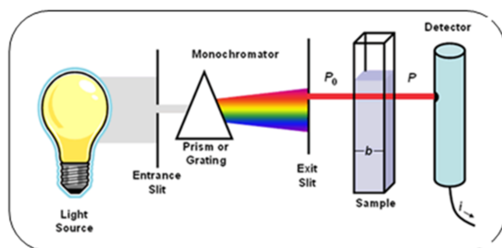
Dades: R (contant gasos)= 8,3 J/mol·ºK= $8{,}3\cdot10^7$ erg/mol·ºK (1 erg=1g·cm^2/s^2).
1 Poise (1 P)= 1 g/cm·s; 1 cP= 0,01 g/cm·s; $\eta_{20,w}$= 1,009 cP; $\rho_{20,w}$= 0,998 g/mL
S: a) s^0= 10S. MM= 280000, hi ha que tenir amb compte la llei de Fick que relaciona el coeficient de difusió, D, amb el coeficient de fricció, f; b) $s^0_{20,w}$= 12S.

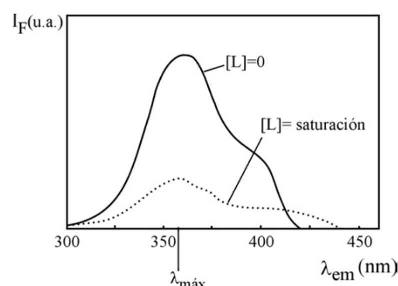
Departament de Bioquímica i Biologia Molecular

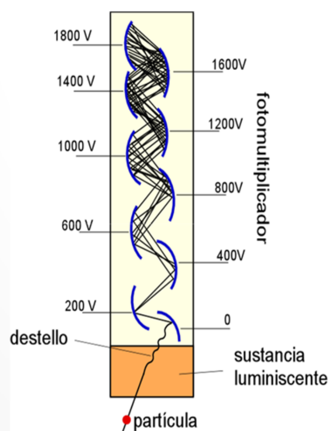
Problemes d'Estudiants

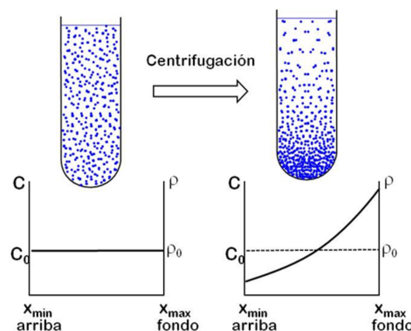
Tema 1e: Caracterització de molècules d'interès biològic
Tema 2e: Espectroscòpia d'absorció
Tema 3e: Espectroscòpia de fluorescència
Tema 4e: Espectroscòpia de ressonància magnètica nuclear
Tema 6e: Radioisòtops
Tema 7e: Electroforesi
Tema 8e: Cromatografia
Tema 9e: Centrifugació

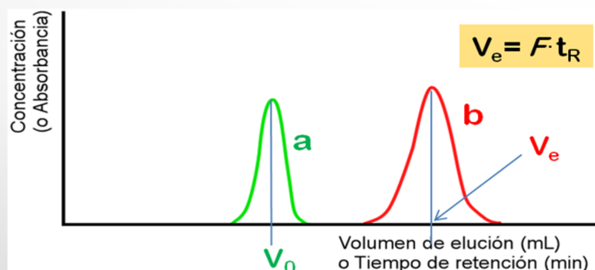


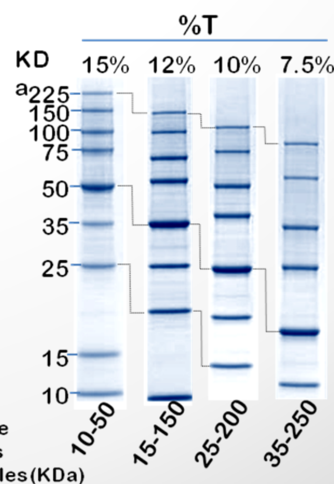












PROBLEMES T1 (Estudiants)
Caracterització de molècules d'interès biològic

1.1e.- La purificació d'un enzim es produeix en els següents passos:

	Volum (mL)	Conc. proteïna (mg/mL)	Activitat (ua/mL)
Extracte cru	15	1.22	254
Pas 1	4	3.15	798
Pas 2	6	1.53	437
Pas 3	10	0.61	241
Pas 4	2	1.75	1158

Quin és el rendiment i el factor de purificació del procés global?
Es considera que el procés és massa llarg i es contempla la possibilitat d'eliminar un dels passos. Quin dels passos caldria eliminar si es pretén mantenir el màxim factor de purificació possible? Quin seria el nou factor de purificació del procés?
S: rendiment, 60,8%; f=3,18. Caldria eliminar aquell pas que produeix un menor factor de purificació particular.

1.2e.- La força iònica d'una dissolució que conté ions es defineix com a $I = (1/2)\cdot\Sigma z_i^2\cdot c_i$, on en el sumatori per a tots els ions, z_i, és la càrrega de cada ió i c_i la seua concentració molar. Calcula la força iònica a la qual es troba una proteïna dissolta en aigua amb NaCl 120 mM abans i després de dialitzar-la exhaustivament enfront d'una dissolució aquosa amb $MgCl_2$ 20 mM.
S: $I_{inicial}$= 0,12 M; I_{final}= 0,06 M.

1.3e.- El sulfat amònic es dissol en aigua a 0 ºC fins a una concentració màxima de 0.5 g/mL. El volum específic parcial (volum ocupat per la unitat de massa) del sulfat amònic en aigua és de 0.567 mL/g. Calcula quant de sulfat amònic (en grams) cal afegir a 20 mL d'extracte aquós per a portar-ho al 20 % de saturació. Quant més cal afegir per a portar-ho al 40 %?
S: fins 20%, 2,12 g; ara fins 40%, 2,39 g.

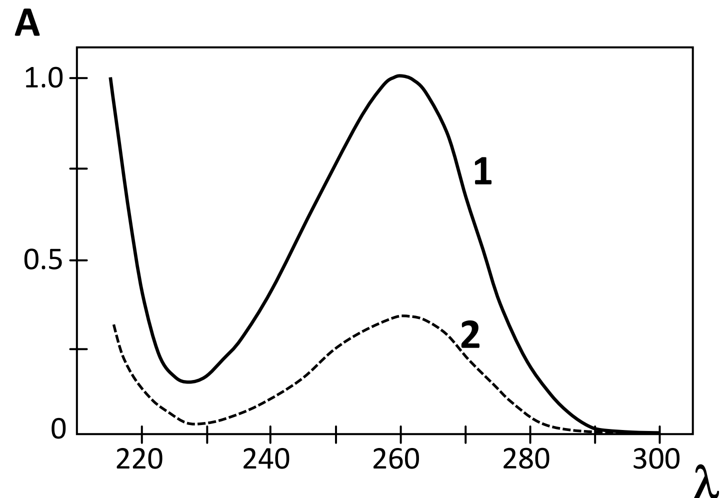
PROBLEMES T2 (Estudiants)
Espectroscòpia d'absorció

2.1e.- La transmitància d'una dissolució del compost A, a concentració C_A, és del 40 % i la d'un compost B, a concentració C_B, és del 50 %. Quant seria la transmitància d'una dissolució que continguera A i B a les concentracions C_A i C_B respectivament?

S: T_{mescla}= 20 %.

2.2e.- En la figura adjunta es recullen els espectres UV de dos dissolucions d'ATP. Calculeu la concentració de la mostra 2 sabent que la de la mostra 1 es de 39 mg/mL.

Quina suposició és necessària per poder fer el càlcul?



S: 11,7 mg/mL. Compliment de Lambert-Beer.

2.3e.- Un indicador és un colorant l'espectre d'absorció del qual canvia amb el pH. Les següents dades corresponen al valors del coeficient d'extinció molar de la forma ionitzada (IH^+) i no ionitzada (I) d'un indicador el qual té un pKa=4.0.

	ε (cm^{-1}·M^{-1})	
λ (nm)	**IH^+**	**I**
400	10 000	0
420	15 000	2 000
440	8 000	8 000
460	0	12 000
480	0	3 000

Els valors d'absorbància d'una dissolució del indicador en una cubeta d'1 cm de pas de llum fou:

λ (nm)	**A**
400	0.250
420	0.395
440	0.400
460	0.300
480	0.075

a) Calculeu la concentració total del indicador i el pH de la dissolució
b) Calculeu l'absorbància a 400 i 440 nm que tindria aquesta dissolució a pH 5.4

S: a) $5 \cdot 10^{-5}$ M; pH= 4,0. b) A_{400}= 0,019; A_{440}= 0,400

2.4e.- La ribonucleasa conté 6 residus de tirosina per molècula i no té triptòfan. Tal i com calia esperar el seu espectre d'absorció és molt semblant al de la tirosina en la regió del UV proper. La identitat no és, però, absoluta, ja que a pH neutre el màxim d'absorció de la proteïna es troba a 277.5 nm. Quan s'incrementa el pH del medi, el màxim d'absorció es desplaça cap a longituds d'ona majors. Aquest desplaçament és reversible fins a pH 12.3; és a dir, el màxim torna a la seu posició original (277.5 nm) quan s'acidifica el medi. Però per damunt de pH 12.3, el màxim d'absorció de la proteïna dissolta va desplaçant-se fins arribar a coincidir amb el de l'anió tirosinat i aquest desplaçament és irreversible. La neutralització de la solució no regenera l'espectre original, sinó que condueix a l'aparició d'un pic ample.

Una mostra de ribonucleasa s'ha dissolt en NaOH 0.1 M i s'ha mesurat l'absorció a 295 nm en funció del temps. Els resultats són els següents:

t (min)	2	3	4	5	6	7	9	13	19	36
$\mathbf{A_{295}}$	0.331	0.351	0.375	0.390	0.400	0.409	0.418	0.429	0.439	0.439

Per altra banda s'ha realitzat un experiment per tal de determinar la influencia del pH sobre l'absorció de la proteïna a 295 nm. Els valors s'expressen en termes de coeficient d'extinció molar i s'han determinat 30 min després d'ajustar el pH:

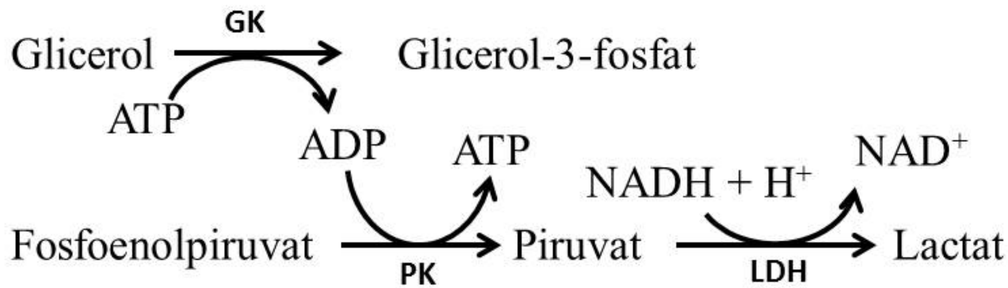
pH	7.2	9.4	9.9	10.4	10.7	11.4	12.3	13.0
$\boldsymbol{\varepsilon \cdot (10^{-3})}$	0.9	2.1	3.4	5.0	6.3	7.5	9.5	15.5

El pKa mitjà dels residus fenòlics de tirosina en polipèptids sintètics és de 10.4.

Busqueu i dibuixeu de forma aproximada els espectres d'absorció de la tirosina (Tyr) i del tirosinat ($TyrO^-$, forma completament desprotonada de la tirosina). Comenteu els resultats obtinguts dels experiments descrits amb la ribonucleasa i deduïu les conclusions possibles.

S: Dibuixar les gràfiques de A_{295} davant temps, per deduir l'increment màxim d'absorbància, i de ε_{295} davant pH, en aquest cas s'observarà una corba bifàsica la qual cosa està relacionada amb la diferent localització (interna o superficial) dels residus de tirosina. Es pot fer una estimació de la fracció de residus de tirosina en cada localització. La irreversibilitat està relacionat amb la desnaturalització de la proteïna.

2.5e.- Per tal de mesurar l'activitat de l'enzim gliceroquinasa (GK) en un extracte purificat es proposa el següent sistema de reaccions:



Es prepara una cubeta d'espectrofotòmetre amb 2 mL d'una mescla dels reactius necessaris (incloent glicerol 0.2 mM) i 0.5 mL d'extracte enzimàtic. Es registra el canvi de D.O. a 340 nm amb el temps, obtenint-se la següent Taula:

Temps (s)	**D.O. (340 nm)**
10	0.572
20	0.564
30	0.537
40	0.510
50	0.483

a) Calculeu l'activitat de l'extracte especificant les unitats en les que s'expressa.
b) Quins reactius deuen estar presents inicialment en la cubeta?, i quin ordre és més convenient afegir-los?
c) El valor de la Km de l'enzim per al glicerol s'ha obtingut de la bibliografia, però per una errada d'escriptura hi han dubtes de si el valor de Km és $1.6\cdot10^{-3}$ o $1.6\cdot10^{-5}$ M. Pot proporcionar informació sobre aquest punt l'experiència anterior?

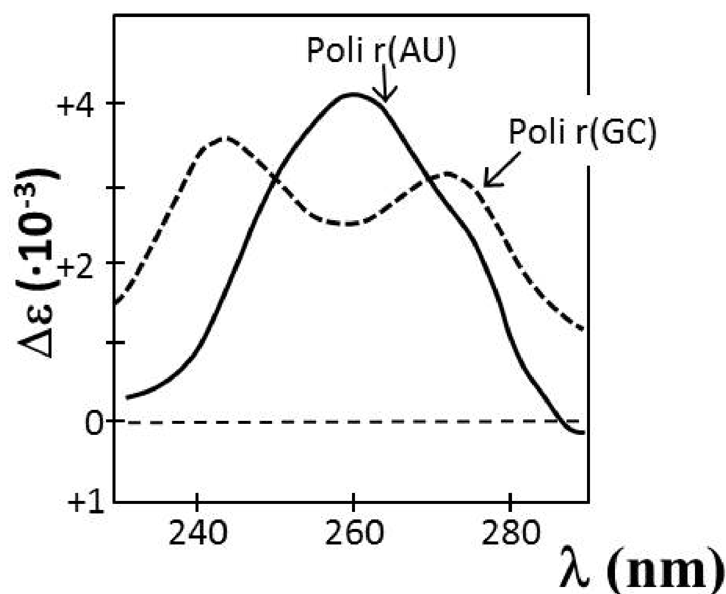
Dades: ε_{340} (NADH)= $6.22\cdot10^{-3}$ $L\cdot mol^{-1}\cdot cm^{-1}$; PK, piruvat-quinasa; LDH, lactat-deshidrogenasa.

S: a) D'una gràfica D.O.$_{340}$ *vs* temps(s) obtenim del tram lineal el pendent i d'aquest, i donada l'estequiometria, l'activitat (Act): Act= $34,8\cdot10^{-5}$ mols/(s·mL$_{extracte}$). c) Si. Com que la concentració de glicerol en la cubeta és de $1.6\cdot10^{-4}$ M, el fet d'obtenir una recta en la gràfica és indicatiu de que eixa concentració és saturant, així que Km no pot ser $1.6\cdot10^{-3}$ M.

2.6e.- Ideeu un mètode per tal d'investigar la relació de residus de cisteïna lliures que es troben a l'interior del plegament natiu de les proteïnes, respecte al que es troben exposats al dissolvent, basant-se en l'intercanvi isotòpic que experimenta el grup tiol al dissoldre la proteïna en aigua deuterada (D_2O), i la seua detecció per espectroscòpia IR. Si la vibració de tensió de l'enllaç S-H produeix una banda d'absorció a $\bar{\upsilon}$= 2200 cm^{-1}, a quin valor de $\bar{\upsilon}$ cal esperar l'absorció de l'enllaç S-D?

S: Amb dos experiments d'intercanvi isotòpic (H→D) dissolvent la proteïna, nativa i desnaturalitzada (per exemple amb urea 8M), en aigua deuterada. Les àrees de les bandes d'absorció IR i la comparació de les dues mostres indicarà la proporció de Cys superficial. $\bar{\upsilon}$ (S-D) = 1580 cm^{-1}.

2.7e.- Un mètode per a conèixer el contingut d'estructura secundària d'un RNA és fer un espectre de diferència en el UV (entre 230 i 290 nm), en el que a l'espectre de la forma desnaturalitzada del RNA es resta l'espectre de la forma nativa. En la figura adjunta es poden veure, superposats, els espectres de diferència corresponents a dos poliribonucleòtids sintètics de doble cadena, el poli r(AU) i el poli r(GC). En aquesta figura el $\Delta\varepsilon$ representat en ordenades ha estat calculat per mol de parell de bases. Perquè són aquests espectres de diferència positius? Explicar detalladament cóm es podrien emprar aquest espectres per a calcular el nombre de parell de bases i la proporció de parells AU i GC que constitueixen l'estructura secundària d'un RNA.



S: Són positius pel fenomen de hipocromicitat. Les mesures de les diferències d'absorbància, natiu i desnaturalitzat, a λ= 250 nm (en la qual els parell AU i GC generen un increment d'absorbància idèntic) permetria l'estimació de nombre de parells de bases. Per al càlcul de les proporcions de parell AU i GC, determinem l'increment d'absorbància a altres dues λ (per exemple, 260 i 240, on els increments són diferents per a les dues parelles). Arribem així a una sistema de dues equacions on les incògnites són les fraccions de AT (f_{AT}) i de GC (f_{GC}).

PROBLEMES T3 (Estudiants)
Espectroscòpia de fluorescència

3.1e.- La durada mitjana de l'emissió fluorescent de l'acridina en dissolució aquosa és de 28 ns quan s'elimina completament l'aire. En presència de 7 mM de O_2 dissolt, la durada mitjana descendeix a 6 ns. Quina pot ser la causa d'aquesta variació? Si la intensitat de la fluorescència emesa en absència d'aire és de 100 unitats arbitràries, quin seria el valor de la intensitat de fluorescència en presència de 7 mM de O_2?
S: Extinció fluorescent. I_f (amb O_2)= 21,4 u.a.

3.2e.- L'ió iodur (I^-) és un extintor molt efectiu del triptòfan. Una proteïna posseeix 8 triptòfans i el seu rendiment quàntic és Q= 0.12 en dissolució aquosa. Al mesurar la fluorescència d'una dissolució de proteïna en presència de diverses concentracions de iodur s'observaren les següents variacions de Q:

I^-, M	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
Q	0.108	0.094	0.091	0.090	0.090

Què es pot dir de la posició dels triptòfans en el plegament natiu de la proteïna?
S: Una representació de Q davant log[I^-] mostra que l'extinció no és completa sinó que es satura a un determinat valor, el qual deu ser indicatiu de la inaccessibilitat d'una fracció (que hi ha que calcular) de residus de Trp, per trobar-se internament en l'estructura nativa de la proteïna.

3.3e.- El fluoròfor extrínsec 8-sulfonat d'1-anilinonaftalè (ANS) es pot utilitzar com un indicador de la unió de lligands a l'enzim glutamat-deshidrogenasa (GDH). L'emissió fluorescent de l'ANS en aigua es modifica notablement al afegir GDH (Fig. 1). A què es deu aquest canvi?
L'addició de NADH (coenzim) o GTP (inhibidor), per separat, no altera la fluorescència de l'ANS unit a l'enzim, però si s'afegeix NADH en presència de GTP (a concentració saturant) s'observa una variació sigmoïdal de l'emissió fluorescent (Fig. 2). Cóm es pot interpretar aquest fenomen?

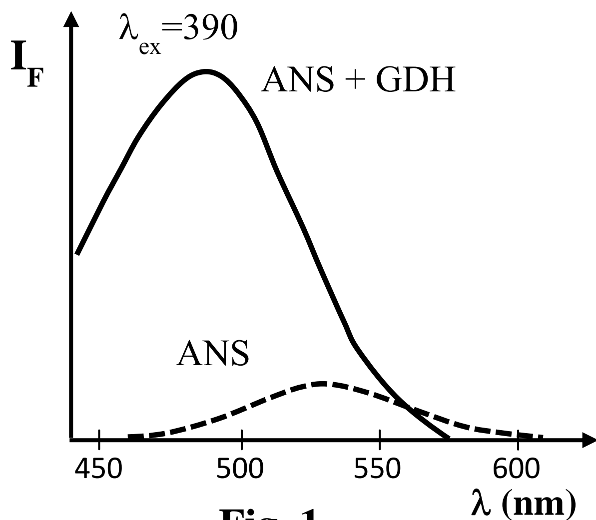


Fig. 1

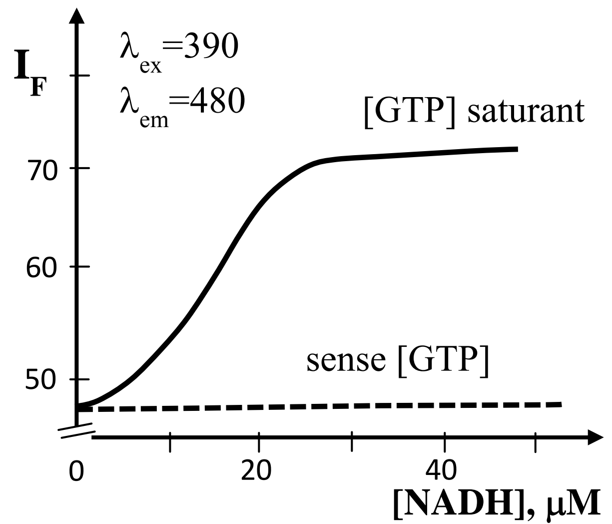
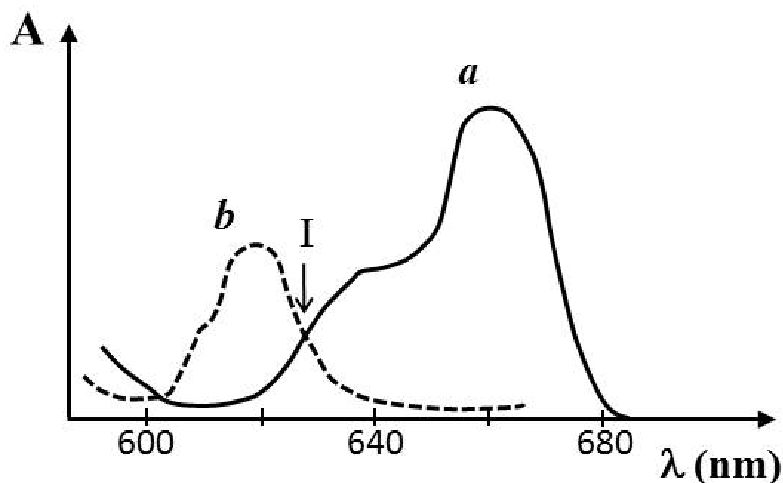


Fig. 2

S: Canvis de l'entorn (polar→apolar) expliquen els canvis en la capacitat d'emissió fluorescent. La unió de NADH (que deu ocórrer en presència de GTP) indueix canvis conformacionals en la proteïna, detectats per la "sonda" fluorescent ANS.

3.4e.- Dos tipus diferents de clorofil·la (***a*** i ***b***) presenten els següents espectres d'absorció en la regió visible:



La clorofil·la ***a*** presenta un màxim d'emissió fluorescent a 740 nm, mentre que la emissió de la clorofil·la ***b***, a aquesta longitud d'ona, és menyspreable.

a) Dibuixa l'espectre d'excitació de la mescla de clorofil·les en el cas de que:
   1) existisca transferència per ressonància induïda entre ambdós
   2) no existisca aquesta transferència

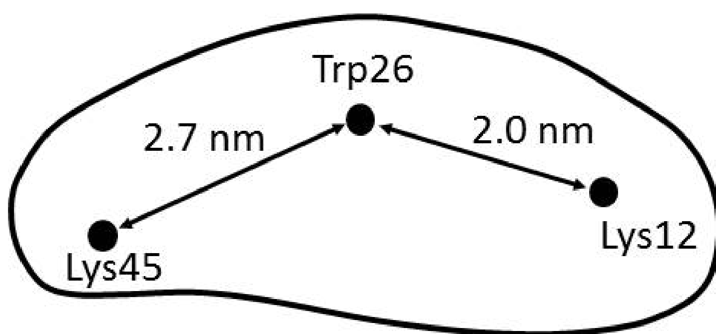
b) És el punt I un punt isosbèstic?

S: a) Amb transferència d'energia es veuria un màxim d'emissió al voltant de 615 nm, que no apareix sense transferència. b) No. Isosbèstic només s'aplica a una longitud d'ona característica en espectroscòpia d'absorció. En el cas de la figura el punt I es podria nomenar com a un punt de "isoemissió".

3.5e.- Una proteïna posseeix tres llocs d'unió de lligands situats, espacialment, en els vèrtexs d'un triangle equilàter. Si s'introdueix un lligand fluorescent (donador) en un dels llocs d'unió, s'observa que el seu rendiment quàntic és Q=0.5. Si s'uneix un segon lligand diferent (acceptor) en un dels llocs vacants (és a dir, s'afegeix acceptor de manera que, en mitjana, un dels llocs està ocupat) el Q del donador es redueix 0.25. Quin seria el Q del donador si se saturaren els dos llocs vacants amb acceptor? Quina seria aleshores l'eficiència de la transferència d'energia (a qualsevol dels dos acceptors)?

S: Amb els dos llocs ocupats per acceptor, Q= 0,166 i Ef= 66 %.

3.6e.- Un investigador intenta confirmar l'estructura tridimensional d'una proteïna que ha esta estudiada per difracció de raig X. Per açò pretén mesurar la distància existent entre l'únic residu de triptòfan (posició 26 de la seqüència) i un acceptor d'energia per ressonància induïda (grup dansil) que ha aconseguit introduir independentment en dos residus de lisina (posicions 12 y 45) que disten (segons el model tridimensional elaborat amb les dades de difracció) 2.0 i 2.7 nm, respectivament, del residu de Trp26. Els resultats obtinguts no pareixen estar d'acord amb l'estructura coneguda, dons, experimentalment s'observa que l'eficiència de la transferència és la mateixa (E= 0.6) quan el grup acceptor (dansil) es troba unit a un o l'altre dels residus de lisina.



L'investigador explica els resultats argumentant que el grup dansil, quan es troba unit a un de les lisines (45), té impedida la lliure rotació per raons estèriques i es troba fix amb l'orientació més favorable per a la transferència, mentre que quan s'uneix a l'altra lisina (12) pot girar lliurement. És consistent aquesta argumentació amb les dades numèriques dels quals es disposa? Cóm es podria confirmar experimentalment la hipòtesi proposada per l'investigador?

S: Amb la teoria de Förster l'eficiència de la transferència depèn de la distància que separa els cromòfors, però també de l'orientació entre ells. Així la interpretació por ser correcta: malgrat distàncies diferents originen una mateixa eficiència per la diferent mobilitat dels acceptors. Aquesta es podria demostrar per polarització de fluorescència.

3.7e.- Es desitja marcar una proteïna amb el fluoròfor extrínsec fluoresceïna. Per açò es mesclen 8 mL d'una dissolució 0.5 μM de proteïna amb 0.1 mL d'una dissolució 0.2 mM de isotiocianat de fluoresceïna. L'avanç de la reacció de conjugació es pot seguir mesurant la polarització de l'emissió de fluorescència que, inicialment, és pràcticament nul·la i va augmentant amb el temps fins estabilitzar-se en un valor de 0.08. Posteriorment la mescla es dialitza per tal d'eliminar l'excés de isotiocianat de fluoresceïna i es torna a mesurar la polarització de la dissolució de proteïna dialitzada observant-se un valor de 0.22. Expliqueu les raons dels successius increments de polarització. Es pot calcular, amb les dades anteriors, el valor mitjà d'unitats de fluoresceïna units per cada molècula de proteïna?

S: L'augment de polarització de fluorescència (p) durant la reacció resulta de la unió del fluoròfor a la proteïna i durant la diàlisi és per l'eliminació del fluoròfor lliure, no unit. Unitats de fluoresceïna/unitat de proteïna= 1,81.

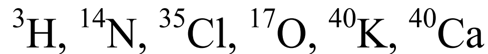
3.8e.- El Indo1 és un és un fluoròfor que uneix Ca^{2+} (estequiometria 1:1, constant d'unió= $1.8 \cdot 10^{-7}$ M) i per això es pot utilitzar com a un indicador de la concentració intracel·lular d'aquest catió. Les dues formes possibles, amb calci i sense calci unit, del Indo1 tenen el mateix coeficient d'extinció molar a λ= 352; però en absència de calci el fluoròfor emet al voltant de 480 nm amb un rendiment quàntic de Q= 0.5, mentre que quan es troba saturat de calci l'emissió es desplaça a 405 nm i el rendiment quàntic augmenta a Q= 0.8. Ambdues emissions no es solapen.

Un suspensió de cèl·lules es tracta amb Indo1 AM (acetoximetil èster) durant 30 min. A continuació, les cèl·lules es renten i es resuspenen en un medi sense Indo1. 2 mL de la suspensió cel·lular es col·loquen en la cubeta de l'espectrofluorímetre i es mesura l'emissió a 480 nm (I= 54 unitats arbitràries) i a 405 nm (I= 102 u.a.). Quina és la concentració intracel·lular de Ca^{2+}?

S: $[Ca^{2+}]_{intracel \cdot lular}$= $6,5 \cdot 10^{-8}$ M

PROBLEMES T4 (Estudiants)
Espectroscòpia de ressonància magnètica nuclear

4.1e.- Quins dels següents nuclis serien detectats per espectroscòpia de ressonància magnètica nuclear (RMN)?


^{3}H, ^{14}N, ^{35}Cl, ^{17}O, ^{40}K, ^{40}Ca

S: ^{3}H, ^{14}N, ^{35}Cl, ^{17}O. Hi ha que discutir perquè.

4.2e.- Quina és la diferència d'energia entre els estats nuclears fonamental i excitat d'un nucli de ^{13}C que ressona a un camp de 15410 G quan s'irradia amb un freqüència de 16.5 MHz? I altre que ressona a 15412 G amb la mateixa freqüència? Què es pot dir sobre l'apantallament relatiu dels dos nuclis? Quin seria la relació entre poblacions nuclears en els estats fonamental i excitat a temperatura ambient?

S: ΔE= 1,09·10^{-19} erg i és la mateixa per als dos nuclis, però sotmesos a diferents camps magnètics. El nucli que ressona a camp magnètic major (15412 G) es troba més apantallat (per què? Explicar-ho). Llei de distribució de Boltzman→n_i/n_0=0,9999737.

4.3e.- Dos pics de ressonància (^{1}H-RMN) d'un compost es produeixen a desplaçaments químics (δ) de 3.2 ppm i 7.1 ppm. Per tal de comprovar que els protons implicats es troben propers es va realitzar un experiment de doble irradiació i observació de l'efecte nuclear Overhauser. A quines dues freqüències caldrà irradiar la mostra si es desitja realitzar l'experiment en unes condicions en les que el patró-referència ressone a 97000 G?

Dada: γ (^{1}H)= 27753 $G^{-1}\cdot s^{-1}$.

S: ν_{m1}= 428,452 MHz; ν_{m2}= 428,453672 MHz

4.4e.- La unió de l'inhibidor glicerol-3-fosfat a l'enzim triosa-fosfat-isomerasa produeix un corriment en el desplaçament químic d'un pic d'histidina, que és funció de la concentració d'inhibidor d'acord amb el següents valors:

Inhibidor (mM)	**δ (ppm)**
0.9	0.048
1.3	0.083
2.7	0.108
3.6	0.125
4.3	0.138
5.2	0.145
6.2	0.158
13.3	0.190

Fer una estimació del valor de la constant de dissociació del complex enzim-inhibidor.

S: La representació de δ front a la [I] origina una corba hiperbòlica, així compleix l'equació: $\delta=\delta_{màx}\cdot[I]/(K_d+[I])$, on K_d és la constant de dissociació. Ara una representació de dobles inversos (1/δ *vs* 1/[I]) origina una recta, l'ordenada en l'origen de la qual és= -$1/K_d$. K_d= 4 mM.

4.5e.- El senyal de ^{31}P-RMN del grup ortofosfat es produeix *in vivo* en una suspensió d'hepatòcits a δ= 5.8 ppm. Quan es simula *in vitro* l'ambient químic cel·lular al voltant de l'ió fosfat, s'obtenen desplaçaments químics de 3.7 ppm a pH 5.0, de 5.3 ppm a pH 7.1 i de 6.5 ppm a pH 9.0. Calculeu el pH intracel·lular de l'hepatòcit.

S: Aplicant l'equació de Henderson-Hasselbalch ($pH= pK_a + \log([HPO_4^{2-}]/[H_2PO_4^-])$, però amb els valors de les diferències entre els δ substituint les concentracions, pH=7,377.

4.6e.- La desoxixilulosa és un anàleg de la xilulosa que pot ser agafat del medi extern i transportat a l'interior de les cèl·lules de safanòria cultivades en suspensió. Una vegada en el citosol, la desoxixilulosa és metabolitzada de forma molt lenta. La $^{13}C_2$-desoxixilulosa dissolta en un medi salí fisiològic dona un senyal en ^{13}C-RMN a 10 ppm, que es desplaça a 16 ppm en presència d'una sal de Gd(III) (ió no transportable a l'interior de les cèl·lules). Quan es col·loquen 10 mL de suspensió cel·lular (10^6 cèl·lules/mL) amb $^{13}C_2$-desoxixilulosa 0.2 mM i $GdCl_3$ 2 mM en un tub d'espectroscòpia RMN i es mesuren les àrees dels pics que apareixen a 10 i 16 ppm s'obtenen les dades adjuntes. Calculeu la velocitat de transport de la desoxixilulosa.

Temps (s)	Àrea (unitats arbitràries)	
	Pic 10 ppm	Pic 16 ppm
0	-	12860
5	940	11912
10	1854	10988
15	2734	10096
20	3574	9244
25	4369	8436

S: El pic 10 ppm identifica la desoxixilulosa internalitzada. Cal representar les àrees dels pics davant del temps i determinar els pendents (són iguals però de diferent signe). Aquest pendent, amb u.a./s, esta relacionat amb la velocitat d'entrada de l'anàleg. Amb el àrea del pic 16 ppm a temps 0 determinem la relació que hi ha entre àrea i mols de desoxixilulosa. Així $velocitat_{transport\ desoxixilulosa}= 29{,}2\cdot10^{-9}$ mol/s. Per cèl·lula, $2{,}92\cdot10^{-15}$ mol/s·cel.

PROBLEMES T6 (Estudiants)
MÈTODES ISOTÒPICS

6.1e.- Calcula la radioactivitat (en Ci) que emetrà una mostra de 10 mg de Na_2SO_4 on tot el sofre present és ^{35}S (radioactiu).
Dades: període de semidesintegració del ^{35}S: 87,1 dies; Massa molecular Na_2SO_4: 142. S: 105,53 Ci.

6.2e.- Es disposa d'una mostra d'ATP marcada amb ^{32}P que el dia 1 de gener tenia 20 μ curies/mg. Què radioactivitat específica tindrà l'1 d'abril?
Dades $t_{½}$ (^{32}P) és de 14,3 dies. S: 0,255 μCi/mg

6.3e.- Una mostra d'àcid fòrmic (CH_2O_2) marcat amb ^{14}C presenta una radioactivitat específica d'1 μCi/mg. No tots els àtoms de carboni són radioactius. Calcula la proporció $^{14}C/^{12}C$ de la mostra.
Dades: el període de semidesintegració del ^{14}C és de 5760 anys; MM (àcid fòrmic), 46
S: $^{14}C/^{12}C$ = 0,00074 (Un de cada 1350 àtoms de C és radioactiu)

6.4e.- Una mostra de timidina tritiada amb un màxim d'un nucli de ^{3}H per molècula té una radioactivitat específica de 6 Ci/mmol, quin percentatge de les molècules són radioactives? Aquesta mateixa timidina s'utilitza com a precursor per a la síntesi d'un DNA ($25 \cdot 10^6$ Da, aproximadament 75700 bases) que posseeix un 50% AT. Si s'utilitza un comptador amb una eficiència del 40 % per al triti, a quina massa de DNA correspon una mesura de 1000 cpm?
Dades: $t_{½}$ (^{3}H)= 12,3 anys; 1Ci= $3{,}7 \cdot 10^{10}$ dps
S: i) 20,57%; ii) 0,25 ng DNA.

6.5e.- Una mostra d'hormona tiroide marcada amb ^{131}I presenta una radioactivitat de 10 μCi. Al cap de quant temps quedarà reduïda la radioactivitat de la mostra a 2 μCi?
Dada: $t_{½}$ (^{131}I) = 8,1 dies.
S: 18,8 dies

6.6e.- Quants àtoms de ^{14}C existeixen en una mostra que emet 4000 cpm mesurada en un aparell de 80 % d'eficiència per a aquest radioisòtop? Calculeu l'interval de confiança al 95 % de la mesura anterior (4000 cpm) sabent que s'ha determinat comptant durant 10 min? Quina aproximació s'introdueix en aquest càlcul?
Dades: λ ^{14}C = $1{,}44 \cdot 10^{-8}$ $hores^{-1}$; *a* (95 %)= 2
S: $2{,}08 \cdot 10^{13}$ àtoms ^{14}C; 4000± 40, o en valor relatiu 4000± 1 %

6.7e.- En la tomba de Ramsés II es va trobar un pergamí la proporció del qual $^{14}C/^{12}C$ era un 68 % de la que tenen els éssers vius actuals. Calculeu l'antiguitat d'aqueix pergamí. Dada: $t_{1/2}(^{14}C)$ =5760 anys.
S: 3205 anys

6.8e.- Si la proporció de ^{14}C en la fusta d'una casa és el 95 % de la que té la fusta d'un arbre recentment tallat, calcula l'antiguitat aproximada de la casa. Dada: període de semidesintegració del ^{14}C: 5760 anys**.**
S: 426 anys.

6.9e.- El ^{45}Ca té un període de semidesintegració de 163 dies. Calcula:

a) La constant de desintegració (λ) en dies^{-1} i segons^{-1}.

b) El percentatge de la radioactivitat inicial que queda després de 90 dies en una mostra que conté ^{45}Ca.

S: a) $4,25\cdot10^{-3}$ dies^{-1}; $4,92\cdot10^{-8}$ segons $^{-1}$; b) 68,2 %

6.10e.- Es té una mostra de fosfat de tiouridina marcat amb ^{32}P i amb ^{35}S, amb una radioactivitat específica total de 9000 dps/mol. Al cab de 45 dies, la radioactivitat específica ha descendit a 4200 dps/mol, quina era la radioactivitat específica per a cadascun dels radioisòtops del fosfat de tiouridina inicial (el que donava 9000 dps/mol)? Com es podria conèixer la radioactivitat deguda a cada radioisòtop sense necessitat d'esperar al fet que la velocitat de desintegració decaiga? Quina fracció de molècules portaven un àtom de ^{32}P?
Dades: λ $^{32}P= 5,65\cdot10^{-7}$ s^{-1}; λ $^{35}S= 9,22\cdot10^{-8}$ s^{-1}
S: ^{32}P: 3559 dps/mol; ^{35}S: 5441 dps/mol. Aproximadament 1 de cada 10^{14} molècules

6.11e.- Es realitza una experiència d'autoradiografia amb ^{32}P. Després d'un temps d'exposició de 350 hores es revela l'emulsió i s'observa que ha sigut sobreexposada no podent-se comptar bé els rastres. Es desitja saber quant temps caldria haver exposat l'emulsió per a tenir exactament un terç dels rastres obtinguts amb 350 hores d'exposició. Dades: λ $^{32}P = 5,65\cdot10^{-7}$ s^{-1}
S: 91,6 hores

6.12e.- Un DNA amb un 40% de parells AT se sintetitza a partir de precursors marcats, concretament [α-^{32}P]*d*ATP, 2 Ci/mmol, i es visualitza per autoradiografia. Després d'un temps d'exposició de 150 hores s'observa que cada molècula de DNA produeix una mitjana de 8 rastres. Si l'eficiència de l'emulsió utilitzada és del 40 % per al ^{32}P, quin és el nombre aproximat de nucleòtids que integren una molècula d'eixe DNA?
Dades. 1 Ci= $2,2\cdot10^{12}$ dpm λ $^{32}P = 5,65\cdot10^{-7}$ s^{-1}
S: $1,5\cdot10^{6}$ nucleòtids/molècula

6.13e.- A fi de mesurar l'absorció de fosfat del medi extern per part d'un determinat bacteri, es va traslladar un cultiu d'aquest bacteri a un medi amb ^{32}P-ortofosfat d'una activitat específica inicial de 0,2 Ci/mmol. En el moment de posar en contacte les cèl·lules amb el medi, es va prendre una mostra de 0,1 mL del medi de cultiu i es va comptar en un comptador de centelleig de 80 % d'eficiència per al ^{32}P, donant 2500 cpm. Les cèl·lules van estar en contacte amb el medi durant 12 hores i, a continuació, es van sedimentar per centrifugació a baixa velocitat prenent-se una alíquota de 0,1 mL del sobrenedant que, a causa d'una avaria en el comptador no pogué ser comptat fins a 68 hores després de la centrifugació. Quan finalment es va realitzar la mesura, en les mateixes condicions que la inicial, es va obtenir un valor de 1200 cpm. Tenint en compte que el cultiu es trobava en fase estacionària (és a dir, que el nombre de cèl·lules no va augmentar durant l'experiment) i a una densitat cel·lular de $2 \cdot 10^6$ cèl·lules/mL, quina va ser la mitjana de molècules de fosfat preses per cada cèl·lula en l'interval de temps considerat?
Dades: λ ^{32}P= $5,65 \cdot 10^{-7}$ s^{-1}
S: $9,2 \cdot 10^6$ molècules fosfat/cèl·lula

6.14e.- En la traducció *in vitro* d'un mRNA es va utilitzar una mescla d'aminoàcids que contenia ^{14}C-Met de radioactivitat específica 1000 dps/mg, com a únic precursor marcat. Com a resultat del procés es va obtenir una proteïna, P, constituïda per una única cadena polipeptídica l'aminoàcid carboxil terminal de la qual era, casualment, Met. Aquesta proteïna va ser sotmesa a un tractament amb bromur de cianògen que va donar lloc a dos polipèptids marcats que posseïen una radioactivitat específica de 840 i 660 dpm/mg. Calculeu el pes molecular de la proteïna P. (Nota: el bromur de cianògen trenca enllaços peptídics en el costat carboxil de residus de metionina; MM Met= 131,1).
S: 21291 g/mol.

6.15e.- En una experiència de doble marcatge amb ^{3}H i ^{14}C es va mesurar la radioactivitat de 0,5 g de mostra en un comptador analitzador d'altura de pols subdividit en dos canals. En les mateixes condicions es va determinar la radioactivitat de patrons de ^{3}H i ^{14}C en tots dos canals i es van obtenir els següents resultats:

	cpm	
	Canal A	**Canal B**
patró ^{3}H	10200	2
patró ^{14}C	4570	8452
mostra	750	782

Tenint en compte que el soroll de fons de l'aparell és de 20 cpm per al canal A i de 2 cpm per al canal B, i que l'eficiència és del 80 % per al ^{14}C i el 20 % per al ^{3}H, calculeu la radioactivitat específica de la mostra per a cadascun dels radioisòtops i determineu l'interval de confiança al 95 % de probabilitat de la mesura del soroll de fons del canal A (20 cpm) sabent que ha sigut comptat durant 500 minuts.

S: ^{14}C: 3000 dpm/g; ^{3}H: 3100 dpm/g; 20± 0.4 i en valor relatiu 20± 2 %

6.16e.- Una proteïna de pes molecular 60000 és capaç d'unir reversiblement GMP cíclic. En una experiència d'equilibri de diàlisi es va col·locar 0,1 mL d'una dissolució de 6 μg/mL de la proteïna en una de les càmeres de la cel·la, mentre que a l'altre costat de la membrana semipermeable es va posar 0,1 mL d'una dissolució de GMP cíclic marcat amb ^{3}H, de radioactivitat específica 10^{6} cpm/μmol. Una vegada aconseguit l'equilibri es van prendre 10 μL de cadascuna de les cel·les i es va comptar 2570 cpm en l'alíquota de la càmera que contenia la proteïna i 1870 cpm en la que no contenia proteïna. Calculeu la concentració de GMP cíclic lliure en l'equilibri i el valor de la funció de saturació.

S: [cGMP] lliure = $1,87 \cdot 10^{-7}$ M; ν = 0,7

6.17e.- Es va fer créixer una planta en una atmosfera que contenia $^{14}CO_2$ ($3 \cdot 10^{8}$ cpm/μmol). Després de diverses setmanes es va preparar un extracte de fulles per a determinar el contingut en glucosa-1-fosfat mitjançant dilució isotòpica inversa. A 20 mL d'extracte se li van afegir 1,5 mmols de glucosa-1-fosfat no marcada. A continuació es va aconseguir aïllar una petita quantitat de glucosa-1-fosfat de l'extracte i es va cristal·litzar i recristalitzar diverses vegades fins que es va determinar una activitat específica constant i igual a $2,6 \cdot 10^{5}$ cpm/μmol. Calculeu la concentració de glucosa-1-fosfat original en l'extracte.

S: 10,84 μM

PROBLEMES T7 (Estudiants)
ELECTROFORESI

7.1e.- Calculeu el punt isoelèctric (pI) de l'àcid *p*-aminobenzoic a partir de les següents dades d'electroforesis:

pH	2,0	3,5	4,0	4,5	6,0	8,0	9,0
Distància migració (cm)	+5,0	+3,6	-0,2	-3,1	-5,2	-5,3	-5,3

7.2e.- En una electroforesi d'alt voltatge en paper a pH 4,0, Quins dels següents aminoàcids migraran cap al càtode i quins cap a l'ànode?

Gly, Cys, Asp, His, Lys

Si es trobaren tots ells formant part d'una mescla, com quedarien distribuïts després de l'electroforesi?

7.3e.- Una mescla conté els següents pèptids, als quals els corresponen els valors de pKa indicats:

	pK_1	pK_2	pK_3	pK_4
Glicilglicina	3,06	8,13		
Histidilhistidina	2,25	5,60	6,80	7,80
Glicilalanina	3,15	8,25		
Aspartilglicina	2,10	4,53	9,07	
Fenialanilarginina	2,66	7,57	12,40	

Discutiu la possibilitat de separar-los per electroforesi en paper a pH 5,65.

7.4e.- La seqüencia amino-terminal de la proteïna P és:

Val-His-Leu-Pro-Glu-Lys-Gly-Ser-Arg-Met….

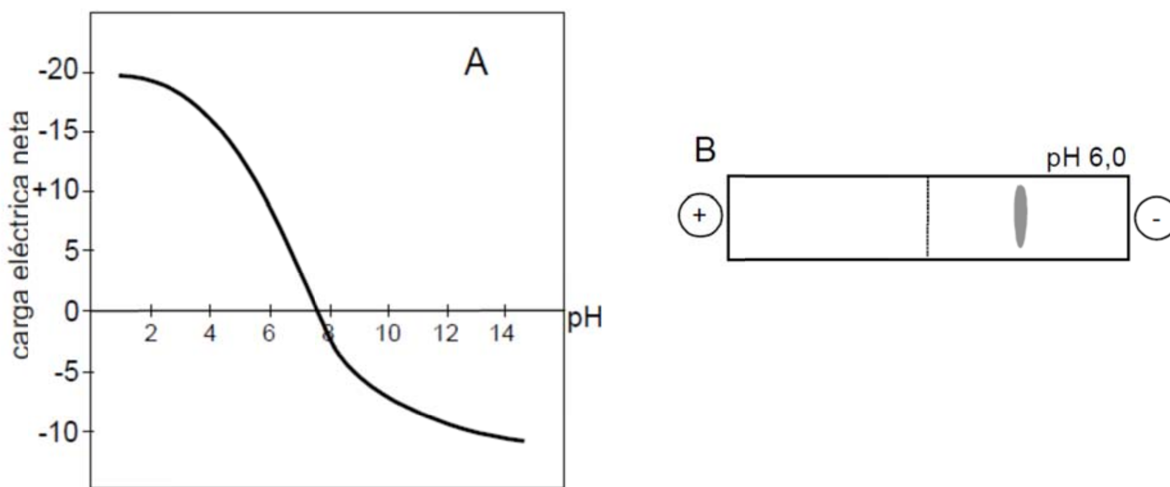
Després de sotmetre aquest pèptid a electroforesi d'alt voltatge en paper a pH 7,0 es va observar una migració de 0,3 cm cap al càtode. El mateix pèptid obtingut d'una proteïna mutant (P*) va migrar, en les mateixes condicions, 2,9 cm en direcció a l'ànode.

Es creu que la mutació de la proteïna P* consisteix en la substitució del residu de Leu de la posició 3 per Asp. Estan d'acord les dades electroforètiques amb aquesta hipòtesi?

7.5e.- S'ha purificat extensivament un enzim, resultant pur segons diversos criteris: un sol pic per cromatografia, electroforesi i sedimentació. No obstant açò, en sotmetre'l a electroforesi en gel en presència de SDS s'obtenen dues bandes, una de doble intensitat que l'altra. Quina informació es pot deduir sobre l'enzim? Podria verificar-se aqueixa hipòtesi emprant cromatografia d'exclusió?

7.6e.-Es pretén separar per electroforesi sobre acetat de cel·lulosa una mescla de les hemoglobines HbA, HbS i HbC. En la HbS un residu de Val substitueix a un de Glu sobre l'hemoglobina normal adulta, HbA, mentre que en la HbC es troba un de Lys. Raona que medi resultaria més convenient per a realitzar l'electroforesi: tampó borat sòdic pH 3,5 o tampó carbonat sòdic pH 8,5. Dibuixa un esquema amb les bandes resultants.

7.7e.- La càrrega neta d'una proteïna depèn dels seus grups dissociables i del pH del medi. La figura A mostra la variació de la càrrega d'una proteïna (MM 17500, pI 7,7) amb el pH.



Per electroforesi en acetat de cel·lulosa, pH 6,0 (120 V, 45 min) es va obtenir, després del revelat, el resultat mostrat en la figura B.

a) Dibuixa, de forma aproximada, el resultat si es realitza l'electroforesi als pH de 4,0; 7,7 i 9,5 (a igual voltatge i temps).

b) A quin pH la proteïna presentaria una major mobilitat electroforètica, a pH molt àcid o a pH molt bàsic? Per què?

c) La proteïna posseeix un residu de Cys, que en condicions oxidants pot participar en un pont disulfur. Si s'analitza per electroforesi en acetat de cel·lulosa a pH 6,0 (120 V, 45 min) en condicions oxidants, quin podria ser el resultat? Fes de nou un esquema.

d) Com serien els resultats de l'apartat c) per SDS-PAGE? Explica les possibles diferències amb els obtinguts en acetat de cel·lulosa.

7.8e.- El revelat específic d'activitat tirosina-aminotransferasa d'una carrera electroforètica corresponent a un extracte obtingut de fetge de rata va mostrar tres bandes (A, B i C). A fi de caracteritzar aquestes activitats, els extractes es van sotmetre a electroforesis en gels de diferent percentatge de acrilamida. Les mobilitats electroforètiques relatives de les tres bandes van ser:

% acrilamida	A	B	C
2	17,78	10,00	8,87
4	11,24	8,13	6,22
6	7,88	6,24	4,73
8	5,53	4,92	3,07
10	3,66	4,00	2,14

A la vista de les dades, es tracta realment d'isoenzims o podrien ser simplement agregats oligomèrics?

7.9e.- Per a esbrinar la massa molecular de l'enzim dihidropterina-reductasa es va sotmetre, juntament amb proteïnes patró de masses moleculars conegudes, a electroforesi en gels de diferent percentatge d'acrilamida i es van determinar els pendents de les representacions de Ferguson resultants. El mateix enzim, i també proteïnes patró, es van sotmetre a SDS-PAGE. Els resultats obtinguts van ser:

Proteïna	MM (KDa)	pendent	Rm amb SDS
Mioglobina	17	0,48	0,46
Prolactina	24	0,55	-
Ovoalbúmina	44	0,66	0,27
Quimotripsinogen	26	-	0,34
Catalasa	58	-	0,21
BSA monòmer	66	0,79	-
Dímer de BSA	122	1,16	-
Dihidropterina reduc.	¿?	0,64	0,42

Discutiu els resultats. Què pots dir sobre l'estructura de l'enzim?

S: MM natiu, 40 kDa; MM desnaturalitzat, 20 kDa

7.10e.- La proteïna A posseeix una massa molecular de 130000. Existeix una forma mutant, A*, en la qual dos residus de Thr estan substituïts per Asp. La proteïna A s'escindeix, per tractament amb bromur de cianogen, en dos fragments: B (MM 50000) i C (MM 80000). Aquests fragments se separen per electroforesi en acetat de cel·lulosa a pH 3.0, sent la mobilitat electroforètica de B major que la de C.

a) Dibuixa la representació de Ferguson (log Rm *vs* %T) que cal esperar per als 4 polipèptids (A, A*, B i C) a pH 3,0. Representa una sola gràfica.
b) Dibuixa (assenyalant els pols) el resultat que cal esperar després de sotmetre una mescla dels 4 polipèptids a:
   I) electroforesi en SDS-PAGE
   II) electroforesi en gradient d'acrilamida (5-30 %) a pH 3,0.

7.11e.- Un virus conté 256 molècules de proteïnes, de les quals 64 tenen una massa molecular de 8100 i les restants 192 de 26000. Dibuixa un esquema del possible resultat que s'obtindria després de la dissociació del virus i electroforesi SDS-PAGE. Cal tenir en compte tant les distàncies recorregudes com les intensitats de les bandes corresponents a cada proteïna.

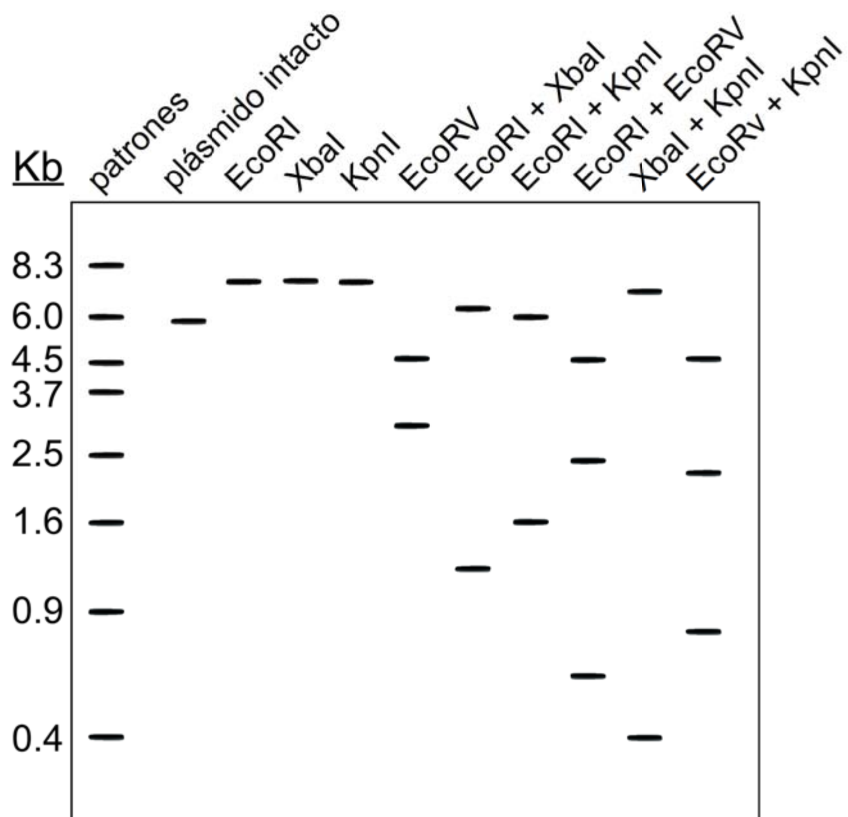
7.12e.- Què posseeix una major migració electroforètica, un plasmidi superenrotllat, circular relaxat o lineal, tots d'igual nombre de pb? Per què? Fes un esquema del resultat d'una electroforesi en gel d'agarosa d'una mostra que continguera una mescla d'un plasmidi present en les tres formes esmentades?

7.13e.- Per què per a la determinació electroforètica de la grandària d'una molècula de DNA (per comparació amb patrons), aquesta ha de ser lineal?

7.14e.- Perquè els gels de poliacrilamida no serveixen per a separar fragments grans de DNA? Perquè els gels d'agarosa no generen bona resolució (almenys no tan bona com els gels d'acrilamida) en la separació electroforètica de proteïnes?

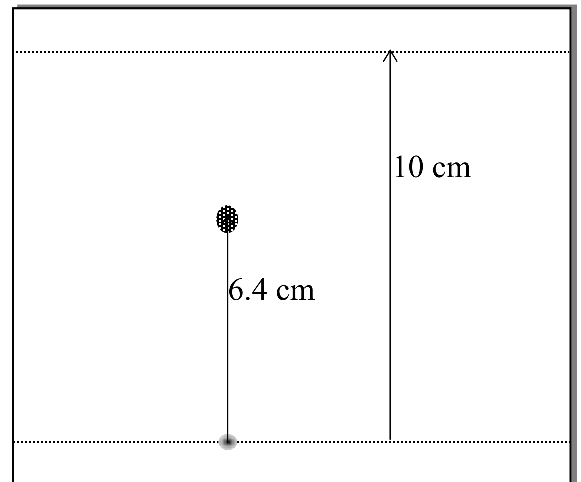
7.15e.- El bromur d'etidi s'empra en la tinció de gels d'agarosa després de la separació electroforètica d'àcids nucleics. Com funciona? Detecta igualment DNA de doble cadena i de simple cadena, RNA, DNA de diferents grandàries? Explica-ho.

7.16e.- La figura adjunta representa el resultat d'una electroforesi en gel d'agarosa del plasmidi circular pTV172 i dels fragments resultants després del tractament amb diferents enzims de restricció. En la línia de l'esquerra es van aplicar patrons de DNA lineal de grandàries conegudes (kb). Calcula la grandària del plasmidi intacte i descriu el seu mapa de restricció.

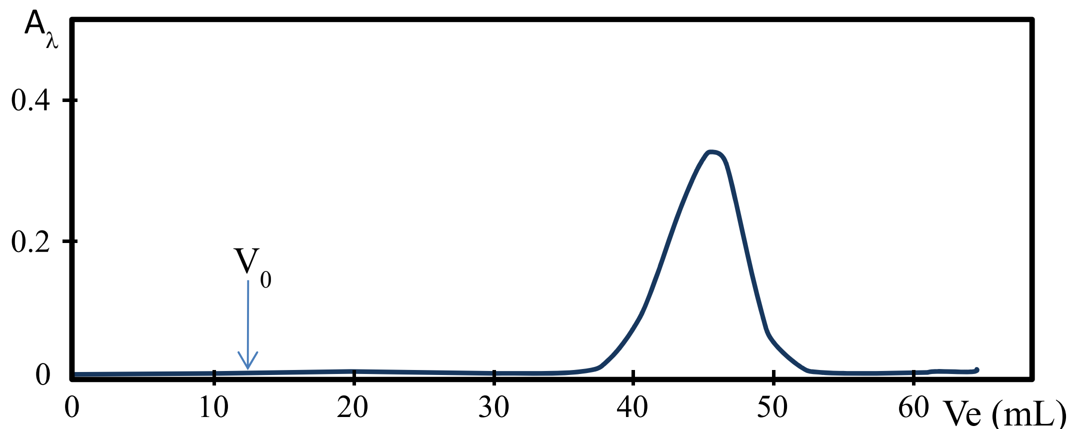

Kb
patrones
plásmido intacto
EcoRI
XbaI
KpnI
EcoRV
EcoRI + XbaI
EcoRI + KpnI
EcoRI + EcoRV
XbaI + KpnI
EcoRv + KpnI
8.3
6.0
4.5
3.7
2.5
1.6
0.9
0.4

PROBLEMES T8 (Estudiants)
CROMATOGRAFIA

8.1e.- Un nucleòtid se sotmet a cromatografia en paper amb doble desenvolupament (monodimensional) de 10 cm (distància aconseguida pel front del eluent, cadascuna de les vegades, des del punt on es va dipositar la mostra). La posició final de la taca es troba a 6,4 cm de l'origen. Quin és el Rf del nucleòtid en aquest sistema cromatogràfic? (Atenció: **no** és 0,32).



8.2e.- Una determinada substància origina el següent perfil d'elució en una columna de cromatografia de repartiment. Calculeu, de forma aproximada, el nombre de plats teòrics de la columna per a aquesta substància en les condicions d'elució corresponents.



S: 278

8.3e.- L'octapèptid AVGWRVKS es va digerir amb la proteasa tripsina. Quin tipus de cromatografia és la més adequada per a separar els productes, la d'intercanvi iònic o la d'exclusió molecular? Si la proteasa emprada haguera sigut quimotripsina, quin haguera estat la tècnica de separació òptima? Justifica la resposta.

8.4e.- S'apliquen 20 mg d'una mescla de proteïna que conté un 30 % d'un enzim, E, en una columna de DEAE-Sepharosa. Després de finalitzar l'elució amb un gradient de força iònica es recuperen, en total, 18,9 mg de proteïna, no obstant açò no es detecta cap activitat de l'enzim E. Dóna una explicació a aquesta absència d'activitat. Què ha pogut ocórrer?

8.5e.- Durant el procés de purificació d'un enzim, un investigador empra un sistema cromatogràfic després del qual s'obté un increment de l'activitat total per a dalt, fins i tot, de l'activitat total present en l'extracte cru de partida. Com pots explicar açò?

8.6e.- Indiqueu l'ordre d'elució dels components d'una mescla dels següents pèptids en una experiència de cromatoenfocament amb una columna equilibrada a pH 9,0 i eluïda amb un tampó multi-iònic a pH 4,0 (en la Taula es proporcionen els pKa dels grups dissociables de cada pèptid). Raona la resposta.

Pèptid	**pKa**			
a) Asp-Gly-Ala	2,1	4,5	9,0	
b) Ala-Val-His-Gly	3,1	6,5	9,7	
c) Ala-Ile-Lys-Gly	3,6	8,0	10,4	
d) Val-Gly-Leu	3,3	9,7		
e) Lys-Gly-Ala-Glu	3,2	4,2	8,3	10,5

S: c>b>d>e>a, d'acord amb el seus pI aproximats.

8.7e.- Es disposa d'una mescla de proteïnes les propietats de les quals són les següents:

Proteïna	**Massa molecular**	**pI**
a	12000	10
b	62000	4
c	28000	7
d	9000	5

Quin seria l'ordre d'elució d'aquestes proteïnes en una columna de DEAE-cel·lulosa eluïda amb un gradient de concentració de sal a pH 8,0? I en una columna de Bio-Gel P30?

8.8e. Un enzim requereix la presència de Mg^{2+} per a la seua activitat. L'eliminació de l'ió condueix a la desnaturalització irreversible de la proteïna. La cromatografia d'intercanvi iònic i la d'exclusió molecular emprats en la purificació de l'enzim porten a la pèrdua de l'activitat. Intenteu explicar a què es deu i suggerir quins canvis podrien fer-se per a millorar la purificació.

8.9e.- Es pretén analitzar l'activitat enzimàtica de les subunitats separades d'un enzim tetramèric després de la seua dissociació. Les subunitats són idèntiques i ha de garantir-se que no existeixen tetràmers sense dissociar en la dissolució de subunitats a estudiar. Quin sistema cromatogràfic es triaria per a separar els monòmers dels tetràmers no dissociats?

8.10e.- S'ha determinat el volum d'elució de diverses proteïnes de massa molecular coneguda en columna cromatogràfica d'exclusió molecular sobre Superdex 200. Els resultats obtinguts són els de la Taula:

Proteïna	**Massa molecular**	**Ve (mL)**
Citocrom *c*	12400	206
Quimotripsinogen	25000	184
Ovoalbúmina	44000	165
Seroalbúmina	68000	153
Lactoperoxidasa	82000	147
Catalasa	240000	115
Fibrinogen	330000	106
Tiroglobulina	670000	90

A continuació es cromatografia sobre la mateixa columna una proteïna problema obtenint un volum d'elució de 131 mL. En repetir aquesta cromatografia havent dissolt prèviament la proteïna en dodecilsulfat sòdic (SDS) a l'1 % i equilibrant la mateixa columna amb la mateixa dissolució amb SDS 1 %, el volum d'elució de la proteïna problema passa a ser 152 mL.

Calcula la massa molecular de la proteïna i comenta els resultats.

S: 133000

8.11e.- Es desitja separar la següent mescla de proteïnes: Albúmina de sèrum (MM 68000), Catalasa (MM 240000), Citocrom c (MM 12400) i β-galactosidasa (MM 520000). Quin subtipus de gel Sephacryl S- es pot utilitzar per a aconseguir una bona separació? Quin serà l'ordre d'elució?

8.12e.- Dues molècules de DNA de massa molecular $6 \cdot 10^6$, una lineal i una altra circular, quin d'elles eluirà en primer lloc en una cromatografia d'exclusió molecular? I entre RNA ribosòmic natiu i desnaturalitzat?

8.13e.- Dues proteïnes de masses moleculars similars presenten, a pH 5,5 una estructura secundària considerable (un 75 % de α-hèlix). A pH 8,5 una d'elles es desnaturalitza i adquireix una conformació en cabdell estadístic, però recupera la seua estructura en tornar a condicions de pH 5,5. Dissenya un procediment que permeta separar aquestes proteïnes.

8.14e.- Moltes drogues, com la morfina, s'uneixen a receptors específics en el teixit nerviós. Dissenya un procediment per a la purificació d'aquests receptors. Proporciona els detalls que consideres importants.

8.15e.- Un investigador està interessat a purificar ràpidament i eficaçment un enzim que uneix ATP des d'un extracte cru contenint moltes altres proteïnes contaminants. Quin sistema cromatogràfic li suggeriries? Proporciona els detalls necessaris per a aplicar el sistema proposat fins a l'obtenció de la proteïna purificada.

8.16e.- Es vol purificar una proteïna que es troba fortament unida a DNA. La dissociació de la proteïna al DNA requereix NaCl 2M i el tractament del complex proteïna-DNA amb nucleases (DNasa) no destrueix el DNA (ha de quedar protegit d'una manera desconeguda). Quin sistema cromatogràfic podria emprar-se per a aïllar la proteïna?

8.17e.- Una certa quantitat d'un pèptid, prèviament purificat, es va sotmetre a hidròlisi àcida total. Posteriorment es va passar l'hidrolitzat a través d'un analitzador automàtic d'aminoàcids amb el que es van obtenir els següents resultats:

	μmols	**Massa molecular**
Glicina	0,248	75
Arginina	0,093	174
Lisina	0,155	146
Metionina	0,062	149
Serina	0,124	105
Triptòfan	Trazas	204

El tractament del pèptid amb tripsina i la posterior separació cromatogràfica dels productes va permetre identificar 9 pèptids més xicotets. El tractament amb bromur de cianògen va donar lloc a 2 fragments a partir del pèptid inicial. El triptòfan, el qual és parcialment destruït en el procés d'hidròlisi àcida, es va determinar espectrofotomètricament. Per a açò es va mesurar l'absorció a 280 nm d'una dissolució aquosa contenint 0,2 mg/mL, en una cubeta d'1 cm de pas de llum, i es va obtenir una mesura de 34,5 % de transmitància (ε_{280} triptòfan= 5550 $M^{-1}cm^{-1}$).

a) Calcula la massa molecular del pèptid

b) Quina de les dues hidròlisis selectives és indicativa del nombre de restes d'aminoàcids que hi ha en el pèptid, i com s'explicaria el resultat de l'altra hidròlisi que aparentment no concorda?

S: a) 2378 g/mol

8.18e.- Per a augmentar la quantitat de material de partida en la purificació de proteïnes per cromatografia d'exclusió, ha d'augmentar-se el diàmetre de la columna?, ha d'augmentar-se la longitud?, o han d'augmentar-se tots dos?

8.19e.- En una columna de sílice per a HPLC es troba que un solut elueix pràcticament amb el front de solvent quan la composició de la fase mòbil és hexà: cloroform 50 % (v/v).

a) Amb quina modalitat de cromatografia líquida està treballant?

b) Com modificaries la composició de la fase mòbil per a obtenir una retenció acceptable?

PROBLEMES T9 (Estudiants)
CENTRIFUGACIÓ

9.1e.- Quin és el camp centrífug aplicat en un punt que gira a 5 cm del seu centre de rotació i amb una velocitat angular de 3000 rad/s^{-1}? S: $45\cdot10^6$ cm/s^2; com a RCF seria: 45900*g*.

9.2e.- Per a la sedimentació de la fracció microsomal d'un homogeneïtzat de fetge s'empra una ultracentrífuga a velocitat de 40000 rpm. Quina és la velocitat angular, ω, en radians per segon? Una partícula situada a 10 cm del centre de rotació en aquesta centrifugació, a quin camp centrífug relatiu, RCF, està sotmesa? S: $175{,}5\cdot10^6$ cm/s^2; RCF= 179000*g*

9.3e.- Calcula la força centrífuga produïda sobre una partícula situada en un rotor de 10 cm de radi quan gira a 1000 rpm. Expressa-ho pel que fa a l'acceleració gravitacional. Quina seria eixa força per a una partícula situada a 20 cm de l'eix de gir? S: RCF_1=111,9; RCF_2=223,8

9.4e.- La variació del coeficient de sedimentació, s, amb la concentració per a l'enzim àcid gras-sintasa (AGS) de glàndula mamària bovina es mostra en la següent Taula:

AGS (mg/mL)	0,44	2,00	3,77	7,44
$s_{20,w}$ (Svedberg)	13,40	13,27	13,06	12,62

La centrifugació es va realitzar a 56000 rpm. Determina la massa molecular de l'enzim assumint un volum específic parcial de 0,72 mL/g i sabent que el coeficient de difusió $D_{20,w}$ es $2{,}12\cdot10^{-7}$ cm^2s^{-1}. Com pots explicar el descens de s amb la concentració?

Dades: R= $8{,}3\cdot10^7$ erg/mol·ºK (1erg= 1g·cm^2/s^2); Tª= 20 ºC; $\rho_{20,w}$= 1,0 g/mL.

S: L'extrapolació a concentració zero proporciona s^0= 13,5S. D'ací aplicant l'expressió derivada de Svedberg: M= sRT/[D(1-$\upsilon\rho_d$)] →MM= 552000.

9.5e.- Raona la validesa de les següents afirmacions:

a) La centrifugació diferencial permet separar la fracció nuclear i mitocondrial de forma eficaç i amb un mínim grau de contaminació.

b) La centrifugació diferencial permet separar les fraccions de peroxisomes i mitocondris.

c) Els àcids nucleics RNA i DNA poden separar-se mitjançant una centrifugació isopícnica en gradient de densitat de clorur de cesi.

S: a) bé; b) correcte però no sempre; c) correcte, la ρ_{DNA}≈1,6-1,7g/mL y ρ_{RNA}≈1,9 g/mL.

9.6e.-. El coeficient de sedimentació de la proteïna hemocianina de *N. antiqua* a pH 5.7 i en presència d'ions divalents és de 101,1S. En presència de l'agent quelant EDTA i a pH 7,5 disminueix el s a 60S. A pH 9,2 resulta ser, tan sols, de 15,9S. Assumint que aquest últim valor correspon al de la subunitat constituent, determinar de forma aproximada el nombre de subunitats de la forma de major s.

S: Aplicant l'aproximació de la dependència de s amb MM, assumint: *i*) forma esfèrica per a totes les formes i *ii*) idèntic υ per totes les formss. Així→ $s=M^{2/3}\{[(4/3)^{1/3}(1-\upsilon\rho_d)]/[N_a^{2/3}\eta\upsilon^{1/3}\pi^{2/3}]\}$ i, tot entre les claus són constants per a totes les partícules → $s= M^{2/3}K$. $s_{101,1}/s_{60}= (M_{s101,1})^{2/3}/(M_{s60})^{2/3}$ y $s_{101,1}/s_{15,9}= (M_{s101,1})^{2/3}/(M_{s15,9})^{2/3}$. D'ací: $M_{s101,1}= 16M_{s15,9}$ → 16 subunitats.

9.7e.- En el fraccionament subcel·lular d'un extracte de múscul es va determinar l'activitat de 4 enzims marcadors en les 4 fraccions (F1-F4) obtingudes, així com en l'homogeneïtzat (H) de partida. Les dades resultants es mostren en la següent Taula:

Fracció	**Proteïna (mg/mL)**	**Succinat-oxid. (u/mL)**	**Lactat-deshidrog. (u/mL)**	**Glucosa-6-fosfatasa (u/mL)**
H	31,00	3,20	4,00	6,00
F1	10,00	2,70	0,10	0,50
F2	6,00	0,01	3,50	0,01
F3	7,25	0,10	0,20	4,00
F4	7,20	0,30	0,01	1,00

Identifica cadascuna de les fraccions i comenta els resultats.

S: S'obté l'activitat específica (activitat/concentració proteïna), observant majors diferències i que identifica les fraccions [mitocondrial, microsomal i soluble (citosol)]

9.8e.- El coeficient de sedimentació de l'enzim dimèric xantosina-5'-fosfat-aminasa (X5P-aminasa), deduït per ultracentrifugació analítica en experiments de velocitat de sedimentació, és de 5,91 S. Després de sotmetre a l'enzim complet i a la seua subunitat, juntament amb proteïnes patró, a ultracentrifugació zonal en gradient de concentració de sacarosa i fraccionar el gradient (de baix cap a dalt) es van obtenir els resultats de la Taula:

Proteïna	**$s^o_{20,w}$**	**Nº de fracció**
Catalasa	11,80	15
Alcohol-DH	7,60	23
BSA	4,31	29
Ovoalbúmina	3,55	31
X5P-aminasa	¿?	27
Monòmer de X5P-aminasa	¿?	28

Calcula el coeficient de sedimentació de l'enzim i de la seua subunitat constituent pel mètode de Martin i Ames. Com expliques la discordança dels valors de s obtinguts amb aquells deduïts per ultracentrifugació analítica?
S: $s^0_{20,w\ (enzim\ natiu)}$= 5,0S; $s^0_{20,w\ (subunitat)}$= 4,2S

9.9e.- La fosfatasa-alcalina d'*E. coli* posseeix un coeficient de sedimentació de 6,0 S. Una mescla de fosfatasa-alcalina i la proteïna lisozima s'ultracentrifuga en un gradient de densitat (isocinètic) de sacarosa. Després de la centrifugació el gradient se fracciona perforant el fons del tub i arreplegant 30 fraccions d'igual volum. En analitzar les fraccions es troba un màxim de fosfatasa-alcalina en la fracció 15 i un màxim d'activitat lisozima en la 25.

a) Calcula el coeficient de sedimentació de la lisozima.

b) Determina la massa molecular de la fosfatasa-alcalina sabent que la MM de la lisozima és 17000.

S: Aplicar el mètode de Martin i Ames en el que sols s'empra una proteïna patró. a) Ací la fosfatasa-alcalina és el patró: $s_{(lisozima)}$= 2S. b) Ací la lisozima és la proteïna patró: $MM_{(fosfatasa\text{-}alcalina)}$= 88000

9.10e.- Calcula la densitat de flotació i el % de parells de bases GC d'un DNA el perfil del qual, després de centrifugació en un gradient de densitat de CsCl, és que es mostra en la Taula:

S: A_{260} front a Nº fracció s'obté el màxim entre las fraccions 40-43. La densitat de flotació corresponent és: 1,665-1,675. De la relació empírica entre densitat de flotació i el % GC s'obté:12 %

Nº de fracció	**A_{260}**	**Densitat gradient (g/mL)**
1	0,01	1,580
10	0,01	1,595
20	0,01	1,620
30	0,02	1,645
35	0,20	1,650
37	0,45	1,660
40	0,50	1,665
43	0,45	1,675
45	0,40	1,680
50	0,10	1,690
60	0,03	1,710

9.11e.- Una fracció ribosomal del bacteri *Acetobacter acetii*, obtinguda per centrifugació diferencial en un medi de densitat 1,04 g/mL, presentava activitat d'enzims oxidasa. Els investigadors van pensar que en aquests bacteris havien d'existir unes partícules ("*oxidosomes*") contenint els enzims oxidasa que sedimentaven com els ribosomes. Mitjançant centrifugació isopícnica en gradient preformat de sacarosa van trobar l'activitat oxidasa en una zona del gradient de densitat 1,23-1,26 g/mL, mentre que els ribosomes sedimentaven en el fons del tub (densitat dels ribosomes estimada per centrifugació isopícnica en CsCl, 1,52 g/mL). Els autors d'aquest treball proposen que el diàmetre dels "*oxidosomes*" (d_{ox}) es pot calcular amb l'equació:

$$d^2_{ox}\cdot(\rho_{ox}-1{,}04) = d^2_{rib}\cdot(\rho_{rib}-1{,}04)$$

on ρ_{ox} i ρ_{rib} són les densitats de "*oxidosomes*" i ribosomes, respectivament.

Es coneix, per microscòpia electrònica, que el diàmetre dels ribosomes (d_{rib}) 70S és de 18 nm. D'ací els autors dedueixen:

$$d^2_{ox}\cdot(1{,}245-1{,}04) = 18^2\cdot(1{,}52-1{,}04); \qquad \text{i així} \quad d^2_{ox} = 28 \text{ nm}$$

Quin és el fonament teòric per a obtenir d'aquesta forma la grandària de la partícula? És una deducció rigorosa o implica simplificacions? Quines crítiques poden fer-se?

El fet que els "*oxidosomes*" i ribosomes hagen sedimentat junts en el medi de densitat 1,04 g/mL, és casual o haguera ocorregut d'igual forma en un altre medi de diferent densitat (sempre inferior a la de les partícules)?

S: De Svedberg, assumint partícules esfèriques, $s= m_p(1-\upsilon\rho_d)/6\pi\eta r$. Com $m_p= Vol_m\rho_m$ i $\upsilon_m= 1/\rho_m$. $\rightarrow s= Vol_m(\rho_m-\rho_d)/6\pi\eta r$. El diàmetre $d= 2r$ i el $Vol_p= 1/6\pi d^3$, així $s= d^2(\rho_p-\rho_d)/18\eta$. Per a ribosomes i "*oxidosomes*", respectivament: $s_{rib}= d_{rib}{}^2(\rho_{rib}-\rho_d)/18\eta$; $s_{ox}= d_{ox}{}^2(\rho_{ox}-\rho_d)/18\eta$. Els autors assumeixen (per centrifugació diferencial en medi de densitat 1,04 g/mL) que $s_{rib}= s_{ox}$ i així $d_{rib}{}^2(\rho_{rib}-\rho_d)/d_{ox}{}^2(\rho_{ox}-\rho_d)$, quedant demostrat. Crítiques: són esferes? La densitat de flotació obtinguda en medis distints no són comparables (distinta solvatació). Com d'exacta és la co-sedimentació en la centrifugació diferencial? I el temps de centrifugació, es va tenir en compte per a assumir que $s_{rib}= s_{ox}$?

Suposant que en aquest medi és cert que: $s_{rib}=s_{ox}$, és casual, a densitat inferior a 1,04 els "*oxidosomes*", per ser més grans serien més ràpids. Amb $\rho_d>1{,}04$ serien els ribosomes més ràpids. (veure solució del problema 9.7 de classe, per a una major explicació).

9.12e.- Expliqueu perquè la presència de bromur d'etidi en centrifugació isopícnica en gradient de densitat amb CsCl modifica la densitat de flotació del DNA i de forma diferent per a DNA circular, DNA superenrotllat i DNA lineal.

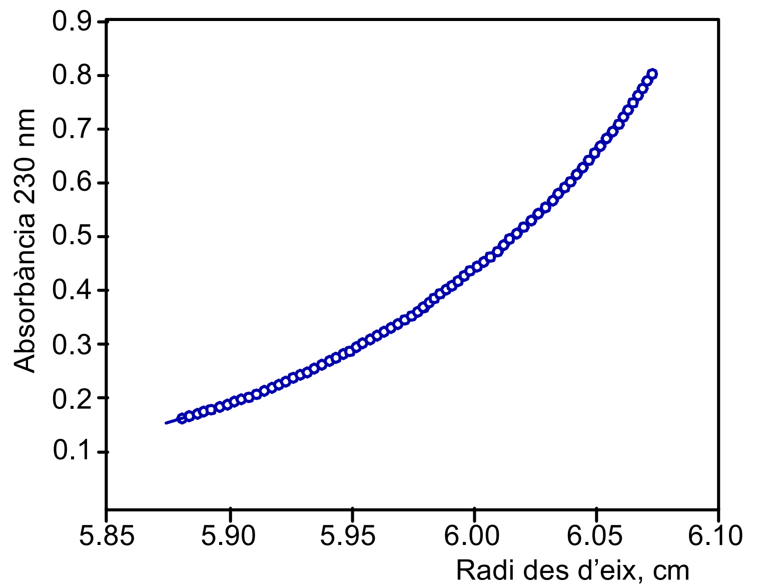
S: El BrEt s'intercala, desenrotllant una mica la doble hèlix, i açò disminueix la densitat de flotació. El DNA circular i el superenrotllat, per restricció topològica, no uneixen tant BrEt com el lineal. En presència de BrEt el DNA lineal disminueix la seua densitat molt, menys el circular i encara menys el superenrotllat.

9.13e.- La densitat del DNA en CsCl 7 M amb $MgCl_2$ 0,01 M és menor que en CsCl 7 M sol. Perquè?

S: Probablement el catió divalent, Mg^{2+}, amb una massa de 24,3 s'uneix als grups fosfat (carregats negativament). En un medi amb només CsCl, és el catió Cs^+, massa de 132,9, el que s'uneix als grups fosfat. Aquesta diferència de masses es tradueix en diferències de densitat de flotació.

9.14e.- El factor de necrosi tumoral α (TNFα) és una proteïna implicada en inflamació i apoptosi. En les cèl·lules es troba en forma de trímer. Quan s'obté en forma recombinant no es coneix si s'organitza en la seua forma trimèrica natural. La cromatografia d'exclusió molecular, donada la seua limitada resolució, no

permet esbrinar la seua massa molecular amb precisió o si la seua elució poguera estar afectada per un plegament incorrecte de la forma recombinant. Així es va decidir estimar la massa molecular mitjançant ultracentrifugació analítica.



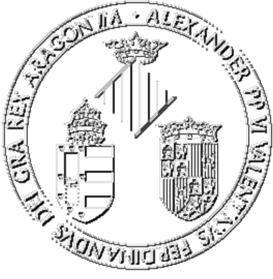
La gràfica mostra les dades de l'equilibri de sedimentació per a aquesta molècula (emprant 10 μg totals) en la qual es representa la concentració de la proteïna (com A_{230}) en funció de la posició en la cel·la de mostra, després de la centrifugació a 16000 rpm durant 18 hores.

La massa molecular del monòmer és de 17000 i el volum específic parcial (υ), estimat a partir dels volums específics parcials dels aminoàcids constituents ($n_i m_i \upsilon_i / \sum n_i m_i$, on n_i, m_i i υ_i són el nombre, la massa molecular i el volum específic parcial de cada tipus d'aminoàcid present en la proteïna, respectivament), de 0,731 mL/g.

És la proteïna recombinant un trímer, igual que la forma natural? Si la resposta és sí, representa de forma aproximada el perfil d'equilibri de sedimentació en la figura adjunta, i també en qualsevol altra gràfica que hages emprat, assumint una forma monomèrica. Si la resposta és no, realitza aquestes representacions assumint una estructura trimèrica.

Dades: R (constant gasos)= 8,3 J/mol·ºK= $8{,}3 \cdot 10^7$ erg/mol·ºK (1erg= $1 g \cdot cm^2/s^2$)
Temperatura 20ºC. Densitat del medi 1,053 g/mL

S: Obtenir de la gràfica diversos valors A_{230} i els seus valors de x (radi des de l'eix). En un experiment d'equilibri de sedimentació ha de complir-se: $\ln C = [M\omega^2(1-\upsilon\rho_d)x^2]/2RT + cte$. En aquest cas és equivalent lnC i $\ln A_{230}$, per la proporcionalitat entre concentració i absorbància. Així obtenim $\ln A_{230}$ i x^2, els representem i obtenim el pendent, en aquest cas 0,675 cm^{-2}. El pendent= $M\omega^2(1-\upsilon\rho_d)/RT$, aplicant υ i ρ_d de l'enunciat: M= 50900 que correspon molt properament a 3 vegades 17000. Es tracta per tant d'un trímer.



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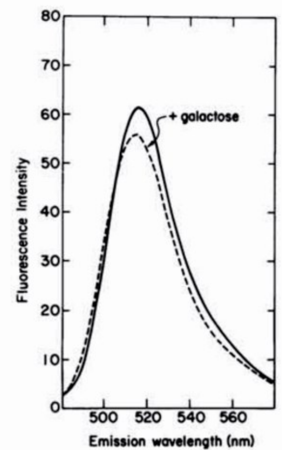

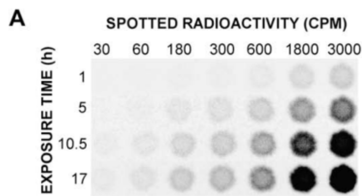

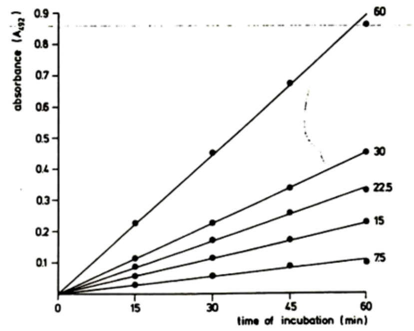

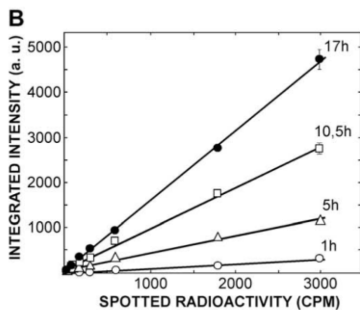

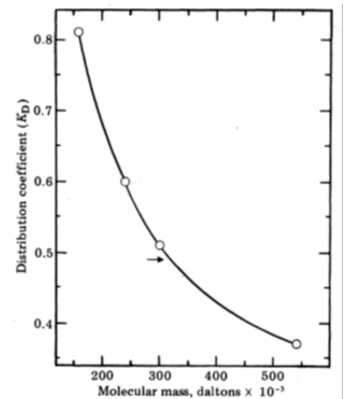

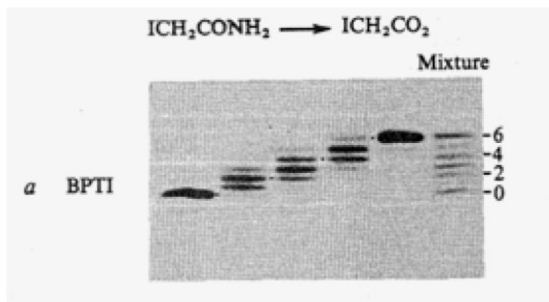

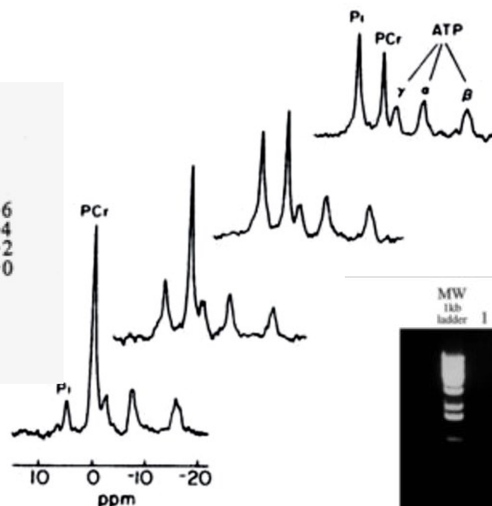

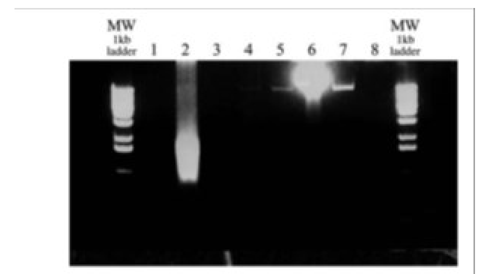

ANALYTICAL BIOCHEMISTRY **180**, 91–94 (1989)

Monitoring of Phenylketonuria: A Colorimetric Method for the Determination of Plasma Phenylalanine Using L-Phenylalanine Dehydrogenase

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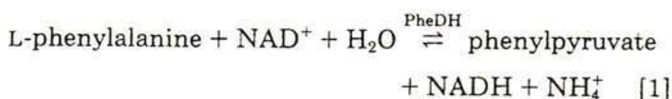
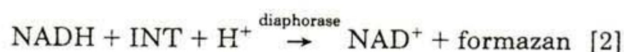
A simple, rapid, accurate, and precise colorimetric assay for the determination of L-phenylalanine in plasma samples using L-phenylalanine dehydrogenase [L-phenylalanine:NAD$^+$-oxidoreductase (deaminating)] from *Rhodococcus* sp. M 4 is described. The enzyme catalyzes the NAD-dependent oxidative deamination of L-phenylalanine. However, the equilibrium of reaction favors L-phenylalanine formation. By stoichiometric coupling of this reaction with diaphorase/iodonitro tetrazolium chloride (INT) the formed NADH converts INT to a formazan whereby the reaction is displaced in favor of phenylpyruvate. Using a kinetic approach the increase in absorbance at 492 nm shows linearity over more than 30 min. Deproteinized standard solutions of L-phenylalanine in the range from 30 to 1200 μmol/liter show a linearity between the $dA_{formazan}$/30 min and the substrate concentration. In phenylketonuria (PKU) plasma samples no interferences caused by L-tyrosine or phenylpyruvic acid are seen. Applicability is demonstrated by comparative determination of plasma L-phenylalanine of treated PKU patients by the colorimetric method and automated amino acid analysis. © 1989 Academic Press, Inc.

Several methods have been reported in the literature for the quantitative determination of L-phenylalanine in physiological fluids, an important parameter in diagnosis and therapy of disorders of phenylalanine catabolism. The Guthrie test (1), which is used worldwide as a semiquantitative test in newborn screening and occasionally also in therapy control in patients with hyperphenylalaninemias, is of low precision. Blood phenylalanine levels in PKU[1] patients on low-phenylalanine diets are most commonly monitored by spectrofluorometric methods (2,3) or by column chromatography using amino acid analyzers (4). Spectrophotometric methods for quantitative estimation of L-phenylalanine using snake-venom L-amino acid oxidase (5) phenylalanine ammonia lyase (6), L-phenylalanine oxidase (7), or derivative spectrophotometry (8) are not routinely applied. Recently Hummel *et al.* (9) reported on an optical kinetic test for the determination of L-phenylalanine by use of a newly detected NAD(H)-dependent PheDH from a strain of *Brevibacterium* sp.

Departing from the latter enzymatic assay we developed a colorimetric method to determine L-phenylalanine in plasma or serum. Our method couples simultaneously the reaction of a NAD(H)-dependent PheDH, an enzyme that catalyzes the oxidative deamination of L-phenylalanine to phenylpyruvate, and a second reaction in which initially formed NADH and diaphorase convert INT to a formazan. This product is measured in the visible range at 492 nm. The catalyzed reactions are



$$\text{L-phenylalanine} + NAD^+ + H_2O \overset{\text{PheDH}}{\rightleftharpoons} \text{phenylpyruvate} + NADH + NH_4^+ \quad [1]$$



$$NADH + INT + H^+ \xrightarrow{\text{diaphorase}} NAD^+ + \text{formazan} \quad [2]$$

This simple, rapid, accurate, and precise photometric method can be applied for clinical routine and gives results identical to those of automated amino acid analysis.

MATERIALS AND METHODS

Reagents

All chemicals were purchased from Merck (Darmstadt, FRG) or Boehringer (Mannheim, FRG). Standard serum (Precinorm S) was from Boehringer.

[1] Abbreviations used: PKU, phenylketonuria; INT, iodonitro tetrazolium chloride; PheDH, L-phenylalanine dehydrogenase.

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TABLE 1

Substrate Specificity and Kinetic Parameters of Partially Purified L-PheDH from *Rhodococcus* sp. M 4 (10)

Oxidative deamination (pH 9.5)		
Substrate	V_{max}	K_m [M]
L-Phenylalanine	100	7.5×10^{-4}
L-Tyrosine	12	3.1×10^{-3}

Note. V_{max} is given in relation to the value for L-phenylalanine. K_m (NAD), 2.7×10^{-4} M; K_i (phenylpyruvate), 7.0×10^{-5} M.

For the enzymatic determination of L-phenylalanine we used solution 1 (potassium phosphate, 25 mmol/liter/triethanolamine 0.2 mol/liter buffer, pH 8.6; Triton X-100, 13.2g/liter), solution 2 (diaphorase from pig heart/NAD; stabilizers), and solution 3 (INT) of the test combination for the colorimetric determination of L-glutamic acid (Cat. No. 139 092, Boehringer).

Enzyme Preparation

PheDH [L-phenylalanine:NAD^+-oxidoreductase (deaminating)] was prepared from a culture of *Rhodococcus* sp. strain M 4 grown aerobically in a bioreactor by the procedure of Hummel *et al.* (10). The crude extract of the cells containing 25–30 U/mg of PheDH activity was purified further by a two-step procedure (liquid–liquid extraction; DEAE-cellulose chromatography) to give a specific activity of more than 1000 U/mg (as described by Hummel *et al.* (10). Less purified enzyme preparations are also suitable, provided the enzyme inducer L-phenylalanine is completely removed. Substrate specifity of PheDH for oxidative deamination reaction is given in the Table 1.

This partially purified enzyme preparation stored at 4°C at a concentration of 3.0 mg of protein/ml (50 U/ml) in 0.2 M potassium phosphate buffer containing betaine (pH 9.5) demonstrated about 35% loss of activity in 8 months. Dilutions were made with potassium phosphate/triethanolamine buffer to give approximately 0.05 U/1.0 ml of assay mixture for measurements of standards and unknowns.

PheDH Assay

The mixture for the assay of reductive amination contained 738 mmol/liter $(NH_4)_2SO_4$ buffer, pH 8.5, 0.1 mmol/liter NADH, 1.0 mmol/liter phenylpyruvate, and limiting amounts of the enzyme preparation. Oxidative deamination was measured in potassium phosphate (8.3 mmol/liter)/triethanolamine (67 mmol/liter) buffer, pH 8.6, with Triton X-100 (4.4 g/liter) containing 1 mmol/liter NAD^+, 2 mmol/liter L-phenylalanine, and limiting amounts of the enzyme preparation.

The reactions were carried out at 21°C and followed by measurement of the absorption of NADH at 340 nm. Enzyme activity is expressed in International Units as the consumption or production of 1 μmol NADH/min using a molar extinction coefficient of 6.22×10^3 liter mol^{-1} cm^{-1}. Activity of oxidative deamination was found about 10 per cent that of reductive amination. In the text PheDH activity is given as reductive aminating activity, throughout.

Automated Amino Acid Analysis

Plasma samples and standards (dilution sequences of phenylalanine dissolved in Precinorm S) were deproteinized with 4 vol of 3.0% sulfosalicylic acid in water and the mixture was centrifuged. Determinations of L-phenylalanine were performed on a Biotronic 6001 amino acid analyzer (Biotronic, Munich) using a short program with the following details: column 0.4 × 30 cm filled with BTC 2710 (Biotronic) resin; column temperature, 46°C; buffer flow, 20 ml/h. Buffers, 0.47 N lithium citrate buffer (pH 3.3) and 0.50 N lithium citrate buffer (pH 4.1).

Phenylalanine retention time was 36 min. The concentrations were quantified with a Shimadzu CR 3A digital integrator (Kyoto, Japan) by comparison to a calibrated commercial standard (Benson H-standard, Biotronic). The sample volume applied to the column was 100 μl. The protein-free supernatant was applied directly to the sample collector or stored frozen (−20°C) for a short time until analysis.

Enzymatic Determination of L-Phenylalanine

Plasma samples and standards (phenylalanine dissolved in Precinorm S) were deproteinized with an equal volume of $HClO_4$ (1.6 mol/liter) and centrifuged and the acid extract was neutralized by the addition of an appropriate volume of $KHCO_3$ solution (6 mol/liter). After centrifugation, 0.1 ml of the supernatant was inserted into the assay. The reaction mixture (1.0 ml) consisted of 217 μl of solution 1 (potassium phosphate/triethanolamine buffer, pH 8.6), 433 μl redistilled water, 100 μl of solution 2 (NAD/diaphorase), 100 μl of solution 3 (INT) of the test combination Cat. No. 139 092 (Boehringer), and 100 μl of the deproteinized sample with a final L-phenylalanine concentration of 1.5–60 μmol/liter. Final concentrations in the assay were for triethanolamine 43 mmol/liter, potassium phosphate 5.4 mmol/liter, Triton X-100 2.87 g/liter, NAD 0.75 mmol/liter, INT 0.08 mmol/liter, and diaphorase 0.15 U. The reaction was started by addition of 50 μl PheDH solution. In general, enzyme activity amounted to 0.05 U/assay. Incubation was at 21°C. Increase of absorbance at 492 nm was measured after exactly 30 min using a Beckman DU-8 spectrophotometer. Appropriate blanks and calibration standards were likewise prepared each day using stan-

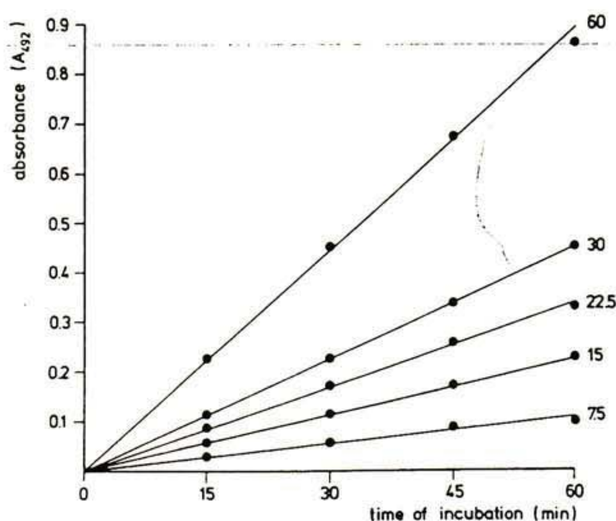

FIG. 1. Time dependence of absorbance (A 492 nm) for assays containing different amounts of L-phenylalanine. L-Phenylalanine concentrations in standard serum were adjusted as indicated. Samples were processed and enzymatic analysis with 0.1 ml serum extract was performed as given under Materials and Methods. After addition of enzyme (0.05 U/assay) absorbance at 492 nm was recorded against time. The amounts of L-phenylalanine in the assay solution (1.0 ml) are given in nanomoles (7.5 nmol/1.0 ml assay solution correspond to 0.15 mmol/liter or 2.5 mg/dl L-phenylalanine in the serum sample). Absorbance (dA/30 min) for blanks (assays without L-phenylalanine) was constantly below 0.01.

dard serum (Precinorm S) containing defined amounts of L-phenylalanine.

Calculations

Plasma concentrations of L-phenylalanine were estimated by the use of appropriate calibration curves. For the determination of recovery, standard serum was spiked with L-phenylalanine to give concentrations of 0.06 and 0.48 mmol/liter, respectively. Results are presented as means ± SD of n determinations. Linear regression (least-square method) was used for statistical analysis.

RESULTS

Enzymatic Determination of L-Phenylalanine

An endpoint method was not feasible because of the limited amount of the PheDH enzyme. We therefore chose a kinetic approach and measured in the linear range of the absorbance change at 492 nm.

The changes in absorbance were linear with reaction time over more than 30 min, at which time about 30% of phenylalanine was converted. A linear relationship was also obtained between the increase in absorbance and the phenylalanine concentration over a range from 1.5 to 60 nmol/ml assay corresponding to 30 to 1200 μmol/liter (0.5 to 20 mg/dl) in the serum sample (Fig. 1). This is the range of phenylalanine concentrations expected in the plasma of healthy individuals and treated PKU patients.

The within-run coefficients of variation (four samples analyzed seven times in succession) for 3, 9, 30, and 60 nmol/ml assay, respectively, were 4.0, 4.3, 2.7, and 2.3%. The between-run coefficients (seven determinations over 2 weeks) for 5, 22, and 42 nmol/ml assay were 5.5, 4.5, and 3.4%, respectively. Recovery of L-phenylalanine from spiked serum was 103 ± 10% (serum concentration, 0.06 mmol/liter) and 98 ± 5% (serum concentration, 0.48 mmol/liter), respectively.

Measurements of a dilution sequence of L-tyrosine in standard serum up to a concentration of 0.55 mmol/liter did not yield any change in absorbance compared to those in the blank. Addition of different amounts of L-tyrosine up to 30 nmol or L-methionine up to 25 nmol in the assay (containing 1, 30, and 60 nmol phenylalanine/ml) did not influence at all the absorbance brought about by L-phenylalanine.

Automated Amino Acid Analysis

Using a short program for the determination of L-phenylalanine the coefficients of variation within and between runs (four standard concentrations from 0.12 to 1.2 mmol/liter, each sample analyzed five times) were below 3 and 5%, respectively. Recovery of L-phenylalanine from spiked serum was 103 ± 4% (n = 12; concentration, 0.48 mmol/liter).

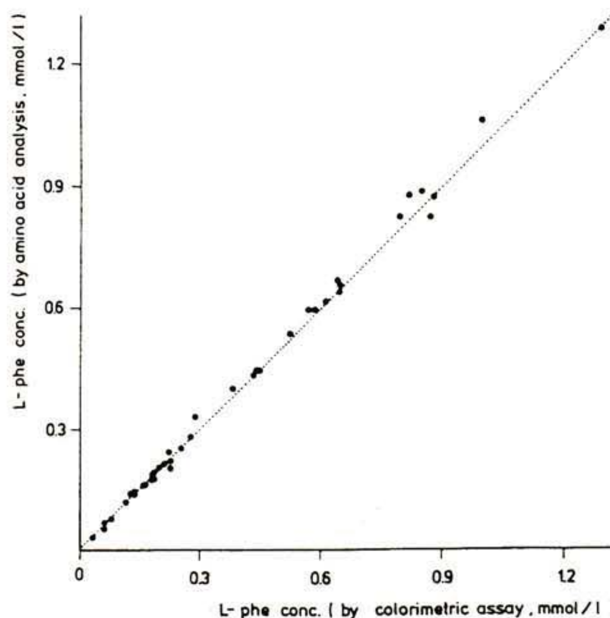

FIG. 2. Correlation between the phenylalanine values obtained by automated amino acid analysis (y-axis) and the colorimetric determination (x-axis) in plasma samples of PKU patients under therapy. Dotted line, linear least-square regression ($y = 0.020 + 1.013\ x$; $r = 0.998$).

Comparison of Methods

Using automated amino acid analysis and our enzymatic method, we estimated in parallel the L-phenylalanine content in 41 plasma samples obtained from 36 treated PKU patients. The data are presented in Fig. 2. In the range from 0.03 to 1.28 mmol/liter plasma the correlation coefficient was $r = 0.998$. With automated amino acid analysis as the dependent variable the regression was $y = 0.020 + 1.013\ x$. The intercept is not significantly different from zero.

DISCUSSION

We describe an enzymatic colorimetric method for the determination of plasma phenylalanine which is based on the oxidative deamination reaction of PheDH. It was our aim to develop this assay as a simple, accurate, and precise method for monitoring PKU patients during dietary therapy. Present-day column chromatographic and spectrofluorometric methods are time-consuming and require high-grade instrumentation and sophisticated technical knowledge. By using one of the just recently isolated L-phenylalanine dehydrogenases (10–12) and by designing the phenylalanine assay according to the principles of commercial Test Combinations, the foundation has been laid for quick monitoring and efficient counseling of children with PKU in an outpatient clinic environment.

The formerly described enzymatic uv method for the determination of phenylalanine (9) is based on the increase in absorbance of NADH during the first minute of reaction. In the present assay the NADH is recycled to NAD with diaphorase and INT. Therefore, the equilibrium which normally lies far toward phenylalanine is displaced to phenylpyruvic acid and the linearity in time of formation of product is much extended. In addition, the sensitivity of the assay is increased by a factor of three, because the product, INT–formazan, has a molar extinction coefficient ϵ of about 20,000 (13).

Compared to the enzyme from *Brevibacterium* sp. (9) the *Rhodococcus* sp. M 4 enzyme has a much higher K_m for phenylalanine. This assures first-order kinetics over a broader range of substrate concentrations and is another advantage of our colorimetric assay. Furthermore, this method compares favorably with automated amino acid analysis. There is no indication of any systematic error in either system and accuracy and precision do not differ significantly for both methods.

Partially purified PheDH from *Rhodococcus* sp. exhibits some catalytic activity on tyrosine (10). However, because of low V_{max} and very high K_m, the integrated rate equation predicts that less than 1% of tyrosine is converted within 30 min under the conditions of our assay. Accordingly, no interference was observed.

Another potential source of interference is endogenous phenylpyruvic acid. Its concentration in PKU sera, however, is very low: 7 ± 6 and 52 ± 28 μmol/liter at 500 and 1100 μmol/liter phenylalanine, respectively (14,15). The 20-fold dilution of the serum samples therefore brings the concentrations in the test solution well below the K_i of 70 μmol/liter. It is for this reason that no systematic deviation is observed in our assay at high phenylalanine levels in PKU sera. Possible interferences by ascorbic acid and pyruvate were shown not to be relevant clinically.

The detection limit of this method for phenylalanine is of the order of 1.5 to 3 μmol/liter in the assay mixture. This is sufficient also for the quantification of the normal plasma level of phenylalanine. The test can thus be used for the quantitative determination of plasma phenylalanine at concentrations ranging from 0.03 to 1.2 mmol/liter without any further modification. It should be applicable to the determination of phenylalanine in whole venous and capillary blood and in paper-dried blood and may also be amenable to automation.

ACKNOWLEDGMENTS

The authors acknowledge the skillful help of Mrs. E. Diekmann, Mrs. H. Göddertz, Mrs. S. Löslein, and Mr. J. Vernau.

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Qüestions proposades de l'article de Wendel *et al., Anal. Biochem*. 180, 91-94 (1989).

1.- Reviseu en llibres de text de bioquímica general les característiques de la malaltia anomenada fenilcetonúria i justifiqueu l'interès de la determinació dels nivell de fenilalanina en la sang dels malalts que la pateixen.

2.- Els autors proposen mesurar la quantitat de fenilalanina mitjançant dos reaccions enzimàtiques acoblades.

a) Quin paràmetre experimental és indicatiu de la concentració de fenilalanina en la mostra? Perquè?

b) Si la primera reacció ja és cromogènica, per a què s'acobla una segona reacció?

3.- En l'article es comenta que la K_m de l'enzim fenilalanina-deshidrogenasa de *Rhodococcus* (que utilitzen els autors) és més gran que la de *Breviabacterium* (utilitzada per altres investigadors). Quin avantatge suposa açò des de el punt de vista de la seua utilització per a determinar fenilalanina?

4.- Interpreteu les Figures 1 i 2. Quina informació es pot obtenir d'elles?

5.- Quines són les principals avantatges del mètode que proposen els autors sobre els altres procediments també citats en l'article?

Proc. Natl. Acad. Sci. USA
Vol. 74, No. 5, pp. 1932–1936, May 1977
Biochemistry

Use of a distant reporter group as evidence for a conformational change in a sensory receptor

(galactose receptor/5-iodoacetamidofluorescein/chemotaxis/spectroscopic rules)

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Contributed by Daniel E. Koshland, Jr., February 28, 1977

ABSTRACT A highly sensitive method for demonstrating ligand-induced conformational changes in protein molecules in solution is described. The method utilizes an environmentally sensitive reporter group that is known to be distant from the active site. In the present application a conformational change is demonstrated in the galactose receptor of *Salmonella typhimurium,* involved in bacterial sensing and transport, by means of an extrinsic fluorophore, 5-iodoacetamidofluorescein, attached at a single methionine residue, and the intrinsic tryptophan fluorophore. Binding of the ligand galactose perturbs the microenvironment of both the fluorescein and tryptophan, as shown by both spectral and potassium iodide quenching changes. The distance between the two dyes is established by fluorescence energy transfer methods to be 41 ± 10 Å. Since only one molecule of galactose binds per molecule of receptor and since the galactose molecule is only about 5 Å in length, changes at one of these sites reflect the result of an indirect effect. Hence, there must be a ligand-induced conformational change that is propagated a minimum of 30 Å through the receptor molecule.

The ability to undergo ligand-induced conformational changes is now an accepted feature of protein molecules. To date, the most widely used method providing unequivocal and detailed evidence of this phenomenon has been x-ray crystallography. Crystallographic studies of hemoglobin (1), carboxypeptidase (2), and lactate dehydrogenase (3) have demonstrated that specific atoms in these protein molecules assume different spatial arrangements in the presence and absence of substrate. Protein crystallography has its limitations, however, which include its inapplicability to the dynamics of molecular interactions in solution, the difficulties of obtaining pure crystals, and the laborious nature of the procedure. Moreover, many protein molecules, of which receptors are a good example, are not available in the quantities required for such an approach.

Other methods that can complement or precede x-ray analysis are needed, therefore, for the study of conformational changes in proteins. One method that has provided definitive but not widely applicable evidence is demonstration of increased reactivity of specific protein residues upon ligand binding (4). Spectroscopic approaches, which include absorption spectroscopy, far-ultraviolet circular dichroism, and fluorescence, are simple to apply and frequently detect changes in the microenvironment of specific protein residues. Such methods as are conventionally used, however, do not distinguish between a direct effect, i.e., a change in the immediate vicinity of the bound ligand, and an indirect effect, i.e., a change in the microenvironment of a residue at some distance from the ligand-binding site.

A method is developed that is applicable to dynamic interactions and that also gives unambiguous evidence of conformational changes. The method utilizes a reporter group (5) known to be far from the active site as a probe of conformational changes that occur upon ligand binding. In the present application, two reporter groups are placed sufficiently distant from one another that the bound ligand cannot interact directly with both groups simultaneously. The distance between the dyes is established by fluorescence energy transfer measurements following Förster's equation (6) and Wu and Stryer's use of fluorescence as a spectroscopic ruler (7). A change in the microenvironment of the fluorescent groups then provides evidence for a delocalized conformational change and a description of the dynamics of the event.

The molecule chosen for development of this method was the galactose receptor of *Salmonella typhimurium* in bacterial sensing. The galactose binding protein of *Escherichia coli* was first isolated by Anraku (8, 9), and was shown to be the receptor in chemotaxis by Hazelbauer and Adler (10). Recently, Strange and Koshland (11) have described a model for receptor action that postulates a ligand-induced conformational change in the galactose receptor as an obligatory first step prior to its interaction with the signalling apparatus of the sensory system. Boos and coworkers (12–15) reported spectral changes associated with one or more tryptophan residues in the antigenically related galactose receptor of *E. coli* upon binding of galactose. These findings, together with the observation that the *S. typhimurium* protein contains a single tryptophan and binds a single galactose molecule (16), led us to utilize as the pair of reporter groups 5-iodoacetamidofluorescein (5-IAF) specifically attached at a single site on the protein and the single intrinsic tryptophan moiety of the *Salmonella* protein. The results and general applicability of the method are discussed.

MATERIALS AND METHODS

The galactose binding protein was isolated by described methods from *S. typhimurium* strain ST1 (16). The protein was determined to be homogeneous by gel electrophoresis under a variety of conditions. Protein concentrations were determined by the method of Lowry *et al.* (17) and binding activities by the nitrocellulose filter assay (18). Liquid scintillation counting was carried out in an Aquasol/toluene 2:1 mixture on a Packard Tricarb 3320 Liquid Scintillation Spectrometer.

The galactose-binding protein was modified by treatment with a 1000-fold excess of 5-iodoacetamidofluorescein (a gift of Richard Haugland, Hamline University) in 10 mM sodium phosphate buffer, pH 7.0/150 mM sodium chloride for 24 hr in the dark. The labeled protein was then purified by passage through a Sephadex G-25 column equilibrated with the same

Abbreviations: 5-IAF, 5-iodoacetamidofluorescein; 5-AF, 5-acetamidofluorescein.

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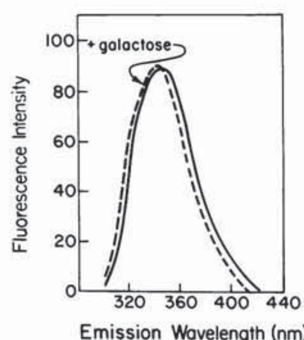

FIG. 1. Fluorescence emission spectra (uncorrected) of the *Salmonella* galactose receptor in the presence and absence of 10^{-4} M galactose. Spectra were recorded on a Perkin Elmer-Hitachi MPF-2A fluorescence spectrometer at ambient temperature with an excitation wavelength of 288 nm. Galactose binding protein (20 μg/ml) was in 10 mM sodium phosphate buffer, pH 7.0/150 mM NaCl.

buffer, and was subsequently dialyzed against this buffer for 5 days at 4° to remove carbohydrate contamination caused by passage through the Sephadex column.

The labeling ratio was determined from the protein concentration and the absorbance of the modified protein at 490 nm. A molar extinction coefficient of 42,000 for the attached fluorescein label at 490 nm was used. This value was obtained by comparison of the absorbance at 490 nm of the urea-denatured 5-acetamidofluorescein(AF)galactose receptor to the absorbance of the native AF-galactose receptor in buffer. A correction was made for the absorbance change of 5-IAF upon addition of urea. A correction for light scattering was made as described by Hartig *et al.* (19).

Steady-state fluorescence measurements were performed on a Perkin-Elmer Hitachi model MPF-2A fluorescence spectrometer at ambient temperature. Polarization measurements were made using the standard polarization accessory for the spectrophotofluorimeter, and were normalized for a constant grating transmission. The extent of energy transfer was calculated from the increase in the fluorescence excitation spectrum of the acceptor 5-AF at 280 nm.

The amino-acid compositions of the native and 5-AF-galactose receptors and their methionine sulfone derivatives were determined by Judy Benson and Marian Koshland using a Beckman model 120C amino acid analyzer. The methionine sulfone derivatives were prepared using performic acid reagent according to the method of Hirs (20).

RESULTS

Effect of Galactose on Intrinsic Fluorescence of *S. typhimurium* Galactose Receptor. In Fig. 1 is shown the fluorescence emission spectrum of the purified galactose binding protein in the presence and absence of saturating galactose. Galactose causes a 5 nm blue-shift in the tryptophan fluorescence spectrum. In contrast, no such shift is observed in the control on addition of sucrose, a sugar that shows no affinity for the protein (11). The spectrum of the tryptophan in the native protein has a maximum emission wavelength that is red-shifted relative to typical protein tryptophan emission, a finding which suggests a hydrophilic environment for this residue (21). The blue-shift that is observed upon addition of ligand usually indicates a change to a more hydrophobic microenvironment (21). A similar shift was observed by Boos in the *E. coli* protein (12).

Labeling of Receptor with a Fluorescent Reporter Group. The native galactose receptor can be reacted with 5-IAF to

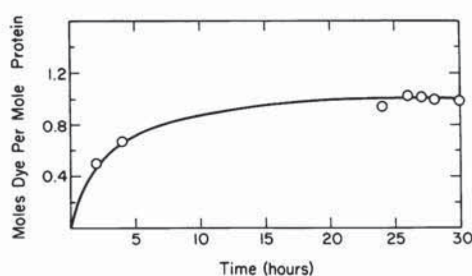

FIG. 2. Kinetics of labeling of the purified *Salmonella* galactose receptor. Galactose binding protein (0.5 mg/ml) was reacted with 5-IAF in 10 mM sodium phosphate buffer/150 mM NaCl at 22° in the dark. At specified times, an aliquot of the reaction mixture was removed and protein was separated from unreacted dye by passage over a Sephadex G-25 column equilibrated with the same buffer. The fraction containing galactose binding protein was assayed for protein by the Lowry *et al.* method (17) and for bound dye by absorbance at 490 nm using the appropriate corrections as described in *Materials and Methods*.

produce a covalently modified protein (5-AF-galactose binding protein), as shown by comigration of fluorescence at 520 nm with the single protein band during sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Modification proceeded to a fixed value, as shown in Fig. 2. At saturation, measurement of the absorbance at 490 nm of the protein in 8 M urea showed one fluorescent group per molecule of protein, as calculated from the molar extinction coefficient of the free dye in urea. Amino-acid analyses of the protein before and after modification established that a single methionine residue, and no other residues, was modified by this reagent (Table 1). Modification of one methionine was shown both by comparison of the methionine contents of galactose binding protein and 5-AF-galactose binding protein and more rigorously by comparison of the methionine sulfone content of the performate-treated proteins. The recoveries of lysine, histidine, and all other amino acids were found to be quantitative, in agreement with the absence of peaks in the positions at elution volumes identified with ϵ-carboxymethyllysine or 1-(or 3)-carboxymethylhistidine (22). Finally, a tryptic digest of the modified protein followed by paper electrophoresis in one dimension and chromatography in the second dimension showed modification of a single peptide. The reporter-modified galactose receptor retained full sugar-binding activity.

Effect of Galactose on Fluorescein Fluorescence of the 5-AF-Galactose Receptor. The fluorescence emission spectrum of the reporter group on the protein showed a 4 nm blue-shift and a 10% quenching upon binding of galactose at saturating

Table 1. Amino-acid analyses of the galactose receptor of *S. typhimurium* and its 5-AF-labeled analog

Amino acid	Galactose binding protein	5-AF-galactose binding protein
Lysine	31.7	31.1
Histidine	3.1	2.9
Aspartate	50.0	49.4
Cysteine	0	0
Methionine	5.7	4.8
Carboxymethyllysine	0	0
Carboxymethylhistidine	0	0
Methionine sulfone*	5.7*	4.6*

* Value reported is for the performate-treated protein.

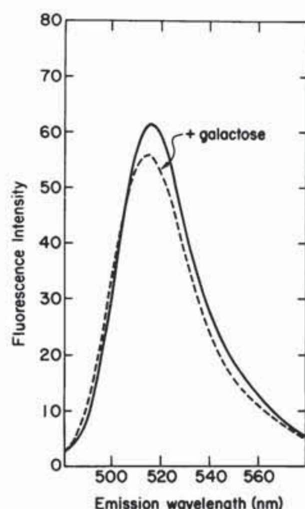

FIG. 3. Fluorescence emission spectra (uncorrected) of the 5-AF-modified galactose receptor from *Salmonella* in the presence and absence of 10^{-4} M galactose. Spectra were recorded at ambient temperature with an excitation wavelength of 460 nm. The 5-AF-galactose protein (10 μg/ml) was in 10 mM sodium phosphate buffer, pH 7.0/150 mM NaCl.

levels (Fig. 3). Changes of this type most likely indicate that the fluorescein residue is shifted to a more hydrophilic environment (19, 23).

KI Quenching. Potassium iodide (KI) quenching (24) of fluorescence emission in the galactose binding protein in the presence and absence of substrate was examined (Fig. 4). In the absence of substrate, KI quenching of the protein fluorescence is observed; a linear response of $F_0/\Delta F$ against 1/[KI] with increasing KI is observed. The presence of 10^{-4} M galactose decreases the rate of quenching by KI, a result which shows that ligand reduces access to the tryptophan residue.

In the case of the modified receptor, a linear quenching of the fluorescein fluorescence is observed and differs little from the quenching behavior of 5-IAF in solution. Addition of saturating amounts of the sugar galactose causes a small increase in the quenching rate (Fig. 4 lower). These findings suggest a very accessible 5-IAF binding site, which becomes even more accessible upon ligand binding.

Distance between the Two Reporter Groups. The distance between two fluorophores that undergo excitation transfer can be calculated from the theory of Förster (6), as demonstrated experimentally by Wu and Stryer (7). The greatest ambiguity in application of this theory is determination of the orientation factor, K^2, which appears in the Förster equation (Eq. **1**).[‡]

$$\frac{9(\ln 10)\phi_D K^2}{128\pi^5 N' n^4 \tau_D R^6} J(\bar{\nu}) = K_t \qquad [1]$$

‡ Symbols are as follows: Φ_D is the quantum yield of the donor in absence of acceptor; N' is Avagadro's number; n is the refractive index of the intervening medium; R is the donor-acceptor separation; $J(\bar{\nu})$ is the overlap integral of the normalized donor emission spectrum with the absorption spectrum; P_{om} is the polarization of a fluorophore bound to a macromolecule when the macromolecule is immobilized, but the fluorophore is free to move within the restricted environment of its binding site; P is the observed polarization; τ is the fluorescent lifetime; ρ_0 is the rotational correlation time of a sphere of equivalent volume to the macromolecule; $N_1 = \rho_1/\rho_0$ and $N_2 = \rho_2/\rho_0$ where ρ_1 is the rotational correlation time for the long axis of the ellipse and ρ_2 for each of the two short axes; P_0 is the immobilized polarization for the fluorophore; and K^2 is dipole-dipole orientation factor (6,7).

Table 2. Polarization of fluorescence values for 5-IAF free and on the modified galactose binding protein*

Theoretical or observed system	Polarization value
5-IAF in H_2O	0.017†
P_0 for 5-IAF	0.43
Theoretical maximum for 5-AF randomly immobilized on protein of molecular weight 33,000 and	
axial ratio: 1:1	0.32
2:1	0.325
4:1	0.36
5-AF-GBP‡	0.11
5-AF-GBP in 5×10^{-4} M galactose	0.12
5-AF-GBP in 10 M urea	0.06
5-AF-GBP in 10 M urea and 5×10^{-4} M galactose	0.06

* Excitation wavelength, 490 nm; emission wavelength, 520 nm.
† From ref. 19.
‡ 5-AF-galactose binding protein.

K^2 can assume any value between 0 and 4, but it is frequently assumed to be 2/3, the value for a donor and acceptor pair that is free to sample all angular orientations by rapid tumbling. The latter assumption cannot be made in the case of fluorescent groups covalently attached to proteins. For the case of fluorophores restricted in their motion, Dale and Eisinger (25, 26) have developed a modeling approach in which each fluorophore is assumed either to be immobilized or to execute rapid motion throughout the defined volume or surface area of a cone. The cone half-angle is calculated from polarization measurements for the dye, and the orientation factor, K^2, is estimated from this computed value.

Our theoretical approach follows that of Dale and Eisinger except that (*i*) from the equations of Perrin (27) and Weber (28), P_{om} has been calculated from the known macromolecular shape and steady-state polarization measurements, and (*ii*) the Weber equation for a dye attached with random orientation to a prolate ellipsoid has been substituted for that used by Dale and Eisinger for a spherical macromolecule. The resulting equation that relates P_{om} to P is given in Eq. **2**:[¶]

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \frac{\left(\frac{1}{P_{om}} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho_0 N_1}\right)\left(1 + \left[\frac{4}{N_2} - \frac{1}{N_1}\right]\frac{\tau}{\rho_0}\right)}{1 + \left(\frac{5}{N_2} + \frac{1}{N_1}\right)\frac{\tau}{2\rho_0} - \frac{9}{8}\left[\frac{\left(\frac{1}{N_2} - \frac{1}{N_1}\right)^2\left(\frac{\tau}{\rho_0}\right)^2}{\left(1 + \left[\frac{2}{N_1} + \frac{1}{N_2}\right]\frac{\tau}{\rho_0}\right)}\right]}. \qquad [2]$$

The measured polarization for 5-AF bound to the galactose receptor protein is 0.11 (Table 2). This value falls between the polarization observed for the free dye in water, 0.017 (19), and the theoretical value, 0.324, calculated for this dye randomly

¶ Deviations from the assumption of an isotropic distribution of 5-AF orientations relative to the ellipsoid axes are not critical in many cases. For example, in this case with a 2:1 axial ratio, the relaxation time calculations are very much like those for a sphere of equivalent volume. Application of a spherical model to 5-AF-labeled galactose receptor yields a conical motion half-angle of 47°, in close agreement with calculations presented herein. A more complete discussion of these models will be presented elsewhere.

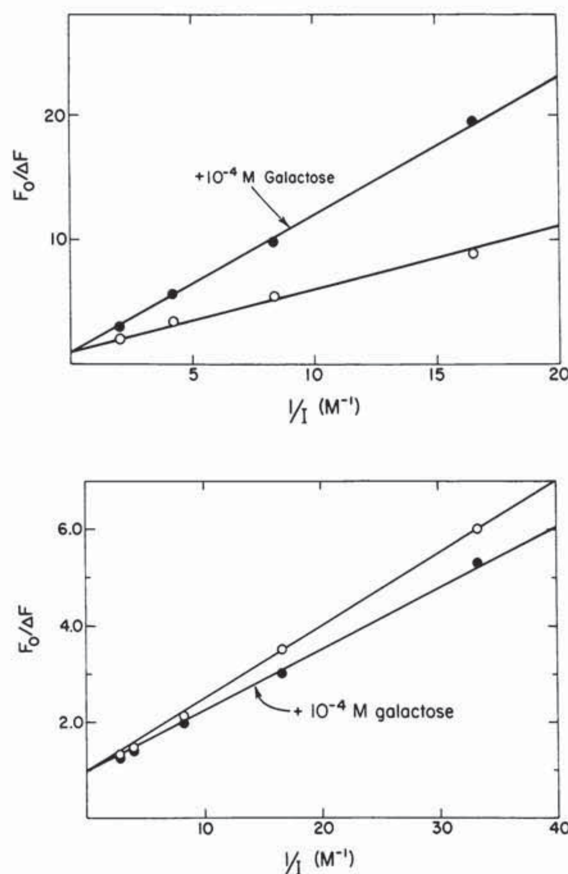

FIG. 4. Quenching of protein fluorescence emission by potassium iodide. To (*Upper*) galactose binding protein (20 μg/ml in 0.01 M sodium phosphate, pH 7.0/150 mM NaCl) or to (*Lower*) 5-AF-labeled galactose binding protein (10 μg/ml in the same buffer) in the presence (●) and absence (○) of 10^{-4} M galactose, increasing amounts of 6 M KI were added. A control cuvet received increasing amounts of 6 M NaCl. F_0, fluorescence in the NaCl-containing control cuvet; ΔF, difference in fluorescence of sample and control cuvet. $1/I^-$ was calculated using the proper dilution factor. (*Upper*) Excitation wavelength, 288 nm; emission wavelength, 340 nm. (*Lower*) Excitation wavelength, 460 nm; emission wavelength, 517 nm.

immobilized on a protein of 33,000 molecular weight with a 2:1 axial ratio for a prolate ellipsoid [the values for the galactose binding protein (29)]. Thus, the reporter group has considerable, but not complete, freedom of motion. These polarization values were then used in Eq. 2 to give a P_{om} of 0.151. The latter was applied according to the Dale and Eisinger theory (Fig. 52 of ref. 25) to give a cone half-angle of 48° for 5-AF. With this value for K^2 of the fluorescein reporter group and assuming that the tryptophan residue is immobilized, the limits of K^2 are calculated to be 0.15–2.82.

The energy transfer efficiency from the donor tryptophan to the acceptor, 5-AF, was determined to be 22% from the excitation spectrum of 5-AF bound to the protein. Using the Förster equation (Eq. 1), and assuming the usual values for other terms (7), an efficiency of 22% and a K^2 value of 2/3 give a distance of 41 Å. The calculated limits of K^2 from the above theory give a range of 32–52 Å for the distance between tryptophan and 5-AF. Since the galactose molecule is only 5 Å in length (Fig. 5) and only one is bound per molecule of protein (16), it is clear that galactose cannot interact directly with both groups.

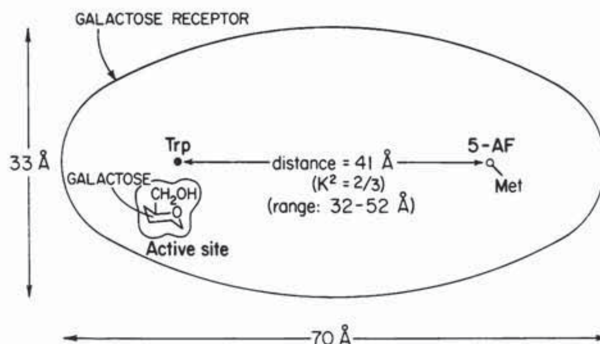

FIG. 5. Schematic representation of the *Salmonella* galactose receptor showing the dimensions of the protein, based on an axial ratio of 2:1, and the distance from the single tryptophan to the 5-AF labeling site as calculated from fluorescence energy transfer measurements. The model includes the single galactose binding site and nearby tryptophan by analogy to the antigenically related *E. coli* arabinose receptor (30).

DISCUSSION

By fluorescence energy transfer measurements we have determined that the single tryptophan residue and the single acetamidofluorescein moiety of the 5-IAF-modified *Salmonella* galactose receptor are 41 ± 10 Å apart. Since (*a*) the galactose molecule is only 5 Å in length, (*b*) only one molecule of ligand binds per molecule of receptor, and (*c*) its binding perturbs the microenvironments of both the tryptophan and fluorescein fluorophores, it is clear that the changes introduced at a minimum of one of these sites are the result of an indirect effect. Hence, there must be a ligand-induced conformational change that is propagated a minimum of 30 Å through the prolate receptor molecule.

This "distant reporter group method" requires the placing of an environmentally sensitive group at a position known to be too distant to allow direct interactions with the ligand. In this application one intrinsic fluorophore, the tryptophan residue, and one dye added to a particular amino acid residue were utilized. However, two added reporter groups or a fluorescent ligand plus a fluorescent reporter group could serve equally well. For example, many enzymatic reactions involve substrates or allosteric effectors that are fluorescent or that have analogs that compete for the binding site that are fluorescent. Establishing that the reporter group is distant from the binding site by energy transfer between the reversibly bound ligand and the reporter group would be sufficient. The donor-acceptor pair must be chosen to allow the fluorescence transfer measurements and the distant group must show microenvironment changes on ligand binding. If two reporter groups are introduced, it will usually be convenient to use different covalent linkages, e.g., one alkylating and one acylating, to attach the dyes to different amino acid residues. The use of fluorescent reporter groups was a particularly attractive feature of the method since the great sensitivity of fluorescence facilitates subsequent dynamic studies of such labeled receptors. However, any method that can establish distances unequivocally would satisfy the procedure.

This approach is less demanding than mapping positions on protein surfaces by fluorescent energy transfer measurements. In the present application only a minimum distance need be established. If that minimum (in this case, 30 Å) is far greater than the size of the ligand (in this case, 5 Å), the chance for an erroneous conclusion is very remote. As the size of the ligand increases and the distance between reporter groups decreases the chance for error will increase, but this problem can be

controlled by the investigator since there are many different reporter groups that can be attached to proteins.

The above derivation makes a number of assumptions (more detailed calculations and theory will be published elsewhere), but all are based on widely observed experimental results or affect the final numbers in minor ways. For example, it is assumed that the tryptophan is immobile, a fact supported by the confined space of the tryptophans so far studied in protein structures and their line widths in nuclear magnetic resonance studies, e.g., the histidine receptor study of Robertson *et al.* (31). The 5-AF is assumed to execute rapid motion in the cone of its oscillation, but deviations from isotropic motion do not invalidate the method since the calculation of the cone half-angle and the K^2 values are affected in the same way by deviations from isotropic conical motion. Similarly, the calculated distance is not very sensitive to the shape of the protein. Calculated ranges of K^2 vary from 0.16 to 2.73 for a 4:1 prolate ellipsoid and from 0.15 to 2.82 for a sphere. A mobile tryptophan would in fact diminish the range error.

Several pieces of data independently corroborate the finding of a ligand-induced conformational change in the labeled galactose receptor. First, modification of a methionine with the large, bulky fluorescein moiety results in no detectable loss of biological activity. If sugar binding involved direct interaction with this amino acid, a substantial loss of activity would probably be associated with its chemical modification. Second, the finding that the fluorescent group becomes more accessible to potassium iodide upon ligand binding demonstrates a conformational change, since a direct interaction would be expected to bury this residue. Third, the distance between the groups is consistent with the dimensions of the protein molecule. A prolate ellipsoidal protein of molecular weight 33,000 and axial ratio 2:1 has dimensions of 33 Å × 33 Å × 70 Å as a prolate ellipsoid. Dimensions of *68* Å × *38* Å × *30* Å are observed from crystallographic data of the similar arabinose binding protein (30, 32). Hence, a distance of 41 ± 10 Å for the two reporter groups is in agreement with the independent limitations of protein size. Finally, the previous work of Boos and coworkers, in which various spectroscopic tools were used to demonstrate conformational changes in the *E. coli* galactose receptor, was subject to the ambiguities described above, but, nevertheless, is entirely consistent with an induced conformational change. Such a conformational change is, moreover, supported by the finding that only one molecule of galactose binds per molecule of receptor (16).

It is generally assumed that effectors induce conformational changes in the receptors to which they bind. The evidence for such a mechanism is usually circumstantial, however, both because of the difficulty of isolating receptors and the ambiguity of the experimental tests. The finding that the purified *Salmonella* galactose receptor must undergo a conformational change on binding of its ligand is, therefore, although not surprising, important support for a widespread belief. It is of particular interest in this case because previous studies of this sensory system had led to the hypothesis that a conformational change must be induced in the free receptor prior to its association with the first component of the signaling system. The demonstration that the isolated purified protein does undergo such a conformational change, therefore, adds further support for such an association mechanism for receptor action.

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Qüestions proposades de l'article de Zukin *et al., Proc. Natl. Acad. Sci. USA* 74, 1932-1936 (1977).

1.- Resumiu els continguts de l'article indicant els següents aspectes:

a) Objectius que es persegueixen

b) Esquema del procediment experimental seguit

c) Resultats més rellevants aconseguits

d) Interpretació dels autors del resultats

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2.- L'article descriu la introducció d'un fluoròfor extrínsec (5-acetamidofluoresceïna) en la proteïna estudiada (el receptor de galactosa).

a) Cóm s'explica que el fluoròfor s'introdueixca en una sola de les metionines que conté la proteïna? Cóm es comprova açò?

b) Cóm es fa el càlcul del nombre mitjana de fluoròfors units per molècula de proteïna? Tenint amb compte que aquest fluoròfor es va a utilitzar posteriorment en un procés de transferència d'energia per ressonància induïda, quina importància té que el nombre de fluoròfors units siga el màxim possible (un per molècula)?

3.- Quins grups actuen com a donadors i acceptors en la transferència d'energia? Quin dels dos es troba més prop del lloc d'unió de la galactosa? Perquè?

4.- Els autors interpreten que un desplaçament de l'espectre d'emissió cap al blau (longituds d'ona més baixes), en presència de galactosa, pot ser degut al pas d'un fluoròfor cap a un entorn més hidrofòbic (cas del Trp en la Fig. 1), o més hidrofílic (cas de la 5-acetamidofluoresceïna en la Fig. 3). Cóm es pot explicar aquesta diferència de comportament?

5.- Per a què serveixen les experiències d'extinció amb iodur potàssic? Quina informació proporcionen?

6.- El receptor de la galactosa uneix solament una molècula de galactosa per molècula de proteïna. Quina importància té aquest fet en la interpretació dels resultats? Què ocorreria si hi haguera més d'un lloc d'unió per molècula de receptor?

7.- Els autors proposen el procediment experimental descrit en aquest article com a un mètode general per a la detecció de canvis de conformació en proteïnes. Quines podrien ser els principals avantatges i limitacions d'aquesta tècnica?

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Biochemistry

Energy metabolism of the untrained muscle of elite runners as observed by ^{31}P magnetic resonance spectroscopy: Evidence suggesting a genetic endowment for endurance exercise

(exercise physiology/muscle metabolism/NMR)

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Contributed by Charles R. Park, June 29, 1988

ABSTRACT The purpose of this study was to investigate whether genetically determined properties of muscle metabolism contribute to the exceptional physical endurance of world-class distance runners. ATP, phosphocreatine, inorganic phosphate, and pH were quantitatively determined by ^{31}P nuclear magnetic resonance spectroscopy in the wrist flexor muscles of elite long-distance runners and sedentary control subjects. These muscles had not been exposed to any specific program of exercise training in either group of subjects. The "untrained" muscles were examined at rest, during two cycles of three grades of exercise, and in recovery. The flexor muscles of the athletes had higher concentrations of phosphocreatine and ATP than did those of the control subjects at rest and during exercise. The athletes' muscles possessed a higher capacity for generation of ATP by oxidative metabolism than did control subjects' muscles according to the following criteria: (*i*) high force output, 60% of maximum voluntary contraction, was more easily reached and better maintained in both exercise cycles; (*ii*) the ratio of inorganic phosphate to phosphocreatine rose less during exercise and recovered faster in the postexercise period; (*iii*) there was no loss of adenine nucleotides or total phosphate from the athletes' muscles but significant losses from the control subjects' muscles; and (*iv*) the pH decreased no more than 0.1 unit in the athletes' muscles during exercise, attesting to a relatively slow glycolysis and/or a rapid oxidation of lactate. In the muscles of the control subjects, on the other hand, the pH decreased nearly 0.4 unit early in the first exercise cycle, indicating a relatively fast glycolysis and/or slower oxidation of lactate. In the second exercise cycle, the pH returned to near normal in the control subjects' muscles, reflecting diminished lactate formation because of glycogen depletion and lactate washout by the high blood flow induced by exercise. By the end of the exercise program, the maximum voluntary contractile force for the control subjects had declined to <60% of the initial value. This decline could be explained best by exhaustion of the glycolytic contribution to muscle contraction. Therefore, the residual maximum strength provided a measure of the oxidative capacity to support contraction, as is discussed. In conclusion, we suggest that a greater oxidative capacity relative to glycolytic capacity for support of contraction in untrained muscle of world-class runners reflects a genetic endowment for physical endurance. Additional systemic effects of training cannot be completely excluded. ^{31}P magnetic resonance spectroscopy provides a noninvasive method for assessing this endowment.

This study compares the metabolic characteristics of untrained muscles of elite long-distance runners with those of nonathletic control subjects. The wrist flexor muscles of the forearm were chosen for study because they were "untrained"; that is, no specific exercise program involving these muscles was undertaken. The muscles were examined by ^{31}P magnetic resonance spectroscopy (*in vivo* NMR) at rest, during two cycles of graded exercise, and in recovery. It seemed possible that constitutional (genetic) differences in metabolism between the athletes and the controls as distinct from effects of training might be revealed in these untrained muscles. These differences could help to explain why world-class runners can reach levels of physical endurance unattainable by all but a very few persons regardless of the training program.

There are no earlier measurements of metabolites in untrained muscle of athletes to our knowledge. However, Jansson and Kaijser (1) found an unexpectedly high percentage of oxidative [type I (slow twitch)] fibers in the relatively untrained deltoid muscle of elite orienteers¶ which led them to suggest that this could be a constitutional rather than a training attribute of endurance athletes. Most prior studies of metabolites in exercising muscle required repeated biopsies (for review, see ref. 2). The variety of metabolites that can be determined in biopsied material by chemical analysis is greater than that by ^{31}P magnetic resonance spectroscopy. However, the latter procedure is noninvasive, allows an unlimited number of *in vivo* measurements during prolonged exercise (3–5), and can be used to estimate ATP and phosphocreatine (*P*Cr) and to determine the physiologically relevant concentrations of P_i and free ADP more reliably than by chemical analyses. Magnetic resonance spectroscopy has the further advantage that a relatively large and therefore more representative mass of tissue is examined. The technique is also uniquely capable of measuring tissue pH noninvasively, a particularly valuable parameter for this investigation.

Some of the methodology in this work was developed in our earlier studies in which steady-state exercise was quantified and the recruitment of oxidative fibers [mostly types I and IIA (intermediate fast twitch)] was differentiated from recruitment of glycolytic fibers [type IIB (fast twitch)] by ^{31}P magnetic resonance spectroscopy during exercise in certain cases (4, 6). An approach to quantitating by this technique the relative maximum oxidative and glycolytic capacities to support muscle contraction is proposed here.

METHODS

Subjects. The athletes were screened by an expert track coach (R.L.B.). They were world-class long-distance runners, three males and one female, ages 26, 28, 28, and 35

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Abbreviations: MVC, maximum voluntary contraction; *P*Cr, phosphocreatine.
†To whom reprint requests should be addressed.
¶Orienteering is a sport that demands a high level of endurance running.

years. One was a recent world champion in the 1500-m race; another was an Olympic alternate for the United States in the marathon, and the others were close to these individuals in performance. They were all in training programs consisting essentially of running from 55 to 100 miles per week (1 mile = 1.609 km).

The control subjects were 4 men and 1 woman, age range 20–23 years. All were sedentary but healthy and nonobese. Only one subject engaged in any routine exercise. This consisted of walking 2 miles per day and playing 1.5 hr of tennis per week.

Instrumentation. The forearm of the seated subject was held by Velcro straps on a platform in the 27.6-cm bore of a 1.9-Tesla superconducting magnet. Spectra were acquired with a Fourier-transform magnetic resonance spectrometer [TMR 32 (Oxford Research Systems, Oxford) or PE 280 (Phosphoenergetics, Philadelphia)]. The wrist flexor muscles of the upper forearm were held firmly by Velcro straps against an inductively driven coil, 4 cm in diameter (7, 8), in the homogeneous region of the magnet. The subject depressed an exercise bar by flexion of his wrist, and the force was recorded by a Cybex ergometer (Lumex, Ronkonkoma, NY) (3, 7). For all subjects, data from both dominant and nondominant arms have been averaged in the presentation of results.

Exercise Protocol. To determine the initial maximum voluntary contractile force (MVC), the subject exerted his maximum effort to depress the exercise bar. This effort was repeated three times at 1-min intervals, and the highest force was noted. After a rest period of 5–8 min, the subject began the first cycle of exercise (1–18 min). He depressed the bar by flexing his wrist once every 5 s for a 6-min interval, adjusting the force to 20% of his initial MVC. Without intervening rest, the subject continued for two further 6-min intervals, increasing his force output to 40% and then 60% of initial MVC, respectively. Exceptions will be noted. Without interruption, a second 18-min cycle (19–36 min) of 20%, 40%, and 60% MVC was executed, giving a total exercise time of 36 min. The subject then rested for a recovery period of 3 min.

Magnetic Resonance Spectroscopy Measurements. At the outset of each exercise trial, the magnetic field was adjusted for maximal proton resolution (≈0.4 ppm). The proton spectrum obtained at 80.3 MHz was used to calculate the percent lipid content from the total water and lipid protons in the tissue examined. Spectra of ^{31}P metabolites were acquired at 32.5 MHz, with a pulse of 45 μs and a repetition time of 5 s. The pulse was timed to occur 4 s after each contraction. First, a spectrum was acquired during 3 min with the muscles at rest. Subsequent spectra were accumulated during each minute of exercise, but only the summation of the last 3 min of each 6-min interval has been plotted in the figures. The spectra accumulated during the last 2 min of the post-exercise recovery period were normalized to 3 min. The resonance areas of P_i, *P*Cr, and ATP were determined by a nonlinear least-squares curve-fitting program and/or by triangulation (4, 6). The proportionality factor to convert all resonance areas to concentrations of P-containing metabolites for all subjects was derived by assuming that the area of the β-P peak of ATP for the control subjects at rest was 5.5 mmol of ATP per kg (wet weight) of muscle, as determined in biopsy samples from comparable subjects by Harris *et al.* (9). Concentrations of P_i and *P*Cr were corrected by factors of 1.18 and 1.29, respectively, to account for saturation effects. The chemical shift of P_i, with *P*Cr set at zero, was used to estimate pH (5). An example of the spectra obtained at rest and during the first exercise cycle (1–18 min) is shown in Fig. 1.

Statistical comparisons of the runners and control subjects at a specified time interval were performed by using the two-tailed Student *t* test.

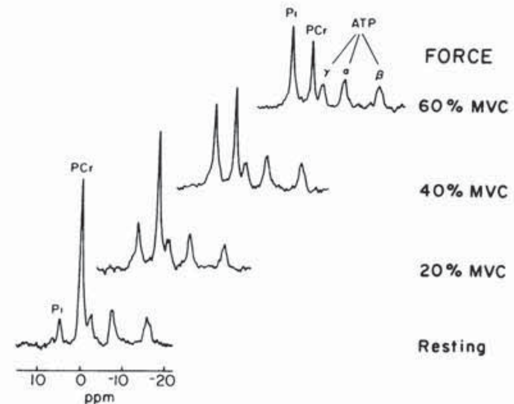

FIG. 1. ^{31}P magnetic resonance spectra and force measurements (% MVC) of wrist flexor muscles of a championship long-distance runner at rest and during contraction at three levels of exercise during the first cycle of the protocol (1–18 min). Ergometer measurements (% MVC) were obtained as described. Spectra were acquired during the last 3 min of each 6-min interval and plotted with 10-Hz line broadening. The times of force measurements and spectral data collection were: 20% MVC, 4–6 min; 40% MVC, 10–12 min; and 60% MVC, 16–18 min.

RESULTS

Contractile Force Exerted During Exercise. The elite runners could maintain without difficulty the highest level of contractile force (60% of the initial MVC) in the final interval of both the first and second cycles of the exercise protocol (13–18 min and 31–36 min, respectively). The control subjects, on the other hand, maintained the level of 60% MVC with some difficulty in the first cycle (13–18 min) as shown by the relatively large standard error (±3.1%) of the force measurement as compared with that (±0.9%) for runners. In the second cycle, half of the trials by control subjects did not reach the 60% MVC level (31–36 min), presumably because of exhaustion of the glycolytic component of muscle contraction (average MVC, 51% ± 4.4%). The performance of one subject who could not achieve 60% MVC is plotted in Fig. 2. After the sharp decline of force output from 55% MVC, a new steady state appeared to be established at 38% MVC.

Concentrations of P Metabolites During the Exercise Protocol. The concentration of *P*Cr in the resting untrained muscles of the athletes was 25% higher than that in the muscles of control subjects (Fig. 3 *Upper*), and it remained higher throughout the protocol ($P = <0.02$ to <0.01). These

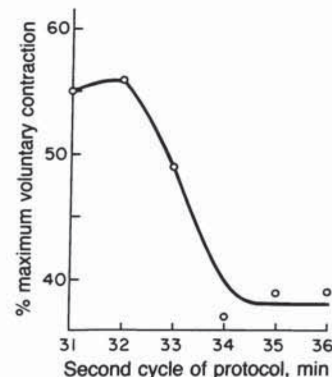

FIG. 2. Decline of contractile force in the wrist flexor muscles of a control subject unable to achieve 60% MVC during the last 6-min interval (31–36 min) of the exercise protocol.

differences were not explained by a difference in lipid content that was 11 ± 5% in the forearm of the athletes and 14 ± 5% in the control subjects. *P*Cr levels decreased about 60% in the first exercise cycle (1–18 min) in both groups. During the first interval of the second cycle (19–24 min), when the force output was decreased from 60% to 20% MVC, the *P*Cr level increased twice as rapidly in the athletes as in the control subjects. Similarly, in the postexercise recovery period, *P*Cr was regenerated 1.5 times faster in the athletes, indicative of a higher oxidative metabolism in their muscles.

The average P_i concentration in resting flexor muscles of the runners was significantly higher than in the control subjects ($P = <0.02$). However, concentrations of P_i during the first cycle of exercise (1–18 min) were the same in both groups of subjects (Fig. 3 *Lower*). In the second exercise cycle (19–36 min), the increase in P_i in the athletes duplicated that seen in the first cycle—evidence of the remarkable uniformity of metabolism that characterized this group. For the control subjects, however, the increase of P_i in their second exercise cycle was significantly less than in the first cycle, indicating a change in metabolism as exercise proceeded. This lower concentration of P_i at the end of the second cycle (31–36 min) correlated with a lower level of total P (see Fig. 5) and a lower percent MVC (53%) in the control subjects (Fig. 3). In the postexercise recovery period, P_i decreased rapidly to the resting level in the athletes' muscles, but remained slightly elevated in the control subjects' tissues.

A lower P_i/*P*Cr ratio was seen in the athletes than in control subjects at all levels of exertion except the last 60% MVC (Fig. 4). The lower ratio was indicative of a greater capacity of the athletes to maintain energy-rich phosphate. For the runners, the changes in the P_i/*P*Cr ratio with time were identical in the first (1–18 min) and second (19–36 min) exercise cycles, a further evidence of their uniform metabolism. At their highest MVC (62%), the P_i/*P*Cr ratio was 1.5. In the case of the control subjects, the P_i/*P*Cr ratio was very high (2.3) at the end of the first cycle (16–18 min). It rose less, however, during the second cycle (19–36 min) possibly because of P loss (Fig. 5) and a decline in the force output to 51% MVC.

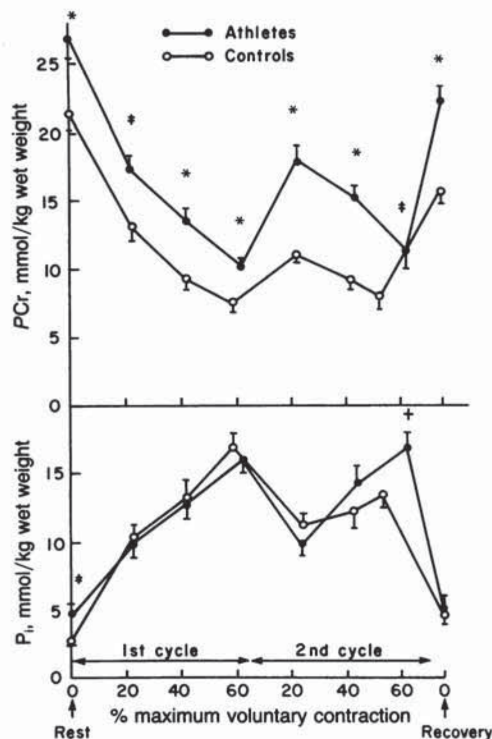

FIG. 3. Mean concentrations of *P*Cr and P_i in the wrist flexor muscles of elite long-distance runners and control subjects at different levels of contractile work during the exercise protocol. *P*Cr and P_i concentrations were determined from the resonance areas as described. The spectral data and force measurements (% MVC) in the figure represent the average values for the last 3 min of each 6-min interval during two exercise cycles. Significant differences between the two groups of subjects are indicated as follows: †, $P = <0.05$; ‡, $P = <0.02$; *, $P = <0.01$. Vertical bars = 1 SEM.

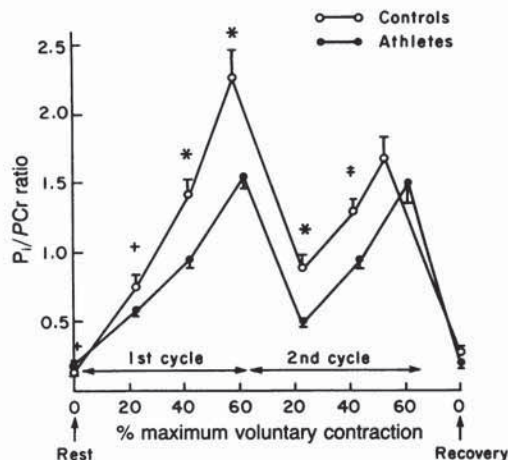

FIG. 4. P_i/*P*Cr ratios in the wrist flexor muscles of athletes and control subjects at different levels of contractile work during the exercise program. The P_i/*P*Cr ratios were calculated from the values in Fig. 3. Significant differences between the two groups of subjects are indicated as follows: †, $P = <0.05$; ‡, $P = <0.02$; *, $P = <0.01$. Vertical bars = 1 SEM.

Resting levels of adenine nucleotide phosphate (sum of α-, β-, and γ-P peaks) and ATP alone (β-P peak) in the untrained flexor muscles of the athletes were 20% higher than in control subjects ($P = <0.02$) (Fig. 5 *Middle* and *Bottom*). In the athletes' first exercise interval (1–6 min), the total adenine nucleotides decreased ≈20% in concentration. The nucleotide levels then stabilized and tended to increase in the second cycle (19–36 min). Likewise, the ATP level in the runners decreased significantly with the onset of exercise ($P = <0.02$) but had regained the initial value by the postexercise recovery period. By contrast, in the controls the adenine nucleotides drifted downward with continued exercise, leading to significant differences in concentrations between athletes and controls during the second exercise cycle and postexercise recovery ($P = <0.02$ to <0.01). For the control subjects, the overall losses of adenine nucleotide P and ATP during exercise were significant ($P = <0.02$).

The sum of phosphate in adenine nucleotides, *P*Cr, and P_i, termed "total P," for athletes and control subjects is shown at the top of Fig. 5. The initial level of total P was 25% higher in the resting muscle of athletes ($P = <0.01$). The value for the runners decreased 15% with the onset of exercise (1–6 min) and thereafter essentially stabilized. In the control subjects, there was an initial decrease in total P of about 8%. A further significant decrease occurred during the second exercise cycle (19–36 min) and the postexercise recovery period ($P = <0.01$). During that time, the concentrations of these metabolites became significantly different between the athlete and control groups ($P = <0.01$).

pH Changes During the Exercise Protocol. The initial pH at rest was 7.04 ± 0.01 in the flexor muscles of the athletes, and it decreased no more than 0.1 pH unit at any time during exercise (Fig. 6). In the controls, the initial pH was 7.03 ± 0.02, but it decreased 0.35 ± 0.07 unit during the first exercise

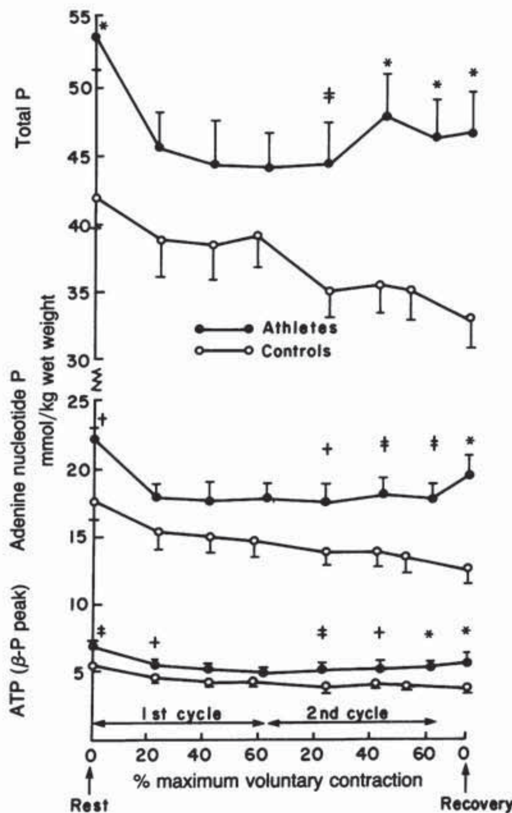

FIG. 5. Concentrations of adenine nucleotide P, ATP, and total P in the wrist flexor muscles of long-distance runners and control subjects at different levels of contractile force during two exercise cycles. Concentrations of total P, adenine nucleotide P (sum of α-, β-, and γ-P peaks, which include ATP, ADP, and NAD), and ATP alone were calculated as described. The data are plotted as in Fig. 3. †, $P = <0.05$; ‡, $P = <0.02$; *, $P = <0.01$. Vertical bars = 1 SEM.

cycle ($P = <0.001$) (Fig. 6). It then increased sharply by about 0.2 unit to pH 6.87 at the start of the second cycle at 20% MVC (19–24 min) and continued to increase thereafter despite the rise in work output to 40% and 51% MVC (25–36 min). These pH changes in the control group suggested a marked dependence on glycolytic metabolism during the first

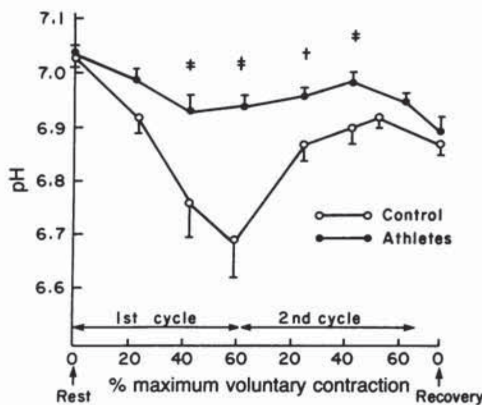

FIG. 6. The pH of the wrist flexor muscles of the runners and control subjects at different levels of contractile force during the exercise program. The pH was determined by the chemical shift of P_i as described. †, $P = <0.05$; ‡, $P = <0.02$; *, $P = <0.01$. Vertical bars = 1 SEM.

cycle of exercise and on oxidative metabolism in the second cycle. In the postexercise recovery period, the pH decreased 0.05 unit in both groups of subjects, consistent with a rapid *P*Cr resynthesis.

DISCUSSION

Concentrations of P Metabolites in Muscles at Rest. The higher concentrations of *P*Cr, adenine nucleotides, and P_i (25%, 22%, and 54%, respectively) in the resting forearms of the athletes as compared with those of the control subjects (Figs. 3 and 5) reflected greater concentrations in the muscle tissue itself since the correction for the greater adipose content of the control tissue was only about 3%. The content of these metabolites in adipose tissue is relatively low. Biopsy determinations in trained muscles of athletes have shown higher (10) or no (11) differences in ATP and *P*Cr concentrations compared to controls, but no measurements have been reported for untrained muscle in athletes. The higher concentrations of ATP and *P*Cr would contribute little to endurance performance because they do not constitute quantitatively significant energy reservoirs. However, higher concentrations may manifest a greater potential for synthesis of high-energy phosphate (3, 4).

Alterations in ATP and Adenine Nucleotide P Levels During Exercise. The initial decreases in the total adenine nucleotide P and ATP concentrations (Fig. 5), which occurred with the onset of exercise at 20% MVC (1–6 min), did not appear to be due to loss of magnetic resonance spectroscopic visibility since they have been noted earlier in biopsy studies of exercise (10, 12). There are several possible explanations for this altered "initial state" biochemistry. The onset of exercise establishes a major, continuing drain on ATP, which induces a lower steady-state concentration of the nucleotide despite ATP resynthesis via creatine kinase, mitochondrial phosphorylation, and/or glycolysis. Furthermore, there may be a lag between the drop of ATP in the myofibrils and activation of mitochondrial ATP synthesis via the creatine shuttle (13); the "Ca^{2+} burst," postulated to occur at the initiation of exercise (14), could preempt ATP for restoration of Ca^{2+} to the threshold concentration. Finally, the microcirculation may not have increased fast enough to keep pace with the O_2 requirement in the mitochondria.

After the initial decrease in ATP concentration, the stabilization of adenine nucleotides in the untrained muscles of athletes, even at high levels of exercise (7–36 min), is evidence for superior oxidative metabolism. The progressive decrease in nucleotide concentrations in the muscles of the control subjects involved equivalent losses in the α-, β-, and γ-P components, indicating that ATP was degraded beyond ADP. These losses could be best explained by a slower oxidative regeneration of ATP, thereby causing accumulation of AMP, which would promote its degradation to adenosine and inosine. The latter compounds pass out readily through the sarcolemma (15). Calculated values for ADP concentrations (3) at 20%, 40%, and 60% MVC in the first exercise cycle were 37, 51, and 82 μM, respectively, in the control subjects' muscles as compared with 20, 38, and 59 μM in the runners' muscles. The lower concentrations are consistent with a larger V_{max} for mitochondrial oxidative metabolism in the runners, assuming $K_{m(ADP)}$ to be 20 μM in all cases (16).

Alterations in Total P and P_i During Exercise. It appeared that total P was lost from the muscle in the first 6 min of exercise (Fig. 5) because there were no compensatory increases in phosphate mono- or diesters as observed by ^{31}P magnetic resonance spectroscopy (data not shown). The loss may have been due in part to the efflux of adenosine and inosine already noted and associated P_i. The continued loss in the control subjects during the second exercise cycle and postexercise recovery period presumably resulted from rel-

atively inadequate ATP resynthesis and accelerated ADP degradation. It was observed (Fig. 3) that P_i was the only parameter that did not differ in value between the two groups during the first 30 min of the protocol. This suggests that there may be physiological constraints on P_i concentration which, in turn, may be related to the partial metabolic control of contraction by P_i (17). However, in the last 6 min of the protocol (30–36 min), the P_i increased significantly less in the control group as compared with the runners, possibly because of excessive loss of P_i and the beginning of the failure of force output by control subjects.

pH Changes During Exercise. The pattern of pH change provided the clearest indication of the greater oxidative capacity of the runners' muscles (Fig. 6). The relatively small pH change in the runners' forearms is consistent with a major participation of oxidative fibers as compared with glycolytic fibers at all levels of exercise. The marked decrease in pH seen with the control subjects in the first cycle of exercise (1–18 min) was doubtless due to greater glycolytic activity, leading to lactate accumulation, and to relatively less oxidative capacity. There is much evidence (1, 18–20) to indicate that glycolysis is activated as oxidative metabolism fails to meet energy needs. In the second cycle of exercise (19–36 min), the return of the pH to near the resting value in the control muscles is best explained by a sharp decrease in glycolysis as a consequence of glycogen depletion (1, 19, 20). The previously accumulated lactate was metabolized to some extent and, more importantly, was washed out by the increase in blood flow engendered by exercise. With little residual glycolysis and substantial *P*Cr and ATP depletion in the fast-twitch fibers (4, 20), oxidative metabolism now provided virtually all of the energy for contraction. It was insufficient in several control subjects to achieve the 60% MVC level. In the runners, however, 60% MVC was not sufficiently high to activate glycolysis, at least to the point of lowering the pH.

The Oxidative V_{max}. Chance *et al.* (3) developed a simple procedure to quantify the fraction of maximal oxidative metabolism (V/V_{max}) that is used at any time during work that employs only oxidative fibers. This condition occurs with exercise at very low work loads, a condition that was most nearly approximated here at the 20% MVC level. Calculations of V/V_{max} based upon the ADP values as calculated above gave values of 50% and 65% for runners and control subjects, respectively (3, 7). Consequently, at a work load of 20% MVC, the runners were able to work at a 15% lower V/V_{max} than the nonrunners. The superior oxidative capacity of the runners compared to nonrunners is thus quantified.

The results in this paper suggest an experimental method for determining the oxidative V_{max} and the glycolytic V_{max} by measurements made at the highest work loads. The oxidative V_{max} can be evaluated in those cases in which the glycolytic capacity has been exhausted and only oxidative capacity remains effective as was suggested for a single subject as shown in Fig. 2. In the control group as a whole, the 60% of initial MVC in the second exercise cycle could not be reached in 4 of 10 trials. The average value reached with maximal exertion (41% MVC) by this subgroup was now a measure of their average oxidative V_{max}. Based on those observations, a more generally applicable protocol could be designed that first would exhaust the glycolytic capacity by a progressive increase in the contractile force to a high level. Immediately thereafter, the force output should be reduced to a level low enough to allow the pH to return to normal but high enough to prevent resynthesis of glycogen. At that point, a MVC measurement would indicate the oxidative V_{max}. The difference between the initial MVC and this oxidative V_{max} would yield the glycolytic V_{max}.

Genetic Endowment for Endurance Exercise. There are two major differences between this investigation and earlier studies of muscle metabolism in athletes. First, the tissue was examined by ^{31}P magnetic resonance spectroscopy rather than by biopsy. Some advantages of magnetic resonance spectroscopy were noted in the introduction, and this study demonstrates particularly its value for P_i and pH determinations. Second, the muscles studied were not those involved in athletic performance and training. The flexor muscles of the wrist remain flaccid during running,[‖] and the subjects engaged in no program that would train them. We have postulated that examination of metabolism during exercise of untrained muscles could reveal some of the constitutional properties that underlie the high performance of elite athletes. The possibility that athletic training could affect untrained muscle cannot be rigorously excluded. It is well known that the endurance athlete increases his cardiac output by training, but the exercise used in our study would put only a very small demand on the circulatory capacity even of the sedentary subjects. The pulse rate, for example, rose only about six to eight points. We are unaware of any evidence that humoral or neuromuscular factors are generated in exercising muscle that increase oxidative capacity in resting muscle. Therefore, evidence from the present study suggests that a superior oxidative capacity for energy production in muscle is an important genetic attribute. Multiple genes determining muscle fiber composition, enzyme makeup, and neuromuscular elements may contribute to increase this capacity.

‖This statement is based on observations by one of the authors, R.L.B., who has many years of experience as a professional coach of elite runners.

These data were presented in part at the meeting of the Society of Magnetic Resonance in Medicine, New York (1987). This work was supported by grants from the National Institutes of Health (HL 31934 and RR02305) and the James S. McDonnell Foundation.

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Qüestions proposades de l'article de Park *et al.*, *Proc. Natl. Acad. Sci. USA* 85, 8780-8784 (1988).

1.- Resumiu els objectius, metodologia i conclusions de l'estudi d'aquest article.

2.- Quins són els canvis principals que experimenta l'espectre de ^{31}P-RMN del múscul durant l'exercici? Cóm es pot mesurar específicament l'ATP evitant la interferència de l'ADP?

3.- Calculeu els camps magnètics utilitzats pels autors per a obtenir els espectres de ^{31}P-RMN y de ^{1}H-RMN. Per a què s'utilitza el ^{1}H-RMN?

4.- Cóm es mesura la variació de pH que ocorre *in vivo* en el múscul? Cóm s'expliquen les variacions de pH durant l'exercici muscular?

5.- Quines són les principals diferencies en les taxes de metabòlits fosforilats i el pH entre els atletes i els individus utilitzats com a controls?

6.- Com a conclusió els autors proposen que els atletes d'elit posseeixen una predisposició genètica que afavoreix un alt rendiment en l'exercici muscular. Està justificada aquesta afirmació en base als experiments realitzats? Quines crítiques y explicacions alternatives es podrien proposar?

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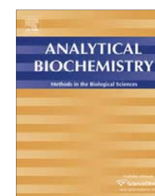

An easy assay for histone acetyltransferase activity using a PhosphorImager

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ABSTRACT

A simple radiometric assay for histone acetyltransferase (HAT) activity employing a PhosphorImager is described. In the proposed procedure, following incubation of [1-^{14}C]acetyl coenzyme A (CoA), histones, and HAT enzyme, radiolabeled histones are fixed on GF/F glass microfiber filter while the excess of acetyl CoA is washed out. Afterward, the filter is exposed to a phosphor-screen and the resulting spot signals are quantified with a PhosphorImager. Given the small volumes required, the new assay reduces reagent consumption and contaminated waste. Moreover, the assay can be performed with a large number of samples simultaneously, is applicable on different protein substrates, and is adaptable to the analysis of other protein modifications.

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Histone acetylation, as well as other posttranslational modifications, is involved in gene expression regulation and other essential processes in the life of eukaryotic organisms [1]. Histone acetyltransferases (HATs)[3] catalyze the transfer of acetate from the substrate acetyl coenzyme A (CoA) to histones and possibly other protein substrates. Because of the increasing evidence revealing that HAT enzymes are important players in human pathologies [2], consistent with their essentiality in diverse cellular functions, the determination of the properties and functions of these enzymes continues to be an area of intense investigation. HAT activity can be detected and quantified by different assays [3]. The most sensitive methods employ radioactive, ^{3}H- or ^{14}C-labeled, substrate acetyl CoA. In classical assays, the radiolabeled products are captured by electrostatic binding on phosphocellulose filters [4] or by precipitation on glass microfiber filters [5] and are quantified by liquid scintillation counting. Another radioactive approach makes use of a biotinylated synthetic peptide corresponding to the N terminus of histone H4. The radiolabeled acetylated peptide is captured onto streptavidin-coated beads and quantified [6]. In another adaptation, which is suitable for high-throughput screening, radiolabeled histones are bound to the scintillant surface into the wells of a microplate (FlashPlate) producing a scintillation signal [7]. Nonradioactive assays have also been described. One of them makes use of the reduction of NAD^{+} to NADH, catalyzed by a dehydrogenase-coupled system, to monitor acetylation reaction [8]. Another employs a fluorescent dye to detect the CoA produced in the HAT reaction [9]. Finally, a recent and novel fluorescence strategy detects acetyltransferase activity by using histone H4 N-terminal synthetic peptides bearing an attached fluorophore; changes in the fluorescence signal, caused by the environmental alteration due to the acetylation, report on HAT activity [10]. Although these different HAT assay procedures have their own particular drawbacks, all of them are adequate for the accurate and reliable determination of HAT activity on wide-ranging biological preparations in vitro. However, these methods either are relatively laborious or require specific materials, and so they are not, in general, easily applicable in nonspecialized laboratories. In this article, we describe the use of a PhosphorImager for rapid and easy determination of HAT activity. The new assay, as a radioactivity-based technique, yields high sensitivity while the amount of radioactive compound and other reagents consumed and the residues and materials contaminated are decreased in comparison with more classical radiometric assays. The procedure is versatile enough for a general application in any laboratory with access to a standard PhosphorImager scanner.

Materials and methods

Substrates and recombinant HAT enzymes

Chicken core histones were isolated from nuclei of erythrocytes as described previously [11]. [1-^{14}C]Acetyl CoA (50 mCi/mmol) was obtained from Moravek. Yeast histone acetyltransferase proteins were recombinantly expressed in bacteria. His6-tagged ryGcn5 and ryEsa1 were purified by affinity chromatography on a Ni^{2+} resin [11] and ryHat1 as described previously [12].

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[3] *Abbreviations used:* HAT, histone acetyltransferase; CoA, coenzyme A; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; CPM, counts per minute; PVDF, polyvinylidene fluoride.

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Preparation of yeast enzymatic extracts and anion exchange chromatography

Whole-cell extracts containing acetyltransferase activity from the yeast *Saccharomyces cerevisiae* were prepared by the salt dissociation/ultracentrifugation method described previously [13]. Extracts were dialyzed against buffer B (15 mM Tris–HCl [pH 7.9], 0.25 mM ethylenediaminetetraacetic acid [EDTA], 5 mM 2-mercaptoethanol, 0.05% [v/v] Tween 20, 10% [v/v] glycerol, and 10 mM NaCl) and loaded onto Q-Sepharose FF columns (1.8×0.8 cm, bed size, GE Healthcare). After washing, bound proteins were eluted with a linear 80- to 400-mM NaCl gradient in buffer B. Fractions were collected and assayed for protein content (A_{280}) and HAT activity.

HAT assays

The PhosphorImager HAT assay was typically performed as follows. First, 12 μl of enzymatic solutions, such as cell extracts, chromatographic fractions, preparations of recombinant enzymes, or their dilutions, was mixed with 4 μg of chicken erythrocyte core histones and 0.005 μCi [1-^{14}C]acetyl CoA in a final volume of 16 μl. After incubation at 30 °C for 20 min, reactions were terminated by the addition of 4 μl of 5 × sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample loading buffer (0.312 M Tris–HCl [pH 6.8], 10% [w/v] SDS, 2.5 M 2-mercaptoethanol, 30% [v/v] glycerol, and 0.005% [w/v] bromophenol blue). Portions (typically 5 μl) of each sample were manually spotted across a piece of Whatman GF/F glass microfiber filter paper at 1-cm intervals. After 5 min of air-drying, filter was soaked in fixing solution (46% [v/v] methanol and 8% [v/v] acetic acid). The same fixative with the addition of 0.1% (w/v) Coomassie brilliant blue R250, which is the solution commonly employed for the staining/fixing of polyacrylamide electrophoretic gels, can also be used. The rinsing off of dye from the filters, except on the spotted positions containing proteins, serves as a visual check of appropriate washing during the subsequent steps. Fixative incubation was carried out with shaking at room temperature for 10 min. The filter was washed twice for 10 min each with a solution containing 30% (v/v) methanol plus 5% (v/v) acetic acid, once with 50% (v/v) methanol for 5 min, and finally once with methanol for 2 min. Glass microfiber filters were then dried at 80 °C for 5 min and were exposed to the BAS-SR IP phosphor-storage image plate (FujiFilm) for variable time periods. Phosphor-screens were scanned, at 50 μm resolution, in a FujiFilm FLA-3000 fluorescent imager analyzer. The integrated intensity, representing the sum of the intensities of the pixels within the selected area, for each spot signal was quantified with Imager Gauge software (FujiFilm). The background measurement, taken from an identical area of a nonspotted region on the same filter, was subtracted from the corresponding spot data. The resulting values, in arbitrary units, represent the relative radioactivity incorporated into the histones retained by the glass microfiber filter in each dot. In some experiments, the radioactivity in the spots was determined by means of the electronic autoradiography system, the InstantImager (Packard).

As a filter binding/liquid scintillation counting HAT activity assay, the procedure described by Lopéz-Rodas and coworkers [5] was employed with minor modifications. Briefly, after incubation of the enzymatic fractions with chicken histones (80 μg) and 0.01 μCi of [1-^{14}C]acetyl CoA, in a final volume of 120 μl, reaction solutions were deposited onto GF/F glass microfiber disks. Disks were air-dried for 5 min and then submerged in 25% (w/v) trichloroacetic acid (TCA) for 20 min; washed twice more with 25% (w/v) TCA for 10 min; washed once successively with ethanol, ethanol/ethyl ether (1:1, v/v), and ethyl ether; and finally dried at 80 °C. Radioactivity on the disks was determined by liquid scintillation counting.

Results and discussion

PhosphorImager systems detect and measure radioactivity on two-dimensional probes and can replace time-consuming film autoradiography. In the course of our studies on yeast HAT(s), we frequently use a PhosphorImager detector for the measurement of ^{14}C-labeled acetylated histones resolved on polyacrylamide gels [12,13]. We wanted to try using the PhosphorImager for the quantification of whole HAT activity, present in diverse preparations, after adsorption of the radiolabeled protein products onto a paper support in a manner similar to the immunological dot-blots. We initiated this work using Whatman GF/F glass microfiber filter paper as an adsorbent, which is habitually used in our laboratory as a retention matrix of protein precipitates [14,15]. The radiolabeled acetylated proteins are readily trapped by precipitation on the glass microfiber filter, whereas the excess of free [^{14}C]acetyl CoA is removed by a washing procedure. In the new assay procedure, the [^{14}C]acetate incorporated into the matrix-retained proteins is then quantified with a PhosphorImager.

To establish the reliability of the new HAT assay procedure, our first focus was to determine the capacity of the PhosphorImager detecting ^{14}C-labeled acetylated histones retained on the GF/F glass microfiber paper and to assess whether the signal intensity obtained is linearly dependent on the radioactivity charged on the filter. With these aims, radiolabeled histones, containing [^{14}C]acetate distributed among the different classes of histones, were prepared by incubation of chicken core histones and [1-^{14}C]acetyl CoA in the presence of a mix of the recombinant yeast enzymes Hat1, Esa1, and Gcn5 and were recovered by TCA precipitation [14]. Equal volumes of a series of solutions of radiolabeled histones, with different specific radioactivity, were spotted onto a GF/F glass microfiber filter, which was soaked with fixative, washed, and dried as described above. Filters were exposed to phosphor-screens for various time periods (1–17 h), and the screens were scanned with a PhosphorImager (Fig. 1A). A good signal/background ratio was obtained at all exposure times. A signal was visualized after 10.5 h exposure over dots with only 30 to 60 counts per minute (CPM). For 1 h exposure, a radioactivity between 180 and 300 CPM per spot was necessary to make the label apparent (Fig. 1A). The integrated intensity of the spot signals was determined and plotted against the deposited CPM (Fig. 1B). A clear linear relationship, for all exposure times, was obtained without reaching saturation within the extent of charged radioactivity. Even after only 1 h exposure, it was possible to quantify the spot signals and to observe a linear increasing of the intensity values with the specific radioactivity. These results show the validity of the glass microfiber filter, in combination with the quantification with a PhosphorImager, to determine the relative content of radioactivity of histones.

When various solutions of ^{14}C-labeled acetylated histones at different concentrations (0.01–1.0 mg/ml) were deposited onto the GF/F filter, remarkably a linear response was also obtained (Fig. 1C). Therefore, the efficiency of histone trapping on the glass microfiber paper is apparently not dependent on the protein concentration, at least within the range of concentrations and under the experimental conditions employed. In contrast, the original report describing the phosphocellulose P81 filter for a HAT assay showed a decrease of the retention efficiency of labeled histones on the P81 filters with concentrations lower than 0.5 mg/ml [4]. The ineffective retention could be palliated by adding cold histones, but not bovine serum albumin, as carrier [4].

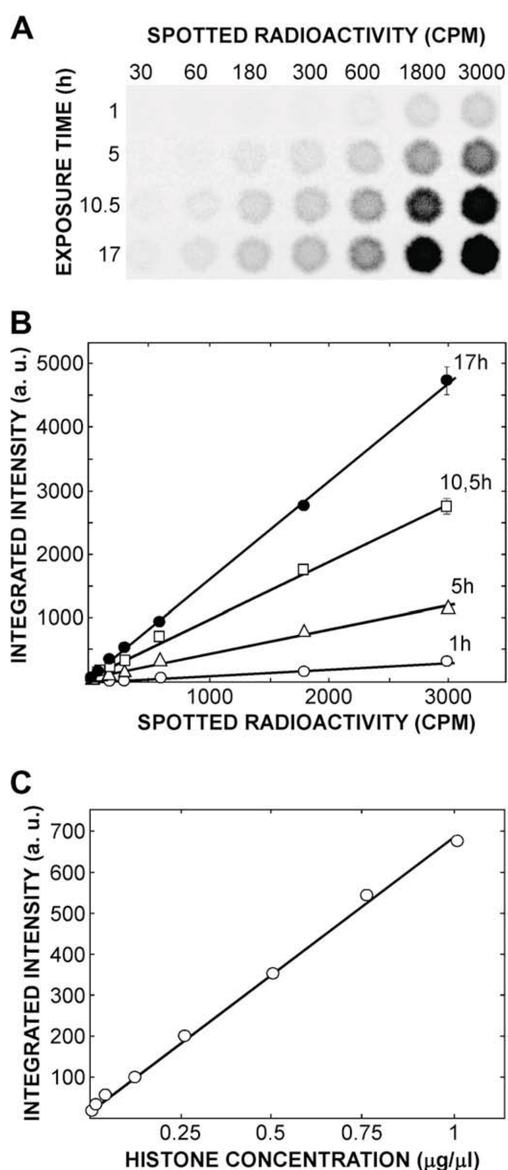

Fig. 1. Quantification of radiolabeled histones after trapping on glass microfiber filter using a PhosphorImager. (A) ^{14}C-labeled acetylated histones, dissolved in sample loading buffer, were mixed with various amounts of nonradioactive histones to obtain solutions with different specific radioactivity but identical concentration. Then 5 μl of each solution (2 μg histones) was regularly spotted across a piece of glass microfiber filter paper (Whatman GF/F). Filters were treated with fixing solution, washed, dried, and exposed to a phosphor-screen for the indicated time periods before visualization using a FujiFilm Fla3000 PhosphorImager. Deposited radioactivity (determined by scintillation counting) in each spot is indicated as counts per minute (CPM). (B) Integrated intensity of the spots in panel A was determined, and the resulting values (indicated as arbitrary units [a.u.]) are plotted against the applied radioactivity. Means and standard deviations of three independent spotting and quantifying repetitions are represented. (C) Radiolabeled histones were efficiently trapped by the glass microfiber paper independent of the concentration. Portions (5 μl) of solutions containing different concentrations of [^{14}C]acetyl histones (~600 CPM/μg) were deposited onto glass microfiber paper. Filters were processed as in panel A and exposed for 2 h. Means and standard deviations of integrated intensity from three independent experiments are graphed against histone concentration.

In some cases, the GF/F filters containing ^{14}C-radiolabeled histones were also quantified with the electronic autoradiography system, the InstantImager (a plate microchannel proportional counter). The sensitivity and apparent dynamic range, detecting radioactivity trapped on the glass microfiber filters, were similar to those of the PhosphorImager (results not shown). As a practical advantage, the electronic autoradiography provides a direct and continuous measurement of radioactivity during the exposure.

Next, different fixing and washing solutions, in addition to methanol/acetic acid mixtures, were tested. Levels of radioactivity comparable to those of the standard procedure were detected when filters were treated with 20% (w/v) TCA as fixative and washed successive with fresh 20% (w/v) TCA and acetone or ethanol (results not shown). We also compared several types of membranes and papers as protein capturing supports. Polyvinylidene fluoride (PVDF, Immobilon-P^{SQ}, Millipore) or nitrocellulose (Protan, 0.2 μm, Whatman) membranes yielded similar results, with regard to the retention efficiency and measurement capability of radiolabeled histones by the PhosphorImager, to those obtained with the glass microfiber filter (results not shown). However, the absorption to PVDF and nitrocellulose membranes occurs slowly; as a result, some dots were distorted. A vacuum-assisted dot-blotting device can be used to shorten the application time on these membranes, but that introduces an inconvenience and an added problem by using an apparatus with radioactive samples. The ease of deposition and the rapid absorption on the glass microfiber paper made its use preferable. On the contrary, when a cellulose fiber paper (Whatman 3MM Chr) was tested, the label obtained with the PhosphorImager was lower than that obtained on the other supports, indicating that radioactive histones were lost from the cellulose paper. In addition, the detected signal was not linearly dependent on the charged radioactivity (data not shown). A previous study by Horiuchi and Fujimoto [4] observed the inefficient recovery of labeled histones by TCA precipitation on cellulose paper. On the other hand, according to the manufacturer's data, the particle size retained by the fibrous matrix of cellulose papers is of several micrometers, whereas the GF/F glass microfiber filter exhibits retention capacity for particles down to 0.7 μm. We consider that the inability of the cellulose paper to retain very fine particles makes it inappropriate as a trapping support of radiolabeled protein precipitates in HAT assays, coincident with the previous report [4].

We next performed two test experiments to show the suitability of the novel PhosphorImager assay to measure HAT activity on distinct samples. First, the linearity of the spot signal with the enzyme concentration was assessed. Increasing amounts of ryEsa1 acetyltransferase were incubated with [1-^{14}C]acetyl CoA and core histones. Reactions were stopped by the addition of 5 × sample loading buffer, and portions of the resulting solutions were spotted on the GF/F glass microfiber paper. As shown Fig. 2A, in the range tested, the PhosphorImager signal was proportional to the enzyme concentration; that is, the dose–response was linear. Moreover, these results also indicate that the excess free [1-^{14}C]acetyl CoA employed in the HAT reaction was efficiently washed out from the filter. Typically, we stopped HAT reactions by adding sample loading buffer (5×) because in some instances the same samples were subsequently subjected to SDS–PAGE for the histone specificity analysis. We also tested the effect of other stop solutions, to terminate acetylation reaction previous to the spotting (e.g., as final concentrations, 0.25 M HCl or 4 M urea/5% [v/v] acetic acid mixture), on the retention of the radiolabeled products by the glass microfiber filter. The direct application of the HAT reaction mixture on the filter, omitting the prior addition of any stop solution, was also tested. In all cases, the same reaction mixture generated similar signal intensity independently on the composition of the application solution (data not shown).

For the second test, a yeast whole-cell protein extract was fractionated by anion exchange column chromatography, and the HAT activity on the resulting fractions was determined by both the novel PhosphorImager assay and a classical filter binding/liquid scintillation counting procedure [5]. As shown in Fig. 2B, the agreement between the two assay procedures was excellent; the acetyltransferase activity profiles obtained with both methods

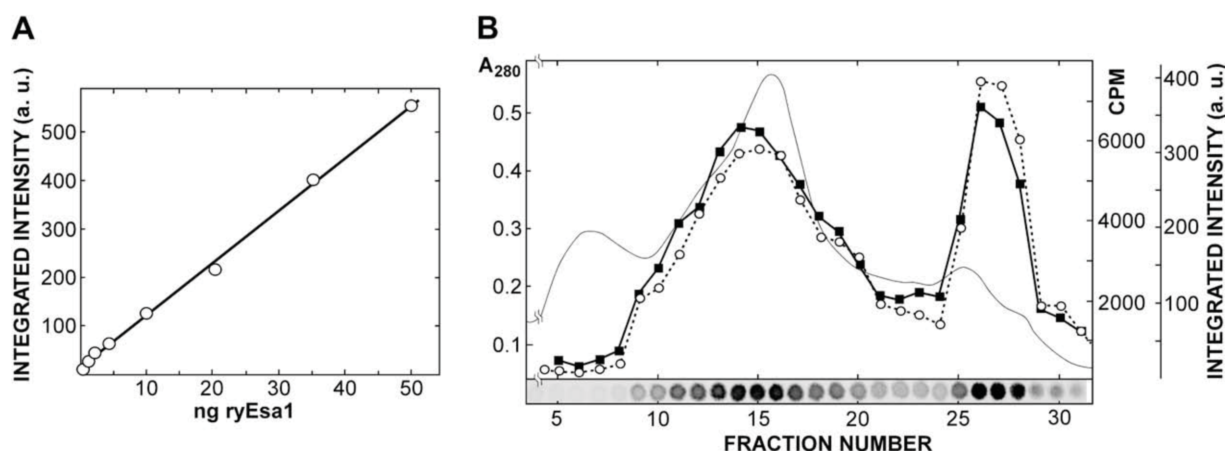

Fig. 2. Tests of validity of the PhosphorImager HAT assay. (A) Increasing amounts of recombinant yeast Esa1 histone acetyltransferase (ryEsa1) were mixed with purified histones and [1-^{14}C]acetyl CoA. After 10 min incubation, reactions were stopped with sample loading buffer (5 ×), and 5 μl of the resulting solutions was deposited onto a GF/F glass microfiber filter. The integrated intensity of the resulting dots after 2 h exposure was quantified. Shown are the means and standard deviations of three independent experiments. Values represented on the abscissa are the amounts of ryEsa1 in the incubation mixtures. (B) A PhosphorImager HAT assay and a standard filter binding/liquid scintillation counting assay generate similar activity profiles during fractionation of HAT enzymes by anion exchange chromatography. A yeast whole-cell extract was chromatographed on Q-Sepharose FF, employing a NaCl gradient to elute retained proteins. Relative HAT activity in the collected fractions was assayed with chicken core histones and [1-^{14}C]acetyl CoA as substrates by both a classical filter binding/liquid scintillation counting method [5] (activity expressed in CPM, ■—■) and the PhosphorImager HAT method (activity depicted as integrated intensity in a.u., ○—○). The inset shows the scanning of the phosphor-screen after 4 h exposure. The solid thin line represents A_{280}. Only the chromatographic elution region corresponding to the salt gradient is shown.

were essentially identical. It should be noted that the PhosphorImager detection method uses significantly lower amounts of reagents, including enzymatic fraction and the substrates, histones, and [1-^{14}C]acetyl CoA, and (more important) produces significantly less radioactive contaminated waste given that scintillation fluid and vials are not used. The standard PhosphorImager HAT assay consumes only 50% of radiolabeled acetyl CoA and 10% of histones used by the filter binding/scintillation counting assay [5] carried out here with a comparative aim. In comparison with other radiometric HAT assay procedures, the [^{14}C]acetyl CoA consumption reaches to be up to 20 times lower [6]. A higher saving in the reagents used is obtained by scaling down the PhosphorImager assay procedure (see below).

Because samples applied to the filter did not excessively diffuse outward, and also because the signal/noise ratio obtained by the PhosphorImager was rather good, it seemed possible that activity could well be measured with much smaller spotted reaction volumes. A small-scale version of the HAT assay would provide even further reduction in the necessary reagents and generated residues. Therefore, we analyzed whether the dotting of small reaction volumes produced a satisfactory response for a precise and reliable quantification of HAT activity. When various volumes of a reaction cocktail, obtained after incubation of [1-^{14}C]acetyl CoA and histones with ryGcn5, were applied on the GF/F glass microfiber paper, a clear linear relationship between the charged volume and the spot signal was observed in the range tested (Fig. 3). Thus, the quantification of the [^{14}C]acetate level incorporated into histones in the HAT reaction was not influenced by the deposited sample volume, and the enzymatic activity could be determined with volumes of only 0.05 to 0.1 μl equally well as with higher volumes. Taking these results into account, it is clear that the reaction volume in the HAT assay can be scaled down significantly; moreover, given that the lowest volumes generate smaller spots, a higher dotting density can be achieved. In conclusion, the proposed PhosphorImager HAT activity assay offers the adaptability to test a large number of samples simultaneously.

We typically use acetyl CoA labeled with the ^{14}C radioisotope because it emits sufficient energy to be readily detected by a standard PhosphorImager. Tritium-labeled acetyl CoA is also commercially available and normally with a higher specific radioactivity

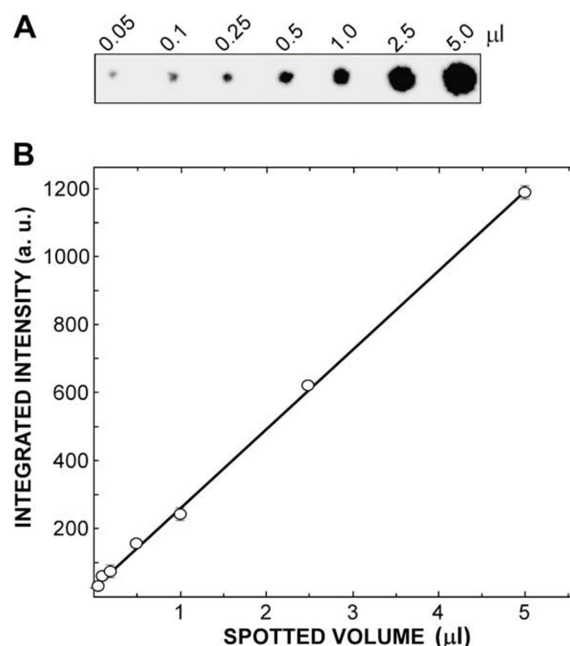

Fig. 3. Effect of the charged sample volume on the efficient quantification of ^{14}C-labeled acetylated product in the PhosphorImager HAT assay. Recombinant yeast Gcn5 acetyltransferase was incubated with histones and [1-^{14}C]acetyl CoA for 30 min at 30 °C. The reaction was stopped by the addition of SDS–PAGE sample solution (5×), and different volumes of the mixture were spotted onto a glass microfiber paper strip. (A) Scanning of the phosphor-screen after 4 h exposure is shown. (B) Integrated intensity of spots in panel A is represented versus the reaction mixture volume deposited onto the filter strip. Means and standard deviations of three repetitions are graphed.

than the [^{14}C]acetyl CoA, making it the first choice for many researchers. Particularly sensitive image recording plates, specially designed for the detection of ^{3}H, are also available from different companies. However, to increase detection of the low-energy particles emitted by tritium, these screens lack protective coating on the phosphor layer. Any contamination cannot be removed easily without damaging the photostimulable phosphor layer; thus, in

practice, imaging plates for ^{3}H are not reusable. Therefore, we consider that the employment of [^{14}C]acetyl CoA is preferable because it demands no particular requirement or care of the phosphorscreens.

The PhosphorImager HAT activity assay described here is versatile and appropriate for largely general use due to the protein retention capacity of the glass microfiber filter. Thus, the technique is applicable to a wide range of HAT enzymes; to test for the presence of activity in different biological samples such as cell extracts, chromatographic fractions throughout enzyme purification protocols, isolated proteins or complexes, and recombinant proteins; and to compare different substrates or reaction conditions. Besides histones, the PhosphorImager assay can be used to analyze HAT activity on nucleosomes, nucleosomal arrays, or chromatin without any adjustment to the protocol. Furthermore, different types of proteins, both basic and acidic, can be assayed with this procedure because all are efficiently trapped by the glass microfiber filter after precipitation with the fixing solution. In contrast, the negatively charged phosphocellulose P81 filter, also used to retain acetylation products, binds proteins or peptides by means of electrostatic interactions; thus, its use is limited to basic proteins [4,8,16]. Moreover, the binding efficiency to the P81 filter is influenced by the reaction buffer composition (e.g., concentration of some salts), which may yield anomalies or variability in the results [16], whereas the retention of proteins onto the GF/F paper is not significantly influenced by the solution components. On the other hand, we must note that synthetic peptides corresponding to the histone N termini, which frequently also are employed as acceptor substrates in HAT activity assays [8,9,16], are not firmly fixed to the glass microfiber filter. Thus, when synthetic peptides are used as substrates in a PhosphorImager HAT assay, we suggest working with PVDF or nitrocellulose membranes.

The renewed interest in histone acetylation and also other histone modifications, particularly on the enzymes responsible, has led to the increasing demand for identifying and measuring acetyltransferase activity in diverse biological samples. Hence, the need exists for easy and reliable assays that can be used in nonexpert laboratories. In this article, we have described a simple, but sensitive, in vitro HAT activity assay by using a PhosphorImager radioactivity detector. The method is amenable to both small and large numbers of samples, for an occasional measurement or for more routine use, in nearly any laboratory with the capability to make use of a standard PhosphorImager. It can be performed using distinct proteins as substrate, and it is potentially applicable for the analysis of enzymatic activity involved in other protein modifications for which a radioactive donor substrate exists.

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Qüestions proposades Article T6 Radioisòtops: Poveda & Sendra (2008) *Anal. Biochem.* 383, 296-300.

1.-Descriu la reacció catalitzada per l'activitat enzimàtica per a la qual es proposa el procediment d'assaig.

2.-Comparat amb altres mètodes descrits en la bibliografia per a aquests mateixos enzims, què és nou en el procediment descrit en aquest treball?

3.-Crees que en aquest mètode la mesura de la radioactivitat pot resultar igual de sensible que l'obtinguda amb un comptador de centelleig? Per que?

4.-Que importància té la dependència lineal observades en les Figures 1C, 2A i 3B? Que intenten mostrar els autors amb aquestes representacions?

5.-Podria obtenir-se valors d'activitat específica de l'enzim emprant aquest procediment? En cas afirmatiu explica com.

6.-Quins poden ser els impediments per a poder emprar aquest mètode? (ací es tracta de discutir, per exemple sobre el tipus d'isòtop, naturalesa dels substrats o de la reacció catalitzada, etc).

7.- Ací s'utilitzen histones com a proteïnes acceptores, però serviria també per a altres proteïnes? Per que? i per a altres modificacions de proteïnes?

Counting integral numbers of amino acid residues per polypeptide chain

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Proteins have integral numbers of each of the 20 amino acids. However, all the currently accepted methods of determining this number measure the ratio of moles of amino acid residue per mole of protein. This value is rarely close to an integer, due to experimental errors in determination of the molar amounts of both amino acid residues and polypeptide chain[1–3]. A simple method which gives integral values of amino acid residues per polypeptide chain, independent of any other properties of the protein, would be useful in characterising proteins. We describe here one method; it is illustrated for the case of Cys residues, although the approach should be useful for many of the other 19 usual amino acids.

The method relies on the charge differences introduced by specific chemical modification of the amino acid. In Cys residues this is readily accomplished by reaction of the thiol group with iodoacetic acid, which introduces acidic carboxymethyl groups. Because of the chemical reactivity of thiol groups, Cys residues not reacted with iodoacetate are blocked with iodoacetamide, a similar, but uncharged, reagent.

A complete spectrum of protein molecules having $0, 1, 2, \ldots, n$ acidic carboxymethyl groups, where n is the integral number of Cys residues per protein molecule, is generated by reacting all the thiol groups with varying ratios of iodoacetamide to iodoacetate, using competition between the neutral and acidic reagents. To make all thiol groups of the protein chemically equivalent, the reaction is carried out in denaturing conditions, in this case, in 8 M urea.

The molecules with varying numbers of acidic groups are separated by an appropriate method; here by electrophoresis in conditions where the electrophoretic mobility is determined by the net charge of the protein. The number of species $(n+1)$, and hence the number of Cys residues (n), is determined by counting the number of bands.

The procedure is shown in Fig. 1*a* with bovine pancreatic trypsin inhibitor (BPTI), a small protein known to have six Cys

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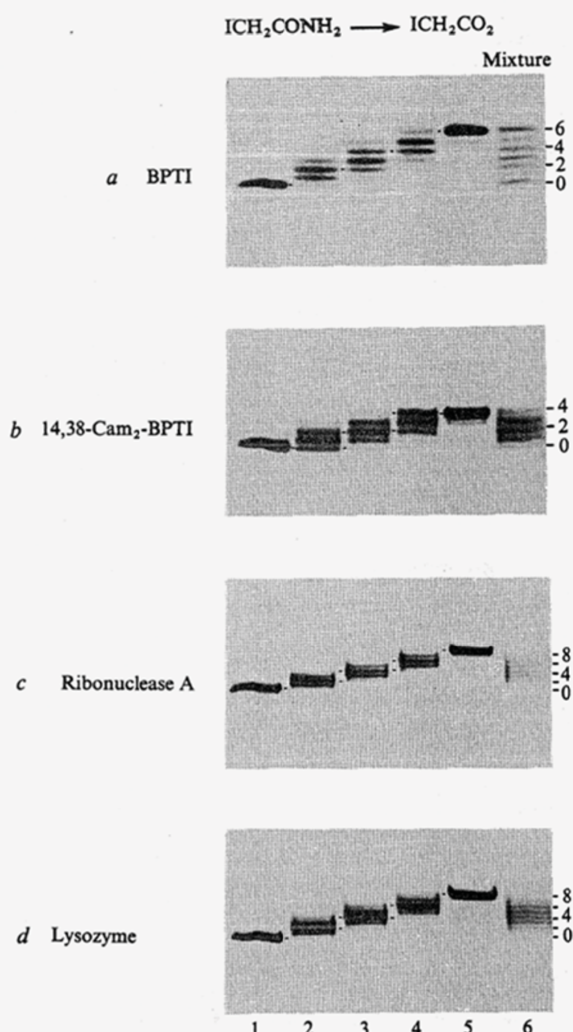

Fig. 1 Electrophoretic separation of protein molecules with 0 to *n* acidic carboxymethyl groups, where *n* is the number of cysteine residues in the protein. The reduced proteins were reacted with the neutral iodoacetamide (channel 1, on left), acidic iodoacetate (channel 5), and 1:1, 1:3, and 1:9 ratios of neutral to acidic reagents in channels 2, 3 and 4, respectively. Channel 6 (right) contains a mixture of equal portions of the samples applied to channels 1–5 in *a*, and of channels 2–4 in *b*, *c* and *d*. The bands are counted on the right by the number of acidic groups, and even-numbered bands are connected by dashes between them. The proteins (0.2 mg) were reduced and unfolded by incubation at 37 °C for 30 min in 1.0 ml 8 M urea, 10 mM dithiothreitol, 10 mM Tris-HCl, 1 mM EDTA, at *p*H 8.0. They were then alkylated by adding 200 µl of this solution to 50 µl of 0.25 M iodoacetamide, iodoacetate (adjusted to *p*H 8.0 with KOH), or mixtures of the two; after 15 min at room temperature, the solutions were placed on ice. Portions of 50 µl were placed on polyacrylamide gels containing 8 M urea, using the low-*p*H discontinuous system of Reisfield *et al.*[17]; electrophoretic separation was at constant current (100–160 V) at 3 °C for 3–4 h. Staining was with 0.1% (w/v) Coomassie brilliant blue in 10% (w/v) trichloroacetic acid and 10% (w/v) sulphosalicylic acid overnight. The gels were destained by diffusion into 7.5% acetic acid and 5% methanol.

residues[4–6]. The reduced protein in which the six thiol groups were blocked with iodoacetate migrates electrophoretically more slowly than that blocked with iodoacetamide, due to the six acidic carboxymethyl groups[7]. Reaction of the six thiols with mixtures of the neutral and acidic reagents in three different ratios generated the seven bands expected with six cysteine residues. Approximately equal competition between the two reagents was observed with a ratio of iodoacetate to iodoacetamide of 3:1, indicating that the latter reacts three times as readily as the former, as has also been observed by others[8]. This mixture produced primarily molecules with 2, 3 and 4 carboxymethyl groups, so the entire spectrum was generated with threefold lower and higher ratios of reagents to generate significant amounts of molecules with 0, 1, 5 and 6 acidic groups. Molecules with 0 and 6 acidic groups were observed when the protein was reacted with just iodoacetamide and iodoacetate, respectively. All seven bands could be demonstrated simultaneously by making a mixture of the above populations.

BPTI, in which two Cys residues (residues 14 and 38) had been blocked covalently by iodoacetamide before competitive modification as described above, so that only four Cys residues were present[9–11], gave the expected five bands (Fig. 1*b*). Reduced bovine pancreatic ribonuclease and hen egg lysozyme, each with eight Cys residues[12,13], gave the expected nine bands in both instances (Fig. 1*c*, *d*). Bovine α-lactalbumin and β-lactoglobulin clearly demonstrated the presence of eight and five Cys residues, respectively, using an electrophoretic system for acidic proteins. Proteins with no Cys residues, such as the penicillinase of *Staphylococcus aureus*[14], gave only a single band in all instances, with the same mobility when treated with either iodoacetamide or iodoacetate.

The procedure developed here for Cys residues is simple, rapid and gives integral values of the number of cysteine residues per polypeptide chain, independent of any other information about the protein, including its molecular weight. The procedure should be useful in determining the number of Cys residues of other proteins. It requires only a nearly homogeneous preparation of protein (which is also necessary, although not sufficient, for the determination of a correct value using currently accepted procedures) and a method of separating molecules with different numbers of carboxymethyl groups. The separation of species could be enhanced by the use of competition between acidic (iodoacetate) and basic (for example, ethyleneimine)[15] reagents. Other means of separating such species, such as isoelectric focusing or ion-exchange chromatography, may be more appropriate with other proteins.

The general approach should also be useful for counting other amino acid residues for which there are specific reagents to introduce differences in net charge or other properties that may be used to resolve the species. Only a single reagent need be used, and the spectrum of molecules with 0 to *n* residues may be generated by varying either the time of reaction[16] or the concentration of the reagent. Where two or more different reagents are available, competition between them may be used, as with iodoacetamide and iodoacetate here. The reaction should be carried out in conditions where the protein is unfolded and where all residues of the protein react at similar rates with the reagent. That this was the case with the Cys residues of the proteins studied here[16] is demonstrated by the close agreement of the distributions of molecules with different numbers of carboxymethyl groups with distributions expected to random reaction of each of the Cys residues with the neutral and acidic reagents. The species generated must also be separated in unfolding conditions, where the modified residues have an equivalent effect on the separation procedure. For example, the band of BPTI with one carboxymethyl group per molecule contains molecules in which that acidic group is on any of the six Cys residues of the protein[16], so all six isomeric species must have the same electrophoretic mobility. The other bands must also contain large numbers of species with the carboxymethyl groups distributed over the six Cys residues. Yet the bands are as sharp as those of the homogeneous species with 0 and 6 carboxymethyl groups and are equally spaced.

Any such modification procedure will depend on the selectivity of the reagent and on obtaining stoichiometric reaction of the residues of the pertinent amino acid in limiting conditions, which should be apparent by comparison of the mixtures at varying extents of reaction. Stoichiometric reaction should be indicated by approaching formation of a single species after extensive reaction, as is the case in Fig. 1. Slower reaction at

other sites should be apparent by the slower generation of additional, multiple bands after very extensive reaction.

The procedure developed here will not replace the standard methods of amino acid analysis, as it is unlikely that specific methods will be available for specifically modifying those amino acids with no reactive groups, for example glycine, proline, valine, leucine. Nevertheless, it should be useful for calibrating the molar ratios determined by amino acid analysis, using the integral values determined for one or a few convenient amino acids.

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Qüestions proposades Article T7 Electroforesi: Creighton (1980) *Nature*, 284, 487-489.

1.- Resumir l'objectiu i procediment experimental d'aquest estudi.

2.- Per què s'empren diverses relacions d'àcid iodoacètic (IAA)/iodoacetamida (IAM)? Es podria haver realitzat l'experiment amb una sola de les espècies?

3.- Tant el tractament amb IAA/IAM com la posterior electroforesi es realitzen en presència d'urea 8M, per què?

4.- Es podria utilitzar en aquest mètode l'electroforesi amb dodecilsulfat sòdic (SDS)? Quines altres tècniques (electroforètiques o no) podrien emprar-se per a separar les espècies proteiques generades per tractament amb IAA*IAM?

5.- Discuteix la possibilitat d'aplicar la mateixa estratègia al comptatge de residus d'altres aminoàcids diferents de la cisteïna. Quines poden ser les limitacions d'aquest procediment?

6.- Es podria adaptar el procediment per a la detecció de ponts disulfur? I per a diferenciar cisteïnes externes (accessibles) de les internes en l'estructura nativa de la proteïna?

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Biochemistry

Evidence for formation of a rabbit liver aldolase–rabbit liver fructose-1,6-bisphosphatase complex

(protein–protein interactions/gel penetration/gluconeogenesis/limited proteolysis by subtilisin/enzyme regulation)

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Contributed by B. L. Horecker, April 7, 1980

ABSTRACT The ability of rabbit liver aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphatate-lyase, EC 4.1.2.13) and rabbit liver fructose-1,6-bisphosphatase (Fru-P_2ase; D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) to partition into the gel phase of Ultrogel AcA 34 is decreased in a mixture of the two enzymes. Titration experiments indicate that a 1:1 complex is formed. The value for the distribution coefficient of the complex corresponds to a molecular mass of 300,000 daltons, the value expected for a dimer containing one mole of each enzyme protein. Complex formation was not observed when either liver enzyme was replaced by the corresponding isozyme from rabbit muscle. The susceptibility of liver Fru-P_2ase to limited proteolysis by subtilisin was reduced in the presence of liver aldolase, but not when the latter was replaced by muscle aldolase, suggesting that the conformation of Fru-P_2ase is altered in the complex. Limited proteolysis of liver aldolase abolishes its ability both to form the heterodimer and to protect Fru-P_2ase from modification by subtilisin.

In mammalian liver fructose-1,6-bisphosphate aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) and fructose-1,6-bisphosphatase (Fru-P_2ase; D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) catalyze successive reactions in gluconeogenesis. Both are found in the cytosol and, like other enzymes of glycolysis and gluconeogenesis, are considered to exist and function as independent entities. In recent years, however, evidence has been accumulating for loose interactions between cytosolic proteins and between these proteins and cellular matrices. Arnold *et al.* (1, 2) have reported that several glycolytic enzymes of muscle, including aldolase and glyceraldehyde-3-phosphate dehydrogenase, tend to associate with muscle actin, and Clarke and Masters (3) have examined these interactions in detail under conditions of physiological ionic strength. In mammalian erythrocytes both aldolase (4, 5) and glyceraldehyde-3-phosphate dehydrogenase (6) bind to the major membrane polypeptide (band 3 polypeptide), suggesting that these enzymes may function as a membrane-bound complex. Kinetic and physicochemical evidence for an interaction between aldolase and glyceraldehyde-3-phosphate dehydrogenase purified from rabbit muscle has been reported by Keleti and his coworkers (7, 8).

It has been proposed (3, 9, 10) that weak interactions between soluble enzymes may play a role in metabolism, either by promoting substrate channeling, in the case of enzymes that catalyze successive reactions, or by the induction of conformation changes that alter their catalytic or regulatory properties. Although direct evidence for subtle interactions has been difficult to obtain, the kinetic evidence for substrate channeling reported by Ovadi and Keleti (7) has been reinforced by the demonstration by Patthy and Vas (11) of the inactivation of glyceraldehyde-3-phosphate dehydrogenase by a labile oxidation product formed by the action of aldolase on dihydroxyacetone phosphate. The efficiency of the inactivation process suggested that it was due to direct transfer of the product from the active site of aldolase to the active site of the dehydrogenase.

We have previously reported (12) indirect evidence for an interaction between rabbit liver aldolase and Fru-P_2ase. In solutions containing mixtures of the two enzymes we observed the following indications of conformation changes: (*i*) in the presence of Fru-P_2ase the fluorescence emission maximum for tryptophan in aldolase was shifted from 336 nm to 330 nm; (*ii*) the addition of aldolase reduced the binding of Zn^{2+} to the two high-affinity sites of Fru-P_2ase. In each case the effect was specific for the two enzymes isolated from rabbit liver; the corresponding enzymes isolated from rabbit muscle did not appear to interact with the enzymes from liver. We also reported preliminary evidence for complex formation based on equilibrium gel penetration experiments, in which the ability of each protein to penetrate into the gel phase of Ultrogel AcA 34 was reduced by the addition of the other enzyme.

On the basis of the titration data reported here, a 1:1 molar complex is formed between rabbit liver aldolase and rabbit liver Fru-P_2ase, with the dissociation constant too small to measure under the conditions employed. Additional evidence for a conformation change in Fru-P_2ase induced by aldolase is presented, based on the decreased susceptibility of the former to limited proteolysis by subtilisin.

MATERIALS AND METHODS

Materials. D-Fructose 1,6-bisphosphate (Fru-P_2) (Na_3 salt), NADH, NADP, and subtilisin Carlsberg type VIII were purchased from Sigma. Glucosephosphate isomerase, glucose-6-phosphate dehydrogenase, and a mixture of triosephosphate isomerase and α-glycerophosphate dehydrogenase (1:10 wt/wt) were purchased from Boehringer Mannheim. Ultrogel AcA 34 was from LKB, Hicksville, NY. The reported effective fractionation range of this gel for globular proteins is 20,000–350,000 daltons. Before use the gel was washed extensively with 5 mM triethanolamine/5 mM diethanolamine buffer, pH 8.5, and allowed to settle.

Rabbit liver and muscle Fru-P_2ases were purified from New Zealand White rabbits by the procedures of Traniello *et al.* (13)

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Abbreviations: Fru-P_2, fructose 1,6-bisphosphate; Fru-P_2ase, fructose-1,6-bisphosphatase.

and Black *et al.* (14), respectively. Rabbit liver aldolase was prepared by the procedure of Pontremoli *et al.* (12), except that the livers were homogenized in a small Waring Blendor by using three 5-second bursts. Rabbit muscle aldolase was purified by the method of Taylor (15) as modified by Lai (16). The purified enzymes were stored at 2°C as suspensions in 80% saturated $(NH_4)_2SO_4$. As required, aliquots of the suspensions were centrifuged and the precipitates were dissolved in 5 mM triethanolamine/5 mM diethanolamine buffer, pH 8.5, to final protein concentrations of 8–15 mg/ml. Prior to use, the aldolase and Fru-P_2ase solutions were dialyzed against the same buffer. Specific activities (μmol of substrate utilized per min per mg of protein) at pH 7.5 and 25°C were 15.0, 22.7, 1.7–2.0, and 12.0, for liver Fru-P_2ase, muscle Fru-P_2ase, liver aldolase, and muscle aldolase, respectively. Specific activities at pH 9.2 and 25°C were 3.25 and 6.9 for muscle and liver Fru-P_2ases, respectively.

Subtilisin-digested aldolase was prepared by treating a solution of the enzyme (2 mg/ml, specific activity 1.8 μmol/min per mg) in 20 mM triethanolamine/20 mM diethanolamine buffer, pH 8.5, containing 60 mM $(NH_4)_2SO_4$ with subtilisin (2 μg/ml) at 22°C. After 20 min, when the specific activity of the liver aldolase had reached a constant value of 1.0 μmol/min per mg, the aldolase was recovered by adsorption on phosphocellulose P-11 and elution with Fru-P_2 (12).

Methods. Fru-P_2ase activity was assayed spectrophotometrically at 25°C by following the rate of reduction of NADP at 340 nm in the presence of excess glucose phosphate isomerase and glucose-6-phosphate dehydrogenase (13). The reaction mixture (1.0 ml) contained 20 mM triethanolamine/20 mM diethanolamine buffer, pH 7.5 or pH 9.2 as indicated, 0.1 mM NADP, 0.1 mM Fru-P_2, 2 μg each of glucose-6-phosphate-dehydrogenase and glucosephosphate isomerase, 2 mM $MgCl_2$ for assay of liver Fru-P_2ase or 5 mM $MgCl_2$ for assay of muscle Fru-P_2ase, 0.1 mM EDTA, and 40 mM $(NH_4)_2SO_4$.

Aldolase activity was assayed spectrophotometrically at 25°C in the direction of Fru-P_2 cleavage by measuring NADH oxidation at 340 nm in the presence of excess α-glycerophosphate dehydrogenase and triose-phosphate isomerase (17). The reaction mixture (1.0 ml) contained 20 mM triethanolamine/20 mM diethanolamine buffer, pH 7.5, 0.5 mM Fru-P_2, 0.1 mM NADH, and 20 μg of the triose-phosphate isomerase/α-glycerophosphate dehydrogenase mixture.

Protein was determined spectrophotometrically at 280 nm, assuming absorbances of: 0.84 (18), 0.91 (15), 0.63 (19), and 0.70 for solutions containing 1 mg/ml of rabbit liver aldolase, rabbit muscle aldolase, rabbit liver Fru-P_2ase, and rabbit muscle Fru-P_2ase, respectively. The value for specific absorbance of muscle Fru-P_2ase was determined on a sample hydrolyzed in alkali and analyzed with fluorescamine (20), using crystallized bovine serum albumin (Miles) as the reference. The values of M_r were taken as 158,000 and 160,000 for liver (18) and muscle (21) aldolases, respectively, and 140,000 and 142,000 for liver (22) and muscle (14) Fru-P_2ases, respectively.

Gel penetration experiments were carried out as described by Ackers (23) as applied by Fahien and Smith (24) to studies of the glutamate dehydrogenase–aspartate aminotransferase complex.

Digestion of Fru-P_2ase with subtilisin was carried out at room temperature (22°C) in 0.5-ml reaction mixtures containing: 20 mM triethanolamine/20 mM diethanolamine buffer, pH 8.5, 60 mM ammonium sulfate (including the contribution from the Fru-P_2ase solutions), 0.5 mg of Fru-P_2ase, and aldolase to a final molar ratio to Fru-P_2ase as indicated in the legends. The solutions were incubated for 30 min at 22°C and the reaction was begun by the addition of subtilisin as indicated in the figure legends. The progress of digestion of Fru-P_2ase by subtilisin was followed by measuring the increase in activity of Fru-P_2ase measured at pH 9.2.

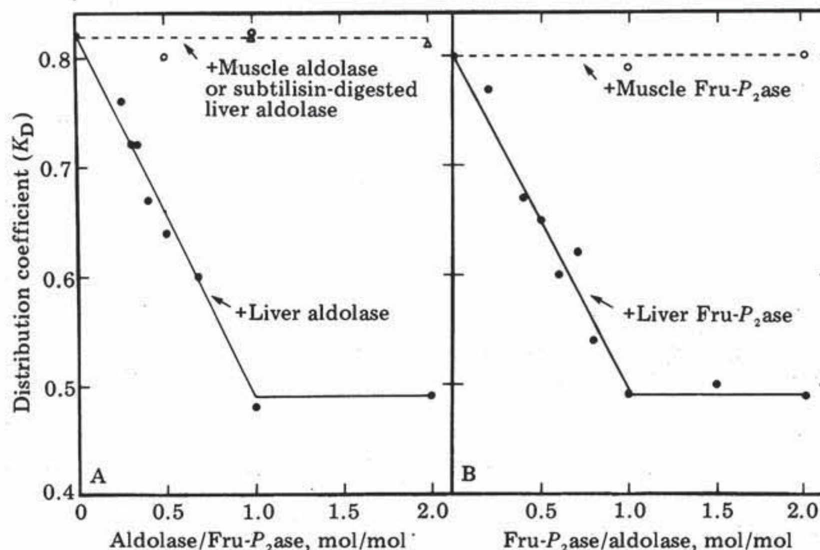

FIG. 1. Equilibrium distribution coefficients into Ultrogel AcA 34 for mixtures of Fru-P_2ase and aldolase. (*A*) Effect of aldolase on the distribution coefficient of liver Fru-P_2ase. (*B*) Effect of Fru-P_2ase on the distribution coefficient of liver aldolase. Incubation mixtures contained 2.0 ml of settled gel, dialyzed solutions of Fru-P_2ase, aldolase, or both as indicated, and 5 mM triethanolamine/5 mM diethanolamine buffer, pH 8.5, to a final volume of 4.0 ml. In *A*, each vial contained 0.22 mg (1.5 nmol) of liver Fru-P_2ase and, in *B*, 0.24 mg (1.5 nmol) of liver aldolase. Other proteins were added as indicated to the required ratios. The suspensions were incubated in 10-ml screw-cap vials at 4°C with gentle skaking for 2 hr, after which the vials were centrifuged at 500 × *g* and the clear supernatant solutions were assayed for Fru-P_2ase or aldolase activity. The distribution coefficients (K_D) were calculated from the relationship $K_D = (V_{prot} - V_{aq})/(V_{tot} - V_{aq})$, in which V_{prot} is the penetration volume, equal to total units added divided by the units/ml in the supernatant solution, V_{aq} is the volume of the aqueous phase excluded from the gel, determined in parallel experiments under the same conditions with blue dextran 2000, calculated from the total amount added divided by the concentration in the supernatant solution. The blue dextran concentrations were calculated from the absorbance at 280 nm. V_{tot} is the total volume of the system. A value of K_D = 1 would correspond to complete penetration of the protein into the gel; a value of K_D = 0 would represent complete exclusion. In *A*, △s represent addition of subtilisin-digested liver aldolase; ○s represent addition of muscle aldolase.

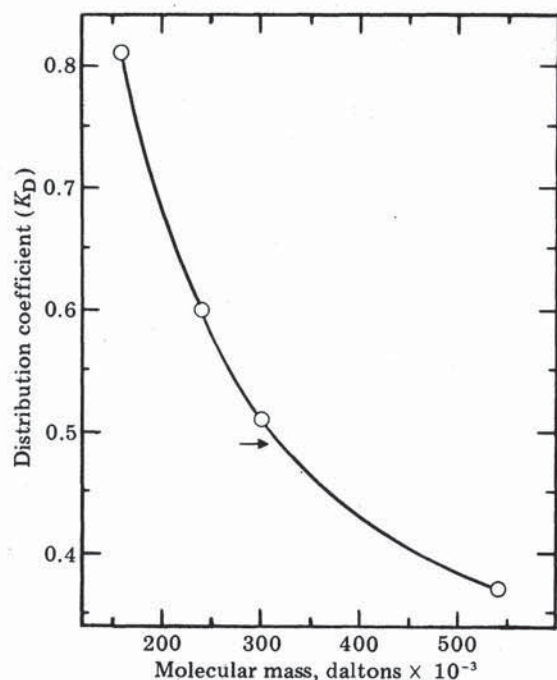

FIG. 2. Estimate of molecular sizes of liver aldolase, liver Fru-P_2ase, and the complex (arrow). Distribution coefficients for the standards—muscle aldolase (160,000 daltons), beef liver catalase (240,000 daltons), horse spleen ferritin (540,000 daltons), all from Pierce, and hemp seed edestin (300,000 daltons) from Sigma—were determined as described in the legend to Fig. 1, except that protein concentrations were determined by analysis with fluorescamine after alkaline hydrolysis (20).

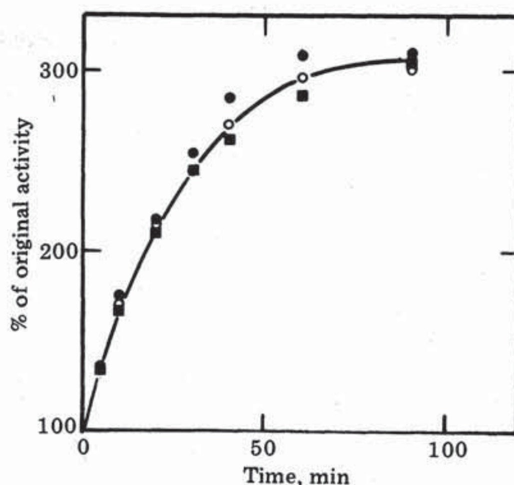

FIG. 4. Activation of liver Fru-P_2ase by subtilisin in the presence of modified liver aldolase. The preparation of subtilisin-modified aldolase is described in *Methods*. Digestion of Fru-P_2ase in the absence or the presence of subtilisin-modified liver aldolase was carried out as described in the legend to Fig. 3. ●, Liver Fru-P_2ase alone; ○, aldolase-to-Fru-P_2ase ratio = 1:1; and ■, aldolase-to-Fru-P_2ase ratio = 2:1.

RESULTS

Gel Penetration Experiments. The addition of increasing concentrations of rabbit liver aldolase to a solution containing a fixed concentration of rabbit liver Fru-P_2ase resulted in a linear decrease in the value of the distribution coefficient, K_D, in Ultrogel AcA 34 for the latter enzyme from 0.81 to 0.49 (Fig. 1*A*). The lower value was obtained when the ratio of aldolase to Fru-P_2ase reached 1.0; further additions of aldolase were without effect. No change in the distribution coefficient was observed when rabbit muscle aldolase was added instead of the liver isozyme. A similar result was obtained when rabbit liver aldolase was titrated with rabbit liver Fru-P_2ase (Fig. 1*B*). The initial K_D for aldolase was 0.80 and the final K_D was 0.49. Rabbit muscle Fru-P_2ase did not alter the value of K_D for liver aldolase.

Prior exposure of liver aldolase to subtilisin, which removes a small peptide from the COOH terminus and decreases the specific activity by one-half (unpublished results), but otherwise leaves the structure intact, abolishes its ability to form the complex with liver Fru-P_2ase (Fig. 1*A*).

To estimate the molecular mass of the complex, we determined the values of K_D for several known proteins. On this basis we estimated the molecular size of the complex to be 320,000 daltons, compared with 155,000 daltons for Fru-P_2ase and 160,000 daltons for liver aldolase (Fig. 2).

Effect of Liver and Muscle Aldolases on the Activation of Liver Fru-P_2ase by Subtilisin. The susceptibility of liver Fru-P_2ase to limited proteolysis by subtilisin was decreased by the addition of liver aldolase (Fig. 3*A*), suggesting that the conformation of Fru-P_2ase is altered in the presence of aldolase. With a molar ratio of aldolase to Fru-P_2ase of 1:1 the effect was seen primarily as a lag in the onset of activation; at higher ratios of aldolase to Fru-P_2ase there was also a marked decrease in the slope of the activation curves. The maximum effect was observed when the aldolase-to-Fru-P_2ase ratio was approximately 2:1. The experiment shown was carried out at pH 8.5, but similar results were obtained at pH 7.5 or pH 6.5. No effect on the rate of activation of liver Fru-P_2ase was seen when liver aldolase was replaced by muscle aldolase (Fig. 3*B*), indicating that the effect was not due to the catalytic activity of aldolase

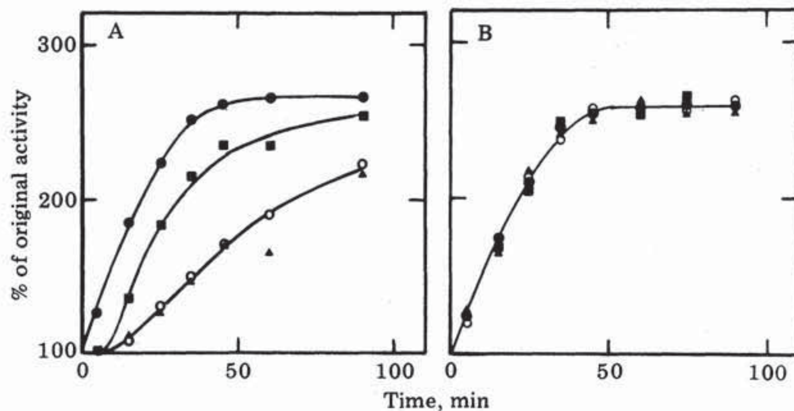

FIG. 3. Effect of liver (*A*) or muscle (*B*) aldolases on the activation of liver Fru-P_2ase by digestion with subtilisin. Rabbit liver Fru-P_2ase (1 mg/ml) alone (●) or mixtures of aldolase (1 mg/ml) and Fru-P_2ase in molar ratios of aldolase to Fru-P_2ase of 1:1 (■), 2:1 (○), or 4:1 (▲) were incubated with subtilisin (2 μg/ml) at 22°C. Aliquots were withdrawn as indicated for assay of Fru-P_2ase activity at pH 9.2.

because muscle aldolase, on an equivalent weight basis, is 6 times more active than liver aldolase.

Effect of Digestion of Liver Aldolase on Its Ability to Protect Fru-P_2ase. The activity of liver and muscle aldolases during digestion with subtilisin was also monitored. Exposure to subtilisin resulted in the rapid conversion of both aldolases to modified forms having approximately 60% and 5% of the initial specific activities, respectively, with Fru-P_2 as the substrate (data not shown). A 40% decrease in the activity of muscle aldolase was observed with fructose 1-phosphate as the substrate. In each case the conversion was complete within 5 min and was not significantly affected by the presence of liver Fru-P_2ase. The initial lag in the activation of liver Fru-P_2ase by subtilisin, observed when liver aldolase was present, appeared to coincide with the time required for aldolase to be converted to the modified form, suggesting that native liver aldolase would provide complete protection even at the molar ratio of Fru-P_2ase to aldolase of 1:1. The modification of muscle aldolase by subtilisin resembled that previously reported for its modification by carboxypeptidase, in that the activity toward Fru-P_2 was reduced by 95%, while its activity toward fructose 1-phosphate was relatively unchanged (25).

Aldolase, modified by exposure to subtilisin and recovered by adsorption on phosphocellulose P-11 and substrate elution, did not protect Fru-P_2ase from activation by subtilisin (Fig. 4). This was in contrast to the result reported in Fig. 3*A*, in which the rate of activation was only 40% of the control value even after modification of the aldolase was complete. This suggests that modification of aldolase in the complex does not cause immediate dissociation. Dissociation of the complex containing modified aldolase would thus be the rate-limiting step in the activation of Fru-P_2ase by subtilisin.

Effect of Liver and Muscle Aldolases on the Activation of Muscle Fru-P_2ase by Subtilisin. To examine the possibility that liver aldolase was protecting liver Fru-P_2ase by competing for subtilisin, we tested its effect, and also that of muscle aldolase, on the activation of rabbit muscle Fru-P_2ase. No change in the rate of activation of the muscle Fru-P_2ase by subtilisin was observed when an excess of either liver or muscle aldolase was added (data not shown). Thus the protection is specific for the combination of liver Fru-P_2ase and liver aldolase and is not related either to their catalytic activities or to a competition for the active site of subtilisin.

DISCUSSION

The gel penetration experiments provide the most direct evidence yet available for the formation of a specific complex between rabbit liver Fru-P_2ase and rabbit liver aldolase. The titration experiments are consistent with a small dissociation constant, because a sharp break was observed at the equivalence point. It was therefore unexpected that efforts to demonstrate the existence of the complex by equilibrium or velocity sedimentation methods, by light scattering, or by more conventional gel filtration techniques were all negative. It should be pointed out, however, that Fahien and Smith (24) were also unable to detect complex formation between glutamate dehydrogenase and glutamate oxalacetate transaminase by ultracentrifugation techniques.

We have previously shown that conformation changes in rabbit liver Fru-P_2ase can be monitored by measuring its susceptibility to limited proteolysis by subtilisin (26). On this basis, aldolase appears to induce a more resistant conformation in Fru-P_2ase. It also decreases both binding of Zn^{2+} to liver Fru-P_2ase and inhibition by this cation (12). Changes in other kinetic properties have not yet been detected.

We have attempted to demonstrate substrate channeling in kinetic experiments similar to those reported by Ovadi and Keleti (7) for muscle aldolase and glyceraldehyde-3-phosphate dehydrogenase, but these experiments were complicated by the fact that aldolase generates the acyclic form of Fru-P_2, whereas the α-furanose structure is required for its hydrolysis by Fru-P_2ase (27). The specificity of the interaction and the fact that a 1:1 complex is formed support the expectation that it will be found to play a role in regulation of gluconeogenesis.

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Qüestions proposades Article T8 Cromatografia: MacGregor *et al.,* (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3889-3892.

1.- Localitza, en la bibliografia de bioquímica general, les rutes metabòliques on actuen l'aldolasa (ALD) i la fructosa-1, 6-bisfosfatasa (FBPasa-1). Quin significat fisiològic té el que aquests dos enzims formen un complex en el fetge i no ho formen en el múscul?

2.- Expliqueu el significat del paràmetre K_D emprat pels autors per a mesurar la penetració de les proteïnes en el gel (veure peu de Fig. 1). Té alguna relació amb K_{av}? Com es determina experimentalment K_D?

3.- De quina forma es determina l'estequiometria del complex? Discutiu si és aplicable, en general, a qualsevol interacció entre proteïnes. A la vista dels resultats, cal esperar que la constant d'unió de les proteïnes siga: alta o baixa?

4.- La FBPasa és un enzim regulat *in vivo* per proteòlisi limitada. Quines probes aporten els experiments de digestió parcial amb subtilisina (una proteasa) d'aquest enzim i del complex que forma amb l'ALD?

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Isolation of DNA-free RNA, DNA, and proteins by cesium trifluoroacetate centrifugation

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Abstract

The ability to simultaneously isolate intact DNA-free RNA, genomic DNA, and proteins from a biological specimen can be useful in cloning genes and analyzing gene expression. Equilibrium density gradient centrifugation with CsCl is a useful tool for fractionating, quantitatively separating, and characterizing RNA, DNA, and the total quota of proteins, respectively, based on differences in their buoyant densities. In the present study we have reexamined the rarely used cesium salt, cesium trifluoroacetate, for the same purpose. A significant advantage of CsTFA lies in the fact that, unlike in CsCl, RNA can be recovered from a single, soluble fraction of the CsTFA gradient. Furthermore, unlike CsCl, CsTFA is freely soluble in ethanol so that co-precipitation of the salt in the recovered RNA upon alcohol precipitation does not take place. Hence, the RNA is recovered with minimum manipulations. The one-step separation of cellular macromolecule classes free of each other in small amount of starting materials provides a major advantage over other methods currently in use.
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Quantitative and representative RNA isolation in a single step from small amounts of tissue, whole cells, or subcellular organelles requires a procedure in which proteins and nucleic acids are each separated in intact form. Such a goal is difficult to achieve because ribonucleases are very stable and active enzymes that require no cofactors. Only cesium salt centrifugation methods can serve this purpose since they result in suppression of ribonuclease degradation and do not otherwise physically alter RNA. Moreover, they permit quantitative recovery of RNA and are applicable to very small tissue samples, indeed. In our initially published protocol [1], the biological specimens were lysed in Tris–HCl buffer and Sarkosyl, but later another lysing alternative was formulated [2] by using high concentration of guanidine chloride or guanidine thiocyanate. Both these lysing conditions have been favored to instantly and effectively inactivate cellular RNAses by denaturing them. The RNA is then fractionated from other cellular macromolecules in a number of different ways. In the cesium chloride ultra-centrifugation procedure, based on differences in their corresponding buoyant densities, one can quantitatively separate, from each other, the major cellular macromolecules, i.e., RNA, DNA, and protein, respectively [1]. In the CsCl method, the buoyant density of RNA in saturated CsCl solution exceeds the density of this salt at saturation; hence, the RNA is pelleted out in CsCl gradient and total RNA is separated from other cellular macromolecules, such as DNA and proteins. Meanwhile, the cellular DNA (chromosomal and mitochondrial) with an average buoyant density of 1.68–1.73 g/cm^3, and the proteins with their buoyant density of about 1.2 g/cm^3, respectively, can easily be separated in a carefully constructed stepwise CsCl gradient [1]. In the CsCl method, RNA is isolated as a solid form that precipitates out from the salt gradient. There have been reports showing that cesium trifluoroacetate (CsTFA), due to its higher aqueous solubility, can attain a higher density than CsCl [3,4]. This property makes it especially suitable for simultaneous purification of RNA, DNA, and proteins, while RNA remains soluble in a CsTFA gradient formed by centrifugation, instead of in precipitated form as in a CsCl salt gradient. By using the

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same approach as we did with CsCl centrifugation [1] and exploiting the properties of CsTFA, we have optimized CsTFA centrifugation for purification of RNA, DNA, and proteins, in particular RNA.

As an illustration we have used the method to study the fate of transcripts upon induction and to compare the composition of mature, fully processed RNA with intron-containing RNA in the total RNA isolated from intact cells.

Materials and methods

The CsTFA stepwise gradient. Routinely, a stepwise CsTFA gradient was performed in 3-ml Beckman conical polyallomer centrifuge tubes. The bottom layer (cushion) of 300 μl of a density of 1.99 g/cm^3 CsTFA was overlayered with 1.5 ml CsTFA at a density of 1.7 g/cm^3. The lysed cells or tissues were adjusted with CsTFA to a density of 1.5 g/cm^3 by mixing equal volumes of the lysed sample and CsTFA at a density of 1.99 g/cm [5,6]. The sample was placed atop the intermediate layer and centrifuged in a Beckman ultracentrifuge, SW 40.1 swinging bucket rotor, overnight (20 h) at 39,000 rpm. At the end of centrifugation, the total RNA content of the sample was found at the border between the cushion and the intermediate layer, the total DNA of the sample at the border between the intermediate layer and the sample, while the protein floated atop the tube. The protein is so tightly packed at the top that it is readily removed with a needle, almost like a cork from a bottle. Both the total RNA and the DNA are easily visible at the corresponding border zones.

In order to fractionate the separated macromolecules from the stepwise gradient a simple collection of fractions by piercing the centrifuge tube from the bottom is sufficient to assure quantitative and uncontaminated recovery of the corresponding fractions.

Lysis and preparation of tissue or cells prior to centrifugation. Tissue or cells were lysed in a buffer containing 4% Sarkosyl, 10% ethanol, and 2 mM DTT, facilitated with trituration. Completely lysed samples are usually viscous due to the presence of genomic DNA. The lysed material is passed through a 20 G needle four times to shear genomic DNA and thereby reduce viscosity, and followed by CsTFA salt gradient centrifugation. Fractions varying from 5 to 30 were collected after ultracentrifugation depending on need.

Agarose gel analysis of CsTFA density gradient fractions. Nucleic acids were precipitated by ethanol and re-dissolved in 100 μl of DEPC-treated H_2O. An aliquot of 10 μl from each fraction was run on 1% agarose gel.

DNAse and RNAse treatment of CsTFA density gradient fractions. A 5-μl aliquot of each re-dissolved fraction containing DNA or RNA in the volume of 100 μl was incubated with 20 U/reaction mixture of RNAse-free DNAse I (Gibco BRL) at 30 °C for 25 min and then electrophoresed on a 1% agarose gel. A 5-μl aliquot of each re-dissolved fraction containing DNA or RNA in the volume of 100 μl was incubated with 5 U/reaction mixture of ribonuclease H (Gibco BRL), at 37 °C, for 25 min. Reaction mix was run on 1% agarose gel.

Cell culture and induction. THP1 cells were maintained at 37 °C in a 5% CO_2 atmosphere in a culture medium of RPMI-1640 supplemented with 10% fetal bovine serum and 0.02 mM β-mercaptoethanol. THP1 cells proliferated in a nonadherent state. At 60% cell confluence, lipopolysaccharides (LPS) (Sigma) were added to a final concentration of 2 μg/ml. Stimulated cells were harvested at various time points by table-top centrifugation.

Cells (293) were maintained at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cell numbers were determined by hematocytometry and cells were harvested by centrifugation. The accuracy of counting cell numbers by hematocytometry is ±15%.

Real-time RT-PCR. RNA obtained by CsTFA gradient centrifugation was precipitated, washed in ethanol, and re-dissolved in 100 μl DEPC for real-time RT-PCR analysis. Real-time RT-PCR was determined with a AmpliTaq 5700 sequence detection system instrument (Applied Biosystems). In two-step RT-PCRs performed using the Taqman Reverse Transcription Reagents kit (Applied Biosystems), cDNA was synthesized from 1 μg of total RNA in each 100 μl reaction using random hexamers as primers. Real-time quantitative RT-PCR assays were conducted with 10 ng of reverse-transcribed total RNA in a final volume of 25 μl, using 300 nmol forward and reverse primers in the SYBR Green PCR master mix of reagents.

For measurements of yields of some mRNA extracted from a small number of cells the following genes were analyzed: rpL34 (ribosomal protein 34, large subunit); rpS21 (ribosomal protein 21, small subunit); GAPDH (glyceraldehyde-3-phosphate dehydrogenase); and P53 (protein 53).

For measurements of mRNA and intron-containing RNAs, induced IL1-RN and constitutively expressed rpS21 genes were analyzed.

Primer sets were designed using the Primer Express software (Applied Biosystems). The primer sets designed for the real-time RT-PCR analysis on various genes were the following:

IL1-RA′ exon1/exon2 Forward Primer: GACCCTCTGGGAGAAAATCCA
IL1-RA exon1/exon2 Reverse Primer: TGGTTGTTCCTCAGATAGAAGGTCTT

IL1-RA exon3/exon4 Forward Primer: TCCTGTGTCAAGTCTGGTGATGA
IL1-RA exo3/exon4 Reverse Primer: CGCTTGTCCTGCTTTCTGTTCT

IL1-RA exon1/intron1 Forward Primer: CCGACCCTCTGGGAGAAAAT
IL1-RA exon1/intron1 Reverse Primer: CACCCTCACCTTC TCCTCCTT

IL1-RA exon2/intron2 Forward Primer: TTGCAAGGACCAAATGTCAATT
IL1-RA exon2/intron2 Reverse Primer: GTGACGTGATGCCCAGATACA

IL1-RA exon3/intron3 Forward Primer: TCCTGTGTCAAGTCTGGTGATGA
IL1-RA exon3/intron3 Reverse Primer: CGCTTGTCCTGCTTTCTGTTCT

IL1-RA exon4/intron3 Forward Primer: CACCTGCCCATCTTTTGATTTC
IL1-RA exon4/intron3 Reverse Primer: GCGCTTGTCCTGCTTTCTG

S21 5′ exon/exon Forward Primer: CATTTCCGCGGCACGT
S21 5′ exon/exon Reverse Primer: AGCCTCGAAATGCAGAACGA

S21 3′ exon/exon Forward Primer: GATGATTCCATTCTCCGATTGG
S21 3′ exon/exon Reverse Primer: TGTGATTCTCTCCAGTCAAAAGTTCT

S21 5′ exon/intron Forward Primer: AAGGACGGAAGAGAGGCATGT
S21 5′ exon/intron Reverse Primer: AGCCTCGAAATGCAGAACGA

S21 3′ intron/exon Forward Primer: AAATGTGAGCCCCCAACCTT
S21 3′ intron/exon Reverse Primer: CAGATGATTCCATTCTCCGATTG

L34 5′ exon/exon Forward Primer: AAGGTTGGGAAAGCACCAAA
L34 5′ exon/exon Reverse Primer: GGACGAACCCCTCGAAGTTT

P53 Forward Primer: CTGGTTAGGTAGAGGGAGTTGTCAA
P53 Reverse Primer: AGGTTCACCAAGAGGTTGTCAGA

GAPDH Forward Primer: GGATCGTGGAAGGGTTGATG
GAPDH Reverse Primer: GGCCCGTCCACTGTCTTCT

Results

Distribution of RNA, DNA, and proteins along the CsTFA stepwise gradient

A typical profile of the distribution of nucleic acids and proteins along the CsTFA gradient is shown in Fig. 1. In this case human brain tissue (60 mg) was lysed and processed by CsTFA gradient centrifugation. Eight

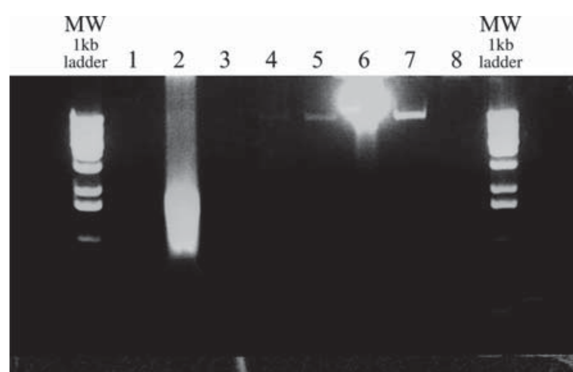

Fig. 1. Distribution of CsTFA salt gradient fractions viewed on agarose gel.

fractions were collected after centrifugation. In the figure, the contents of all fractions were analyzed by agarose gel electrophoresis. Note that in fraction #1, the cushion contained no trace of UV-absorbing material, whereas in fraction #2, the zone between the cushion and the middle layer, there is a strongly UV-absorbing material. Thereafter, there is a faint band in fraction #5, a heavy band in fraction #6, and a small band again in fraction #7. Finally, in the uppermost fraction #8, at the very entrance into the gel, there is an UV-absorbing material. Based on their relative densities these fractions were presumed to contain the total RNA in fraction #2, the majority of cellular DNA in fraction #6 (fractions #5 and #7 also contain some genomic DNA due to the overloaded gradient) and total cellular proteins in fraction #8.

Electrophoretic analysis of CsTFA gradient fractions

Electrophoretic analysis on agarose gels confirmed that fraction #2 contained the undegraded RNA (Fig. 2A). In addition to the distinct rRNA bands of defined molecular weights, diffuse UV-absorbing material corresponding to unprocessed and processed mRNA was also present.

The integrity of this diffused material was further monitored by re-isolating RNA and reanalyzing recovered RNA on gels. The integrity (high and low molecular weight RNA) was thereby reconfirmed. In Fig. 2B, the part of the gel containing RNA larger than the 28S material was cut out and divided into two portions that contained higher and lower molecular weight RNA, respectively. RNA from each portion was recovered and independently rerun on an agarose gel. Again, it was clearly shown that the high molecular weight RNA, as observed in the first lane, retained its higher molecular weight, as compared to the lower molecular weight RNA (Fig. 2C).

DNAse and RNAse treatment of CsTFA fractions

To confirm the identification of fractions #2 and #4 as RNA and DNA, respectively, samples from both were digested with either RNAse or DNAse. (Fig. 3A). Fraction #2 was thereby shown to contain completely RNAse-sensitive material (Fig. 3B) and fraction #4 to be completely DNAse sensitive (Fig. 3C).

The absorption spectra of RNA

To verify the quality and yield of isolated RNAs, the UV spectra of the isolated RNA were determined (Fig. 4). Total RNA isolated from 10, 20, and 30 mg of brain tissue was precisely proportional and each showed the same 260/280 absorbance ratio of 1.8, indicating that the total RNA was recovered consistently. The standardization of gene expression levels based on total RNA present in any given biological specimen is particularly timely and important because normalization to single housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is not recommended, since its

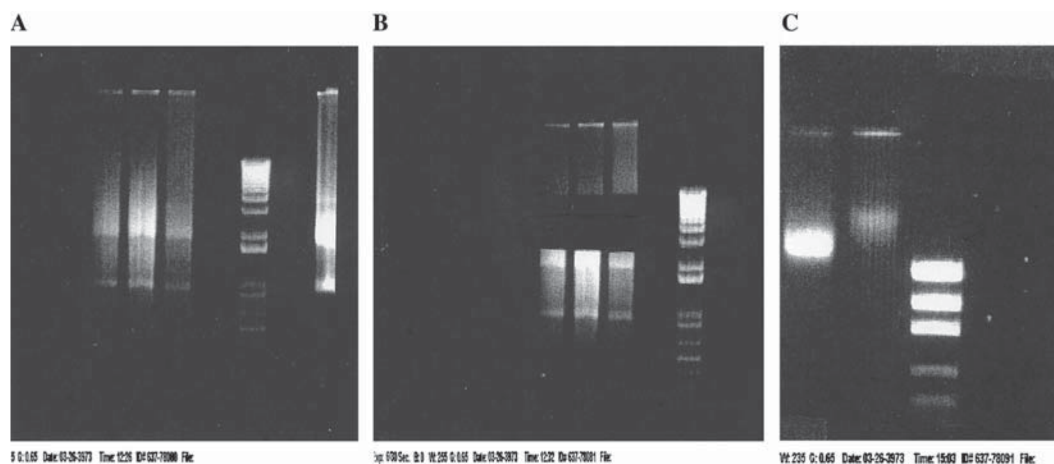

Fig. 2. Agarose gel analysis of isolated RNA fraction.

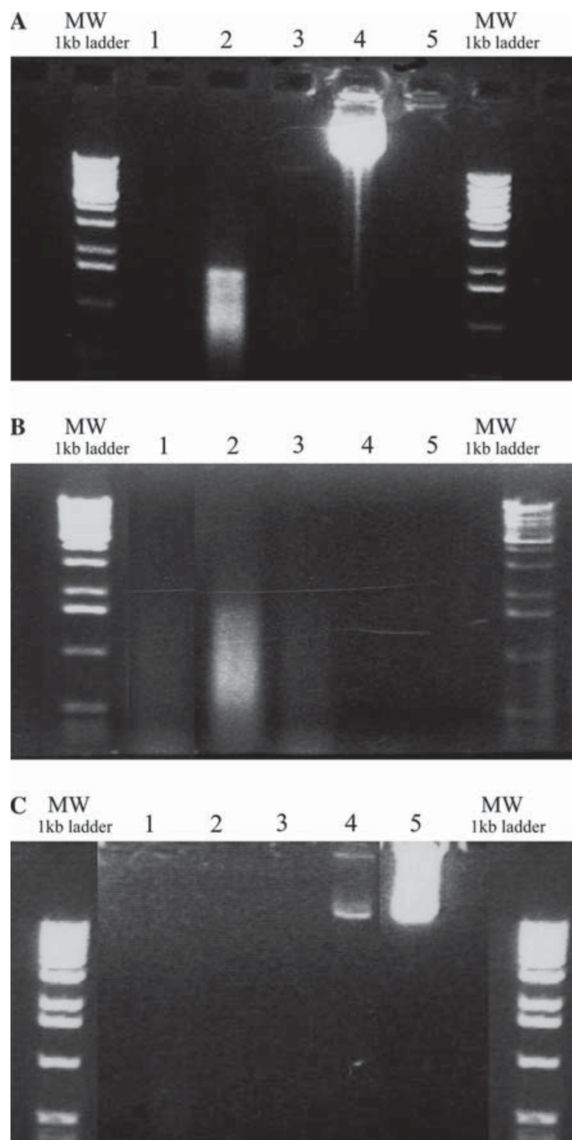

Fig. 3. DNAse and RNAse treatment of CsTFA salt gradient fractions.

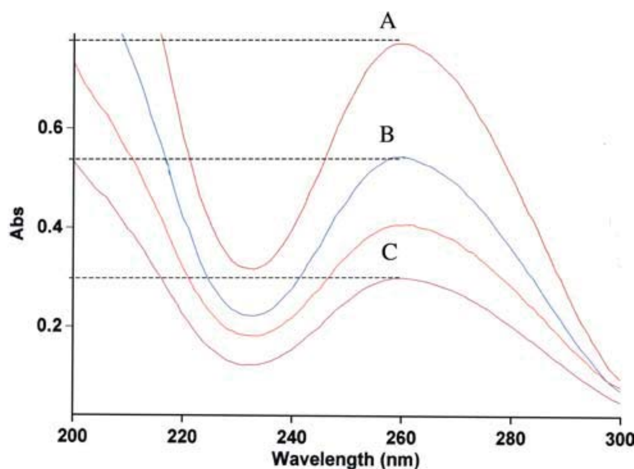

Fig. 4. UV absorbance spectra of RNA.

transcription levels can vary significantly, both in vivo and in vitro [7].

PCR and RT-PCR testing of CsTFA fractions

It was essential, for our purpose, to establish that the yield of the RNA isolated by CsTFA centrifugation is quantitative and that the RNA so isolated is not contaminated by genomic or mitochondrial DNA. Therefore, all the CsTFA fractions were further analyzed either by PCR to detect the presence of DNA or RT-PCR to detect the presence of RNA. After up to 40 PCR thermocycles, amplification of the genomic sequence of two specific genes, GAPDH, and ELF, all showed products in DNA-containing fractions, but none in RNA-containing fractions, indicating the complete absence of contaminating DNA molecules in the RNA fractions (Fig. 5A). At the same time, RT-PCR products with PCR primers that amplify exon sequences, mRNA sequences of these genes were detected in the expected size in the RNA-containing fractions, while no RT-PCR product was detected in the DNA-containing fractions, indicating also that the DNA fractions were free of RNA (Fig. 5B).

Monitoring individual mRNAs in CsTFA-isolated RNA

Assured that total RNA could be reproducibly isolated from biological specimens, we analyzed for the contents of several mRNAs in a variety of physiological settings. By calibration in a GeneAmp 5700 Sequence Detection system, one can quantify the given RNA in a sample. Although we are aware that strict quantitation for any mRNA requires calibration against its own standard, approximate quantitation can be obtained when an unrelated mRNA is used as a reference standard. Therefore, we used the message for the 1.2-kb kanamycin resistance gene prepared by in vitro transcription as a yardstick, in the range of 75–50 thousand kanamycin mRNA copies per sample (well).

Quantitative analysis showed that the lowest C^t value of 27.15 for the 50 thousand copies of mRNA per sample correlates well with the increased C^t value related to the 75 copies of mRNA per investigated sample. Our, experience and that of others are that the reproducibility and reliability of C^t measurement in the real-time RT-PCR assays varies within the range of one C^t cycle. Therefore, all our subsequent evaluations were based on this assumption (results not shown).

Quantitative extraction of total RNA from small numbers of cells

In order to see if RNA from a small number of cells could be quantitatively isolated by the CsTFA method, we extracted the RNA from human THP-1 (acute

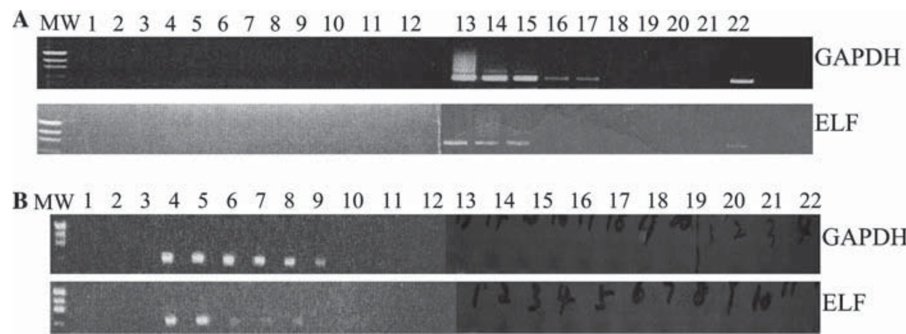

Fig. 5. PCR and RT-PCR testing of CsTFA fractions.

monocytic leukemia) and kidney-transformed 293 cells, starting with a material of 10^4, 10^5, and 10^6 cells, respectively. Since the yield of RNA from 10^4 and 10^5 cells cannot be measured accurately by photometry, we collected the fractions containing the total RNA and quantified its recovery indirectly by measuring the amount of a specific mRNA present in the total RNA by real time RT-PCR. For this purpose, the total RNA-containing CsTFA fractions were dissolved in the same volume of buffer for all three samples, and the presence of four different transcripts, ribosomal proteins S21 and L34, GAPDH, and P53, respectively, and amounts were determined by real-time RT-PCR. The results are shown in Table 1.

Table 1 compares the yield of four different mRNAs in two different human cell lines, when isolated from between ten thousand and one million cells. One order of magnitude difference in the yield of RNA corresponds to differences of 3.2 in terms of C^t value. Considering the accuracy in counting cell numbers (±15%) and of real-time RT-PCR analysis (2-fold), the recovery of RNA extracted from small amounts of cells and the content differences among the four RNAs in the cell lines would appear to be quantitative and representative. This feature of the CsTFA isolation method is particularly advantageous in cases where only localized parts of tissues (in brain, for instance) are biologically meaningful and relevant for gene expression profiling.

Monitoring the fate of transcripts upon induction

Since the CsTFA gradient centrifugation method is able to harvest the total RNA from a biological specimen, it makes it possible to monitor consistently and accurately the fate of transcripts upon induction even when the mRNA is expressed at a low level. As an example we chose to study human interleukin-1 receptor antagonist (IL1-RN) transcripts induced by LPS in THP1 cells. The induction of these transcripts was monitored at intervals between 30 min and 24 h. The mature, fully processed mRNA was monitored by analyzing the presence of the exon/exon junctions of the transcripts of IL1-RN at both 5′ and 3′ ends. However, to obtain a more general picture of the fate of transcripts upon induction, we simultaneously monitored the presence of the intron-containing RNAs by measuring the corresponding intron/exon junction sequences of the primary transcripts at both 5′ and 3′ ends as well as the intermediate intron/exon junctions between these two ends. The results of these experiments are presented in Table 2. The numbers in Table 2 represent C^t values. The threshold cycle C^t is defined as the PCR cycle number at which fluorescence is determined to be statistically significant above background. The overall accuracy of any given measurement is within ±1.0 C^t (i.e., 2-fold). The lower the C^t number, the more abundant the given RNA in the sample investigated. By analyzing the mRNA for IL1-RN at both the 5′ and 3′ exon/exon junctions, one can conclude that upon induction a dramatic increase of mature message of IL1-RN takes place. At 30 min, a 50-fold increase of mRNA over the uninduced level takes place. At 3 h the mRNA increases a 100-fold, and at 24 h, almost a 1000-fold. Here, the expression level of a constitutively expressed housekeeping gene, rpS21, which is assayed as an internal control, remains reliably and expectedly constant during

Table 1
Real time RT-PCR measurements of yields of some mRNAs extracted from a small number of cells

Number of cells	THP1 cell line			293 cell line		
	10^6	10^5	10^4	10^6	10^5	10^4
rpL34	23.5	26.2	30.1	24.5	27.1	31.1
rpS21	21.0	24.1	27.4	21.0	23.5	27.5
GAPDH	28.1	30.3	34.2	25.6	28.2	32.6
p53	29.3	32.5	36.2	23.7	26.6	31.0

The numbers represent C^t values for samples investigated.

Table 2
Real time RT-PCR analysis of mRNA and intron-containing RNAs for induced IL1-RN and constitutively expressed rpS21 genes

	Time			
	0 min	30 min	3 h	24 h
IL1-RN				
Exon1/exon2	29.3	24.7	23.8	21.0
Exon3/exon4	31.1	24.6	23.6	20.4
Exon1/intron1	30.0	28.7	28.7	27.1
Exon2/intron2	31.0	27.4	27.0	24.9
Exon3/intron3	30.7	27.0	26.7	23.7
Exon4/intron3	29.6	23.8	23.1	20.0
rpS21				
Exon1/exon2	24.2	25.3	26.0	26.0
Exon5/exon6	26.0	26.9	27.0	27.5
Exon1/intron1	30.0	31.6	30.7	31.0
Exon4/intron4	29.7	31.0	30.0	30.5

the time course of the induction, as indicated by the similar expression level of the 5′ and 3′ exon/exon junction sequences.

On the other hand, a similar analysis of the status of the premRNA for the same gene within the same time frame shows an interesting deviation from the results obtained with the mature mRNA. Although the presence of both 5′ and 3′ intron/exon junction sequences increases in the total RNA upon induction, it does so unequally. In the un-induced controls, the level of IL1-RN is very low, and both the processed and unprocessed RNA are present in the cell at an equimolar ratio. Upon induction, however, a disproportionate accumulation of unprocessed RNA takes place. By the 24th hour after induction, the 3′ intron/exon junction sequences are as abundant in the cell as the corresponding 3′ exon/exon sequences of the mature mRNA. At the same time the accumulation of the 5′ exon/intron is significantly less pronounced and differs from the corresponding 3′ exon/intron sequences by two orders of magnitude. If one compares the same expression pattern with a housekeeping gene, i.e., the ribosomal protein S21 that was assayed in parallel as an internal control, one cannot observe the discrepancies seen in the IL1-RN expression profile upon induction.

The following conclusions may be drawn from these experiments: (i) The induction of IL1-RN by LSP had, as expected, no effect on the expression levels of the investigated ribosomal structural protein (the control experiment). However, a clear and dramatic increase in the abundance of IL1-RN mRNA was observed during the time interval under investigation, reaching the 1000-fold mark by the 24th hour after induction. The reliability of this result is based also on the fact that both the 5′ and 3′ exon/exon junction sequences of the mature mRNA have increased in parallel. (ii) The picture of the presence of intron/exon junction sequences was quite different. The abundance of the 3′ exon/intron junction sequences over the corresponding 5′ junction was apparent even 30 min after induction. By the 24th hour after induction more than a 100-fold prevalence of the 3′ intron/exon sequences over the 5′ exon/intron sequences was registered. This observation then strongly indicates that the splicing of this premature RNA in the nucleus takes place from its 5′ end. Quantitation of intron/exon junctions between the 5′ and 3′ end, i.e., at exon2/intron2 and exon3/intron3, respectively, showed the tendency in the same direction. (iii) An unexpected result, which is also apparent from our experiments, is that the molar ratio of the unprocessed and processed IL1-RN RNA in the induced cell is equal to or even higher than 1. Since we know now from the human genome analysis that introns represent 24% and exons represent only 1% of the genome, our results indicate that the mass of the unprocessed RNA in the cell may reach the same prevalence as the mature, processed mRNA of any given gene, as is the case in our study. In addition to biological implications of this finding, it is important to note that this very fact may significantly influence the reliability of results obtained by gene expression profiling, regardless of what methods might be later applied in those studies.

Discussion

The CsTFA density gradient centrifugation method for quantitatively isolating an intact, DNA-free RNA is, in principle, analogous to the CsCl centrifugation method [1]. However, the trifluoroacetate anion imparts some properties that result in a higher quality nucleic acid preparation. This is because the maximum molarity of CsTFA at saturation is about 10 M compared to 7.36 M of CsCl, resulting in a maximum density of about 2.6 g/ml as compared to 1.9 g/ml for CsCl. This results in higher hydration of both the DNA and RNA in CsTFA, so that the density of these macromolecules is significantly lower than in CsCl. Thus, the density of DNA in CsTFA is about 0.1 g/ml lower than in CsCl, whereas RNA typically bands at around 1.90 g/ml. In the present work we have particularly evaluated the utility of this method for characterizing and isolating the RNA components of the cell.

In the course of developing the CsTFA method, we were able to isolate total RNA from a variety of biological samples, including cultured cell lines, human and monkey brain tissue, human tumor tissue, and sea urchin eggs, each with quantitative yield.

In summary, we would like also to emphasize that the method has broader applicability beyond RNA, since it provides from one sample, pure DNA and total protein, as well. This can find particular use when wanting to correlate gene expression profiling at the RNA level with profiling at the protein level.

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Qüestions proposades Article T9 Centrifugació: Zhang *et al.,* 2003, *Biochem. Biophys. Res. Comm.* **312**, 131-137

1.- Quina modalitat de centrifugació s'empra ací? Quina és la propietat de les molècules implicada en la seua separació?

2.- Quin és l'avantatge (o avantatges) d'emprar la sal de trifluoroacetat de cesi (CsTFA) enfront de la de clorur de cesi (CsCl)? Podria haver-se obtingut resultats similars amb CsCl jugant amb la seua concentració?

3.- Encara que s'empra un compost, CsTFA, adequat per a la autogeneració del gradient continu de densitat, els autors preparen un gradient escalonat (discontinu) emprant dissolucions amb diferents concentracions de CsTFA, per què? Hi ha algun avantatge per fer-ho així?

4.- Són les mateixes les densitats de flotació de les macromolècules obtingudes en gradients de CsTFA que aquelles descrites per a gradients de CsCl? Per què?

5.- El procediment es planteja com a mètode adequat per a aïllar al mateix temps DNA, RNA i proteïnes. Quina és la raó que açò siga possible en un únic experiment d'ultracentrifugació? Com són detectats cadascun d'aquests components en el gradient? Com és comprovada l'absència de contaminació d'uns components amb uns altres?