Egyértékü ionviszonyok és metabolizmus kapcsolata emlős vörösvértestekben

Ph.D. Disszertáció

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1995

Tartalomjegyzék:

Bevezetés,

A klasszikus enzimológia alapkoncepciója, hogy a kémcsőben, egyszerű modell rendszereken elvégzett kísérletek eredményei választ adhatnak arra a kérdésre, hogy *milyen* biokémiai folyamatok játszódnak le az élő sejtben. Ez az egyszerű feltevés -a maga keretei között- fényesen igazolódott. Ma már kiterejedt tudással rendelkezünk arról, hogy az egyes sejtekben miként épülnek fel vagy bomlanak le a legkülönbözőbb molekulák. Kevesebbet tudunk arról, hogy ezek a folyamatok *hogyan* játszódnak le az élő sejten belül, mennyire felelnek meg a klasszikus enzimológia híg vizes oldatokra, kis számú komponensre vonatkozó leírásainak.

Ugyanakkor korán felismerték, hogy a citoplazma fiziko-kémiai tulajdonságainak jellemzése a klasszikus, híg vizes oldatokra vonatkozó törvényekkel maradéktalanul nem lehetséges; s itt a Donnan és más egyszerű membrán-függő megoszlásokkal nem jellemezhető, egyenlőtlen ion- és molekula-megoszlásokra a sejt és környezete között, a fehérjék nem ideális ozmotikus partikulomokként való viselkedésére, eukarióta sejtekben a diffúzió sebességének elégtelen voltára, a sejten belüli ion aktivitás mérések ellentmondó adataira, a magmágneses rezonancia spektroszkópia által láthatatlan egyértékű ion-frakciókra, stb. utalok (1). Ennek ellenére az alap hipotézis továbbra is az, hogy az élő sejt lényegében egy intelligens plazma membránnal körülvett híg vizes oldattérként viselkedik, *és a citoplazmának nincs lényeges szerepe a sejt belső környezetének* fenntartásában (1).

A jelenleg általánosan elfogadott elképzelés szerint az élő sejt ionegyensúlyát (a kálium és nátrium ionok tekintetében) alapvetően két tényező határozza meg. Az első tényező ezen ionok *sejtmetabolizmustól függő koncentrációgrádiens ellenében történő mozgása, 'pumpálása'*. A második tényező pedig *a metabolizmustól független, de az adott sejt membrán-permeabilitásától függő passzív áteresztés, 'leakage'(2-9)*. A két folyamat *együttesen* alakítja ki az adott sejttípusra jellemző K és Na szinteket. *Általában* ez magas intracelluláris K szintet, s alacsony intracelluláris Na szintet jelent, meglehet jelentős eltérések vannak az egyes szövetek egyértékű kation szintjei között (10,11). Régi megfigyelés, hogy az emberi vörösvértestek K tartalma is körülbelül 6-7-szeresen múlja felül Na tartalmukat (12). Ugyanakkor más emlős fajokban jelentős vörösvértest K és Na tartalom eltérések is lehetségesek. Pl. a

ragadozók vörösvértestjeiben a plazmáéhoz hasonlóan alacsony K és magas Na szintek találhatók (12-16). Az angol szakirodalmi elnevezés szerint ezek "low potassium type" **(LK)** vörösvértestek, míg az emberihez hasonló magas K tartalmú vörösvértesteket szokás "high potassium type" **(HK)** vörösvértesteknek nevezni. (A továbbiakban csak a rövidített elnevezést fogom használni.) Míg ragadozókban csak *kivételesen* -és tulajdonképpen örökletes betegséghez kapcsoltan- fordul elő magas vörösvértest K tartalom (17,18), addig kérődzőkben az egyes egyedekre is *jellemzően eltérő*, recesszív-dominánsan öröklődő vörösvértest K és Na szinteket írtak le (19-24). Már 1937-ben Kerr megfigyelései felhívták a figyelmet a fajok közötti, ill. egyes fajokon belüli különbségekre (19). Leírta, hogy ragadozók /pl. kutya, macska/ vörösvértestjei "alacsony kálium", LK típusúak, azaz K tartalmuk alacsony, míg Na tartalmuk magas. Juhokat vizsgálva azt találta, hogy az egyes állatok vörösvértestjének K,, ill. Na tartalma alapján azok három csoportba sorolhatók. Az állatok vörösvértestjei vagy LK típusúak, vagy HK típusúak, de vannak olyan állatok is melyek vörösvértestjeinek K tartalma az előző két csoport között foglal helyet. Később Evans foglalkozott részletesen a juhokban és más kérődző fajokban fellelhető K és Na polimorfizmusokkal (20). Ennek ellenére meglepően hosszú idő telt el addig, amíg pl. szarvasmarhában is leírhatták a K/Na polimorfizmus jelenségét (22). Magunk azzal járultunk hozzá ezen területhez, hogy leírtuk *az első nem kérődző fajban található vörösvértest K/Na polimorfizmust* (25). (A szamárban (Equus asinus) leírt vörösvértest K/Na polimorfizmust ezért annak ellenére szerepeltetem ebben az értekezésben, hogy valójában egy véletlenszerű felfedezés volt. Ugyanakkor ezt soha nem tettük volna meg a dolgozat fő tárgykörét alkotó vizsgálatok nélkül.)

Az 1950-es években Bernstein számolt be kilenc faj vörösvértestjének K és Na tartalmáról (12), amelyet itt saját és mások időközben mért adataival egészítünk ki (1. táblázat). Harris után ő is kísérletesen bizonyította a glukolízis szerepét az intracelluláris K/Na arány fenntartásában.

Jelenleg nem ismerünk olyan örökletes, sem szerzett betegséget, amelyik megmagyarázhatná a különböző fajok, ill. egy fajon belül az egyes egyedek vörösvértestjeinek eltérő K és Na tartalmát. Az bizonyos, hogy a vörösvértest funkcióival az alacsony K tartalom nem interferál, az állatok tökéletesen.

I. táblázat.

faj	(n)	K mM	Na mM	K/Na	H_2O %
ember	64 **	93.1	15.3	6.08	66.41
	120 #	136.0	19.0	7.16	
majom	56 *	140.0	16.0	8.75	
sertés	5 **	88.3	14.1	6.26	64.05
	7 *	105.9±4.80	15.6±0.68	6.70	
ló	5 **	67.8	15.4	4.40	63.47
	8 *	140.0	16.0	8.75	
	8 #	140.0	16.0	8.75	
szarvas	5 **	67.1	24.4	2.75	65.76
	- *	99.6±1.88	8.85±0.84	11.25	
patkány	5 **	96.9	17.2	5.23	65.56
	36 #	135.0	28.0	4.82	
nyúl	5 **	100.0	14.5	6.90	67.85
	15 #	142.0	22.0	6.45	
	16 *	110.1±1.50	16.8±1.57	6.55	
juh LK (B)	20 **	11.0	111.1	0.099	63.17
(S)	10 **	14.5	83.9	0.17	64.48
	18 #	46.0	98.0	0.47	
	76 *	8-26	79-121		
juh HK (S)	9 **	70.4	25.4	2.77	63.74
	76 *	60-88	10-43		
kecske LK	2 *	26-32	69-79		
kecske HK (C)	10 **	61.1	25.1	2.43	63.17
	68 *	46-74	22-56		
bölény LK	57 *	40.3±0.62	70.0±1.21	0.58	
bölény HK	89 *	91.8±0.84	31.4±0.78	2.92	
marha LK	283 *	7-15	87.0		
szamár LK	1 **	45.7	55.5	0.93	66.57
szamár HK	1 **	86.5	26.0	3.33	64.16
kutya	6 **	6.9	103.0	0.067	65.24
	28 #	10.0	135.0	0.074	
	12 *	5.7±0.29	92.8±3.20	0.061	
macska	5 #	8.0	142.0	0.056	
	4 *	5.9±0.95	105.8±7.20	0.056	
hangyász##		59.5	48.3	1.23	
kacsacsőrű emlős##		71.5	74.0	0.97	
teve	40 *	51.1±0.49	27.8±1.15	1.84	

* mmol/l vvt, 2. ref.; ** mmol/l vvt, saját adatok; # mmol/l sejtvíz, B és S betű Booroola, ill. Suffolk fajtájú juhokat, a C betű Kameruni törpe kecskét jelöl.

egészségesnek tekinthetők. Első táblázatunkban összesítettem az irodalomban található vörösvértest K és Na concentráció értékeit. Saját mérési adatainkkal kiegészítve valószínűleg ez a legteljesebb táblázat, mely emlős fajok vörösvértest K és Na adataira vonatkozik. Fontos kiemelni, hogy a plazma K és Na szintek nem térnek el lényegesen az emberi adatoktól LK típusú állatok esetében sem. Talán érdemes leszögezni azt is, hogy az intracelluláris K/Na koncentrációk egy adott egyedben igen stabilak. Sem táplálkozási szokások, sem a normál viszonyok között változó K vagy Na bevitel nem gyakorol a vörösvértest K és Na tartalmára értékelhető nyomást. Sőt általában csak a vörösvértestek öröklött megbetegedései (26), vagy súlyos szisztémás toxikózisok, pl. urémia okoznak értékelhető eltéréseket (27,28). Saját méréseink szerint azonban a Pécsi Nephrológiai Centrumban hemodialízissel kezelt betegek esetében *nem találtunk* értékelhető vörösvértest K vagy Na tartalom eltéréseket, meglehet az anémią és az anisopoikilcytosis továbbra is, a korszerűbb kezelési lehetőségek ellenére állandó szövődményei az urémiás állapotnak. Komplex, részben a mi megatív eredményeinket igazoló értékelésekkel találkozunk az újabb irodalomban is (29,30). Meglehet a hemodalízis kezelés korszerűsödése hozza azt, hogy a korábban leírt vörösvértest K és Na tartalom eltérések eltűntek, s a korábbiaknak ellentmondó adatokat mérünk.

Mint azt fentebb kiemeltük, minden sejt, így minden vörösvértest is *permeabilis* a K és Na ionokra nézve, ezért a környezettel szembeni equilibráció ellen valamilyen mechanizmusnak dolgoznia kell, hogy a Donnan megoszláson túl net ion akkumulációt vagy kizárást kapjunk. Továbbá, ennek a mechanimusnak ***különbséget*** kell tudni tenni a kémiailag igen hasonló K és Na ionok között is.
Meglehetősen régen, az 1950-es években fedezték fel a Na és K -ionok, valmint Mg ionok által aktivált ATPáz molekulát, amelyet az "ion-motiv ATPase" molekulák közül a legelsőként jellemeztek. Elsődleges szerkezetének a földerítéséhez meg kellett várni az 1980-as évtizedet (31,32), működési mechanizmusára vonatkozólag azonban mind a mai napig csak hipotézisek vannak .
Ennek ellenére a sejt és a környezete közötti ion gradiensek, s a membrán permeabilitásokból következtetni lehet arra, hogy mennyi energiát igényel a sejt és környezete között fennálló gradiensek fenntartása. Ezt az emberi vörösvértestek esetében az összes elhidrolizált ATP molekula mintegy 30-50%-ában adják meg (33).

A ragadozók LK típusú vörösvértestjeiről pl. leírták, hogy membránjukból szinte teljesen hiányzik a ouabainnal bénítható K-Na-ATP-áz (13). A juhokban pedig, bár vörösvértestjeik ouabain-kötő helyeinek száma alig marad el a HK típusú sejtekre jellemző érték mögött, membránjuk K és Na áteresztésében /"leak"/ *jelentős eltéréseket* találtak (3,21,34,35). Tehát magas K-Na-ATP-áz szint mellett is találhatunk alacsony K tartalmat egy vörösvértestben, ha a membránja fokozott mértékben engedi ki a K ionokat. Érdekes módon az LK típusú juhok intakt vörösvértestjeinél tapasztalt fokozott membránpermeabilitást az un. "szellemsejtekben" már nem láthatjuk (36).

Ugyanígy a vörösvértestek ős- és elősejtjei a retikulocytáig bezárólag *HK típusúak, LK típusú állatokban is*. A hemoglobin és vas akkumulációjához időben kapcsoltan a retikulocyták elveszítik magas K tartalmukat (16). Fontos hangsúlyozni azt is, hogy közvetlen a születés után pl. a szarvasmarha borjakban *mindig* magas a vörösvértest K tartalom, s csak a 2.-3. hét táján történik rapid csökkenés a vörösvértest K tartalomban az LK típusú állatokban, ahogy azt saját magunk demonstráltuk (1. ábra). Tehát a miénkhez hasonló elemzés során fontos figyelemmel lenni az állat életkorára, a kiválasztott populáció nem tartalmazhat nagyon fiatal állatokat.

Míg a csaknem általánosan elfogadott "membrán-oldat" hipotézis egy klasszikus *nem equilibrium* állapotot feltételez, amit energia befektetésével lehet csak fenntartani, addig az úgynevezett szorpciós hipotézisek azt feltételezik, hogy a sejtre jellemző iongradiensek fenntartása *nem igényel energiát*. (9,37,38).

Ezen utóbbi hipotézisek lényege, hogy a sejt és környezete között fennálló ion koncentrációgrádiensek nem valódi koncentrációgrádiensek, amelyek folyamatos energia-invesztálást követelnek, hanem a citoplazma *equilibriumban van a környezetével* amiatt, hogy a sejten belüli kismolekulák és ionok nem annyira szabad oldat, mint inkább kötött formában vannak jelen. Az ionaktivitások K és Na tekintetében is lényegesen kisebbek, mint 1. A membránnak inkább árnyékoló szerepe lenne, ugyanis a citoplazma nem érintkezhet teljes felületén a minőségileg is oldatként viselkedő környezetével.

A K és Na ionok aktuális *koncentrációja itt egy kizárási, s egy kötési mechanizmus eredője*. A kizárási mechanizmus lényege, hogy a sejten belül az erősen hidratált kismolekulák és ionok kevésbé jól jutnak be, mint azok, amelyek hidratált rádiusza kisebb. Ezek szerint a kisebb ionátmérőjű, de nagyobb hidrációs burokkal

1. táblázat.

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* mmol/l vvt, 2. ref.; ** mmol/l vvt, saját adatok; # mmol/l sejtvíz, B és S betű Booroola, ill. Suffolk fajtájú juhokat, a C betű Kameruni törpe kecskét jelöl.

rendelkező Na már a sejtbe való bejutás tekintetében is hátrányban van a K ionokkal szemben. Az igen jól ismert, hogy a K és Na ionok vizes oldatban a legtöbb anionnal ionos kötést képeznek. *Ugyanakkor szelektíven megkötheţők ioncserélő oszlopokon* (38). Az élő sejten belül egyedül a fehérjék glutaminsav és aszparaginsav oldalláncai kerülnek szóba egy ilyen kötési mechanizmus szempontjából. Valójában az emberi vörösvértesteken belül a hemoglobin laterális carboxyl csoportjai mennyisége mintegy 14-szeres a K+Na ion tartalomhoz képest (saját számítás, nem közölt adat). Természetesen a szorpciós hipotéziseknek is választ kell adniuk arra a tényre, hogy miért eltérő az egyes sejttípusok, vörösvértest típusok K és Na tartalma. Az elképzelés lényege, hogy a fehérjék ion-kötő képességeit az egyes metabolitok, különösen az ATP alterálják. Ezek szerint itt *nem az ATP turnovertől, a glukolízis sebességétől függ a relatív K-Na arány, hanem a steady-state ATP szinttől.*

Saját kutatási vezérlő elvemnek e területen azt tekintem, hogy az egyes emlős vörösvértest típusokat összehasonlítva a metabolizmus, s az ion szintek szempontjából, a membrán-oldat és a szorpciós hipotézisek helytálló elemeit felderítsem, kiegészítsem és összerakjam.

A kérdés kritikus vizsgálata magában kell, hogy foglaljon minden olyan ismeretet, amely a vörösvértest energia *generálásáról* rendelkezésre áll. Ugyanígy lehetőség szerint figyelembe kell venni minden egyéb energia *felhasználó* tényezőt is.
Szerencsés módon az érett emlős vörösvértestek nemcsak struktúrálisan egyszerű sejtek, hanem metabolizmusuk is igen egyszerű (39,40). Természetesen ez csak egy relatív egyszerűség, amennyiben a glukolízis, amely az ATP egyedüli forrása vörösvértestekben, a körülményektől (pH, ionerősség, hőmérséklet, redox állapot stb.) még egy adott faj vörösvértestjeiben is jelentősen *eltérő* lehet. Ezért vannak az egyes irodalmi közlések között jelentős különbségek. Ezek részletes elemzése önmagában messze meghaladná a jelen mű kereteit, de kellően figyelmeztet arra, hogy csak a kísérleti körülmények *kellő standardizálása* ad lehetőséget az adatok összevetésére. Szintén röviden reflektálni kell arra a tényre is, hogy az egyes fajok vörösvértestjei más-más szubsztrátot használhatnak fel (pl. a sertés vörörsvértestek a máj által termelt *inozitolt* metabolizálják, amely a ribóz-5-foszfáton keresztül képes elérni a pentóz foszfát shuntöt, s belépni a glikolízisbe) (41-44). Érdekes módon ezek

a kísérletek vezettek arra a felvetésre, hogy esetleg az emberi vörösvértestek számára is fontos szubsztrát lehet pl. az *adenozin* (45).
Az energia (ATP) generálás oldalán tehát, mindezen eltérések ellenére, relatíve egyszerű a helyzet. Igen jól ismert, hogy a glukolízis (Embden-Meyerhof pathway) net ATP produkciója glukózonként kettő. A glukolízis sebessége könnyen mérhető a glukóz fogyásának meghatározásával, vagy pedig a tejsav produkciójának a mérésével.
A vörösvértestek energia felhasználásának az oldala jóval bonyolultabb, s nehezen quantifikálható (40). Mai tudásunk mellett az elektrolit egyensúly fenntartása mellett a glutathion szintézis, valamint a sejtalak fenntartása használ ATP-t (46-49). Sajnos az utóbbi körül sok a bizonytalanság. Míg az ötvenes évek végén, s a hatvanas években a japán Nakao meggyőző bizonyítékok sorával állt elő a a vörösvértest morfológia, s az ATP szintek összefüggéseinek mérésével (47), addíg később kiderült, hogy egyáltalán nem mindegy, hogy milyen módon depletáljuk a vörösvértesteket ATP-ben. Pl. a jódecetsav, a glicerinaldehid 3-foszfát dehidrogenáz inhibitora drasztikus ATP-depléciót okoz, de alakváltozás, duzzadás nem következik be (40).

Magunk munkálkodásunk egyik fókuszába azt a kérdést helyeztük, hogy *miként függ össze a vörösvértestek metabolizmusa ion-tartalmukkal.* S meglehet, még nem adhatunk konkrét választ a feltett kérdésre, néhány szerény megállapítást mindenképpen tehetünk. Ugyanígy, alkalmunk volt néhány olyan "melléklelet" felismerést is tenni, amelyek tudományos jelentőséggel bírnak más, meg nem célzott területeken (25). Vizsgáltuk mind az ATP turnover, s az ionszintek, mindpedig a kulcs purin-nucleotidok, s az ionszintek összefüggéseit, ahogy azt a kérdésfeltevések részben még bővebben kifejtem.

Értekezésem második felében is főként vörösvértesteken, s szemlencséken tett megfigyeléseket ismertetek. Itt azonban azt az érdekes jelenséget vizsgáljuk, hogy miként alakulnak a sejtek ion, s metabolikus viszonyai, ha *nem ionos detergensekkel* kezeljük őket. Az egyes *nem-ionos detergensekkel kezelt sejtek ionviszonyai meglepően stabilak, még akkor is, ha a plazma membrán biztosan minden sejtben átjárhatóvá vált.*
Ennek az érdekes jelenségnek az eredeti leírása laboratóriumunkban történt meg, s témavezetőm, dr. Kellermayer Miklós nevéhez fűződik (50-53). Magam elsősorban

azokat a *detergens tulajdonságokat* vizsgáltam, amelyek a sejt és környezete közötti elektrolit szint kiegyenlítődés *sebességét* befolyásolják. Ugyancsak vizsgáltuk a jelenség *reverzibilitását,* valamint progresszióját, ahogy ezeket a pontokat a kérdésfeltevések részben még bővebben kifejtem.

Nem-ionos detergenseket, digitonont, NP-40-at, Triton X-100-at, s újabban a Brij valamint a Tween család detergenseit is széleskörűen használják a legkülönbözőbb sejtfrakcionálási eljárásokban. Csaknem mindíg a detergens lipolitikus tulajdonságát használjuk ki, mikor is a detergenseket a kritikus micelláris koncentráció (CMC) szintje feletti tartományban használjuk. A fő szempont általában funkcionálisan intakt fehérjék nyerése. Mivel egyes lipid elemek igen sok fehérje funkcionálisan esszenciális részei, valóságos művészet a megfelelő detergens kiválasztása. E tekintetben a legfontosabb a megfelelő hidrofil-lipofil balance szám (HLB) (54). A fentebb említett népszerű detergens családok leggyakrabban használt tagjainak az esetében a HLB szám általában 12 és 16 között van. Ebben a tartományban a detergensek általában egy percen belül megnyitják, 'fenesztrálják" a plazma membránt, de a sejtek megfigyelhető ion-equilibrációja igen eltérő. Dr. Kellermayer Miklós kimutatta, hogy mind a Triton X-100, mind pedig a Brij 58 effektíven távolítja el a plazma membrán lipidjeit, de az előbbi esetében a sejt igen gyorsan dekomponálódik, míg az utóbbi esetében egy meglepően hosszú ideig -mely az adott sejttípustól is függ- a sejtek megtartják magas K tartalmukat egy alacsony iontartalmú médiummal szemben is. (50-52,55,56).

Meglehet ez a típusú megközelítés és módszer laboratóriumunkhoz fűződik, a plazma membrán szelektív eltávolításának az ötlete nem előzmények nélküli. Béka sartorius izom harántszekciójával a hatalmas izom-sejtek felszínének egy jelentős hányada megnyitható a környezet felé, s pl. izotóp-tartalmú Ringer-oldatból a K és Na bejutása nyomon követhető. Ezen mérések egyértelműen ugyanazt a következtetést engedik levonni, amit mi a detergens kezelt sejtek esetében tehettünk meg, ti. hogy az oldott anyagok híg vizes oldatokra jellemző diffúziós hányadosainál jóval lassúbb az equilibráció, tehát valamilyen módon a citoplazma erőteljes retenciós képességgel rendelkezik (57). Itt természetesen triviálisnak tűnhet, hogy az egyes subcelluláris kompartmentek valameddig a plazma membrán átjárhatóvá tétele után is intaktak maradhatnak. Ezt azonban megnyugtatóan kizárhatjuk, mikor a jelenséget érett emberi vörösvértesteken vizsgáljuk, s lényegében ugyanezt találjuk (58,59).

De nem csak a CMC feletti detergenshatás jelenségei bizonyultak érdekesnek, hanem kiterjedt tanulmányokat kezdtünk a CMC alatti detergens hatás elemzésére is. Meglehet a CMC a detergensek lipid oldó képességével társult, a CMC alatti detergens hatás eseteiben is gyorsult a sejtek, s környezetük között az ion kicserélődés (60,61). Fontos megjegyezni, hogy a detergens hatások "tiszta" elemzésére csak itt van lehetőség. Ennek oka, hogy a leggyakrabban használt nem-ionos detergensek a CMC felett hemolítikusak.

Érdekes ugyanakkor, hogy sok nem ionos detergens a CMC alatti koncentrációban is hemolítikus, s ezért szerencsésebb volna egy nem hemolizáló, általában a CMC-nél alacsonyabb detergens koncentráció értéket is megadni. Sajnos azonban itt további nehézséget jelent, hogy az adott detergenssel szemben az egyes donorok vörösvértestjei eltérő rezisztenciát mutatnak. Az egyetlen használható "modus operandi" az, ha olyan alacsony detergens koncentrációt alkalmazunk, mely biztosan, az egyes vérmintákon sem hemolytikus.
Vizsgálataink során a detergenseknek mind a *poláros "head group", mind pedig az apoláros "tail" része szerepét vizsgáltuk a K és Na kicserélődések tekintetében*, olyan körülmények között, amikor aktív transzport nem lehetséges. Ugyanígy, vizsgáltuk azt, hogy meg lehet e "menteni" a detergenssel kezelt sejteket? S, ha igen milyen mechanizmusok révén áll helyre a sejtek elektrolit egyensúlya?
Eredményeink számos érdekes adattal egészítették ki korábbi vizsgálatainkat, s ahogy az gyakran történni szokott, még több új érdekes kérdést vetettek fel.

Kérdésfelvetések:

Van-e összefüggés érett emlős vörösvértestekben a metabolizmus sebessége (glukolízis sebessége) s az egyértékű ionok szintje között? Van-e összefüggés a vörövértestek ATP (ADP, P) tartalma és az ionszintek között? Miként alakulnak a vörösvértesben az ionszintek a sejtek öregedése folyamán? Létezik-e vörösvértest polimorfizmus nem kérődző fajban?

Ezen belül vizsgálni kívántuk a különböző fajok vörösvértestjei közötti eltéréseket, valamint egy fajon belül a különböző egyedek vörösvértestjei közötti eltéréseket.

Amint azt a bevezetőben már részben kifejtettük, a "membrán-oldat" elmélet szerint a pumpaműködés fenntartása energiát (ATP-hidrolízis) követel, s ennek megfelelően összefüggés várható a glukolízis sebessége és vörösvértest valamint a plazma között fenálló K-Na gradiensek mértéke között. Mivel a "pumpa" működtetése bármely nullánál nagyobb ATP szint mellett is létrejöhet, ha az ATP megfelelő sebességgel pótlódik, s mivel más ATP molekulákat hidrolizáló aktivitások is vannak a vörösvértestekben, az ATP szintek és K-Na gradiensek nagysága között *nem* várunk ezen elmélet alapján összefüggéseket. Más a helyzet a szorpciós hipotézis szempontjából, mely szerint a K-Na gradiensek fenntartása nem követel energiát, de a steady state ATP szintnek szerepe van a sejtre jellemző ion-koncentrációk kialakításában.

Vizsgálni kívántuk továbbá az ember eltérő denzitású (korú) vörösvértestjeinek ionszint eltéréseit. Itt a várakozás elsősorban az, hogy az idősebb vörösvértestek K szintje csökken, míg a Na szint emelkedik. Ezt azért gondoltuk így, mert érett vörösvértestekben nincs fehérjeszintézis, s ezért a "pumpa molekulák" utánképzésére sincs lehetőség.

A nem kérődző szamárban (Equus asinus) észlelt K és Na polimorfizmust azért vizsgáltuk, mert a fentebb említett, a vörösvértest metabolizmus és K-Na szintek kapcsolatának vizsgálata során két szamártól is vettünk vérmintákat, s jelentős eltéréseket találtunk.

Mivel nem kérődző fajban korábban nem írtak le K-Na polimorfizmust, jó helyzetbe kerültünk, hogy elsőként közöljük ezt az érdekes jelenséget még akkor is, ha nem kapcsolódott szorosan eredeti célkitűzéseinkhez.

Miként alakulnak az ionviszonyok nem ionos-detergensekkel kezelt vörösvértestekben, különös tekintettel a Brij szériájú polyoxyethylene-származék detergensekre?

Ezen belül kiegészítő adatokkal kívántunk szolgálni ahhoz a megfigyeléshez, hogy a *CMC-feletti* koncentrációnál Brij 58 detergenssel kezelt sejtek miként tartják meg meglepően hosszú ideig magas intracelluláris K tartalmukat. Ugyanezt a folyamatot vizsgálni kívántuk szöveteken is.
Vizsgálni kívántuk a K vesztési folyamat *reverzibilitását.* Lehetséges-e albumin hozzáadásával eltávolítani a detergens molekulákat a sejtmembránból úgy, hogy azok visszanyerjék életképességüket?
Vizsgálni kívántuk a *detergensek azon tulajdonságait, amelyek a környezettel történő ion-equilibráció szempontjából fontosak.* Milyen hatással van a hidrofób "tail" és a hidrofil "head" rész növelése a kicserélődési folyamatra?

Ismeretes, hogy a detergens molekulák sajátosságait leginkább a hidrofób és hidrofil részek aránya (HLB szám) határozza meg. A Brij szériájú detergensekben a hidrofób rész egy zsírsav (eltérő méretű, telített vagy telítetlen), míg ismétlődő oxyethylén egységek képezik a vízoldékony részt (ezek száma is meglehetősen eltérő lehet).
Nemrég nyílott csak lehetőség arra, hogy aránylag olcsón nagyobb Brij detergens szériák elérhetők legyenek.

Megközelítések-módszerek:

Vizsgálatainkhoz általában heparinos vért használtunk (ca. 125NE Na-heparin/ml vér). Az emberi mintákat egészséges önkéntesektől a Klinikai Kémiai Intézet Ambulanciáján nyertük. Állati véreket többnyire az Egyetem Állatházából, vagy a PATE farmjáról szereztünk be. Ugyanígy a kutya véreket az Egyetem Kísérletes Sebészeti Intézetéből szereztük be. Macska vért az Egyetem Élettani Intézetéből szerezhettünk be. Mindezeket az intézményeket és a közreműködő személyeket köszönet illeti.

A steady state metabolitszintek vizsgálatára szánt mintákat általában a vérvétel után azonnal folyékony nitrogénban fagyasztottuk a megfelelő kezelés, pl. perklórsavas kicsapás után. Az elektrolitviszonyok vizsgálatához vagy a kinetikus metabolikus vizsgálatokhoz a mintákat általában jégen szállítottuk. Törekedtünk arra, hogy a vérmintákat a lehető legrövidebb időn belül fel is használjuk. Ez maximum 120 percet jelentett, kivéve a szamár vérmintákat, melyek néha igen messziről érkeztek.

ATP méréseket kétféle módon végeztünk. Vagy a Boehringer cég által fejlesztett luciferin/luciferase kitt segítségével mértünk, vagy pedig HPLC metodikát használtunk.
A biolumineszcens ATP mérésekhez teljes vért használtunk, a hematokrit értékeket vagy hematokrit kapillárisban történő centrifugálással, vagy pedig sejtszámlálóval (Coulter Counter, Cell-Dyn-3500) határoztuk meg. Ezt követően a vérmintákat a Kőszegi által leírt módon higítottuk a biolumineszcens méréshez (62). Ez eseteinkben általában két lépcsőben történő 4625-szörös higítást jelentett. A higított vérmintákat ezt követően 0.1ml frissen elkészített regenssel hoztuk össze, a kibocsájtott fényintenzitást Berthold luminométerrel mértük. Ismert koncentrációjú és mennyiségű ATP standard hozzáadása után minden minta fénykibocsátását újra mértük. Egy vérmintát általában háromszor mértünk le, s az eredményt e három mérés átlagaként adtuk meg. A minta és a standard+minta mérési eredményéből számolható az ismert mennyiségű és koncentrációjú standardra vonatkozó fényintenzitás, s ebből valamint a higítási és hematokrit értékekből a vörösvértestekre jutó ATP tartalom.

Az ATP szintek HPLC rendszer segítségével történő meghatározása során a mintákat előbb perklórsavval csaptuk ki, s neutralizálás után Stocchi és munkatársai módszerét használva mértük az ATP szinteket (63). (Ezekben az esetekben is additív standardokat használtunk, s a görbe alatti területekből a Beckman Gold System típusú HPLC szoftver megfelelő programozásával végeztük számításainkat.)

A kálium és nátrium szintek meghatározásához a vérmintákat kétféle módon kezeltük. 1.: 1 ml vért microcentrifugában centrifugáltunk 5 percig 18000g-nél, majd a plazmát és 'buffy coat"-ot eltávolítottuk. Ezt követeüen a vörösvértesteket további 10 percig centrifugáltuk, s a maradék plazma cseppeket is eltávolítottuk. Ezzel az egyszerű módszerrel a reziduális sejtek közötti víztér 2,2-3.1%. (Referencia: szilikon olajban centrifugált vörösvértestek víztartalma.) Ezt követően, amennyiben csak K és Na méréseket akartunk végezni, legegyszerűbb a vörösvértestek direkt bemérése volt a lángfotométer higító oldatába (50µl vvt. 5ml Li higító), amit egy Braun automata higítóval végeztünk el. A lángfotométer belső standardjaként használt híg lítiumos oldatban a vörösvértestek azonnal szétesnek, s megfelelő standardizálás mellett azonnal mérhetők az elkektrolittartalmak is. Méréseinket egy OMSZÖV lángfotométeren végeztük. 2.: Azokban az esetekben amikor más komponenesek, pl víztartalom, Cl, P, Mg, Ca tartalmak is érdekeltek bennünket, a fentebb leírt centrifugálásokat analitikai mérlegen lemért mikrocentrifuga csövekben végeztük. A centrifugálási lépések után a cső + vörösvértestek tömegét ismét meghatároztuk, majd a mintákat egy Savant SC-110 gyorsvákuum rendszeren súlyállandóságig szárítottuk. Ezt ismét gravimetriás mérés követte, majd a mintákhoz nagyságuk szerint 0.5-1ml 1M HCl-t tettünk. A lezárt csöveket rázás mellett 24 óráig tártuk fel, majd mikrocentrifugában centrifugáltuk (14000g 5 perc). A K és Na meghatározásokat a felülúszóból végeztük. A K és Na szinteket pedig a súly és higítási adatokból számoltuk.

A Mg, K és Rb méréseket egy Varian AA-20 atomabszorpciós spektrofotométeren végeztük megfelelő kalibrálás után. A mérések a fentebbi bekezdésben leírt sósavas felülúszóból készültek. A Cl és P méréseket ugyaninnen, az intézeti rutin kolorimetriás mérésekkel együtt végeztük.

A vörösvértestek glükóz fogyasztásának meghatározása:

A heparinozott teljes vér egy részét steril minta csövekben 37°C-on inkubáltuk és a glükóz szinteket a 3 órás inkubáció előtt és után is meghatároztuk. Egy másik részét a vérnek centrifugálás után 5 mmol/l glükóz tartalmú Hanks' oldatban reszuszpendáltuk. A glükóz szintet glükóz oxidáz-peroxidáz módszerrel (Reanal, Hungary) mértük. A hemoglobin (Hb) koncentrációt fotometriásan (Coulter CBC5) határoztuk meg és a glükóz fogyasztást mmol/kg Hb/óra fejeztük ki.

A tejsavtermelés meghatározásához a jól bevált laktát-dehidrogenáz enzimet használtuk. Az eredmények számolásánál hasonlóan jártunk el, mint a glukóz-fogyás mérésénél.

Detergens kísérleteinkhez mosott vörösvértesteket, vagy sertés szemlencséket használtunk. Kísérletezésre alkalmas vörösvértesteket a fentiekben leírt módon nyertünk, ezért itt csak a sertés szemlencsék "előállításáról" emlékezem meg. Sertés szemeket a helyi vágóhídról szereztünk be. A szemeket tiszta üveglapon szikével "szedtük szét", vigyázva arra, hogy a lencsetokot ne sértsük meg. A lencséket nedvesített papírvatta mellett, kis Petri edényben tároltuk 4°C-on, amíg fel nem használtuk. Erre általában egy-két órán belül sor került .

A felhasznált detergenseket vagy a Sigma vagy pedig a Serva cégtől szereztük be. A kísérletek mindig frissen készített detergens oldatokkal történtek.

Statisztikai analíziseinket a Borland Scientific Company Quattro for Windows 5.0 programjával végeztük.

Nem lehet célom itt lefordítani az összes, a tézisek anyagát képező közlemény metodikai részét. A fenti, általában előforduló mérések is jelentős változásokon mentek át, s így az olvasónak mindig helyes a megfelelő közlemény "Materials and Methods" részéhez fordulni.

Számos olyan eljárás van ugyanakkor, amelyet nem alkalmaztunk általánosan, s itt a jobb tájékozódás kedvéért egy rövid keresési útmutatót helyezek el.

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Eredmények és megbeszélés:

Az emberi és kutya vörösvértestek a HK, illetve az LK vörösvértest csoportok tipikus képviselői. Mint az az első dolgozat első táblázatából kitűnik (I./1. táblázat), a plazma K és Na szintek között viszont nincs lényeges eltérés. Amennyiben az emberi és kutya vörösvértesteket egyszerű 5mM KCl-al komplementált fiziológiás sóoldatban tartjuk, a steady-state ATP szintek igen hasonló mértékben esnek (I/1. ábra). A dolgozat legérdekesebb megfigyelése azonban az ATP szintek és a K tartalom közötti összefüggés (I/4. ábra), amelyet részletesen a második dolgozatban elemeztünk.
Emlős állatfajok vörösvértestjeiben (II/1. táblázat) szoros pozitív összefüggés van a K szintek ill. az ATP-tartalom között. Ugyancsak érdekes -meglehet nem meglepő- az ATP és Mg tartalmak közötti összefüggés. Erre utal ugyan az a tény, hogy számos ATPáz szubsztrátja a Mg-ATP, de például nagy klinikai beteganyagokon többeknek nem sikerült az ATP tartalom és a Mg tartalom változásai között összefüggést találni.. Ugyanígy, hasonló öszefüggést találtunk az egyes juhok vörösvértestjeinek ATP és K-Na tartalmát vizsgálva (II/2. táblázat). Nem találtunk összefüggést viszont a vörösvértestek glukolízisének sebessége, s a K-Na tartalom között (II/3. táblázat).

Mióta ez a dolgozat megjelent, számos új mérést is végeztünk. Ezek némelyikében Dr. Robert J. P. Williams, az Oxfordi Egyetem Anorganikus Kémia Tanszékének professzora volt egyik tanácsadóm, akivel érdekes megbeszéléseink voltak. Általában in vitro körülmények között a pumpaműködés sebessége az ATP/(ADP+P) hányadossal függ össze. (Újabb vizsgálódásaink során is lángfotometriás módszerrel mértük a vörösvértest K és Na tartalmakat, de a luminometriás ATP mérés helyett HPLC technikát alkalmaztunk.) Mint az az adatainkból jól kitűnik, újabb megfigyeléseink alátámasztják a korábbiakat, valamint rávilágítanak arra a tényre, hogy nincsen értékelhető összefüggés a vörösvértest K tartalmak és az ATP/(ADP+P) hányados között (2. táblázat).

2. táblázat

	[ATP]	[ADP]	[P]	[ATP]/([ADP]+[P])
$[K^+]$	0.723$^{\$}$	0.456*	0.744$^{\$}$	0.335*
Log $[K^+]$	0.702$^{\$}$	0.439*	0.723$^{\$}$	0.330
$[Na^+]$	-0.400*	-0.232	-0.356*	-0.368*
Log $[Na^+]$	-0.364*	-0.231	-0.315	-0.346*
$[K^+]+[Na^+]$	0.725$^{\$}$	0.466*	0.776$^{\$}$	0.241
Log $[K^+]+[Na^+]$	0.719$^{\$}$	0.468*	0.776$^{\$}$	0.239
$[K^+]/[Na^+]$	0.499*	0.339*	0.466*	0.355*
Log $[K^+]/[Na^+]$	0.536$^{\#}$	0.337*	0.512*	0.366*
Water	0.608$^{\#}$	0.412*	0.583$^{\#}$	0.025

The relationship between ATP, ADP and phosphorus levels, and the elektrolyte concentrations of erythrocytes 13 HK species. Data sets of 34 individual animals are included (n = 34).
Data significant at:

$^{\$}$ $p < 0.0001$
$^{\#}$ $p < 0.001$
* $p < 0.05$

Továbbá, a vörösvértestek ATP, ADP és foszfor tartalma között jó korreláció van. A vörösvértestekre jellemző K + Na tartalmak meglepően állandóak. Újabb adatokkal erősítettük meg azt a megfigyelésünket is, hogy míg a steady-state metabolit

koncentrációk jól korrelálnak, a glukolízis sebessége nem függ össze a K és Na tartalmakkal vörösvértestekben.

Összefoglalva a vörösvértest K és Na szintekkel, a steady-state purin metabolit szintekkel, valamint a glukolízis sebességével kapcsolatos vizsgálatainkat elmondható, hogy **1. Szoros poztív összeföggés van a vörösvértest ATP szintek és a K szintek valamint a K/Na hányadosok között. Hasonlóan szoros a negatív összefüggés az ATP szintek valamint a Na szintek között. 2. Az ATP szintek és a Mg szintek között is szoros pozitív összefüggést találtunk, ami valószínüleg az élettanilag fontos Mg-ATP komplex jelenlétét húzza alá. 3. Az ATP, ADP és P szintek egymással jól korrelálnak, s ezért természetesen jó az összefüggés az ADP, P, valamint a K és Na szintek között is. 4. Az ATP/ADP szintek meglepően állandóak a különböző HK és LK típusú fajokban. Nincs értékelhető összefüggés az ATP/(ADP+P) hányados valamint a K szintek között, ami azért meglepő, mert elsősorban ettől függ in vitro a Na-K-ATP-áz turnovere. 5. Nem találtunk értékelhető eltérést a HK és LK típusú juh vörösvértestek glukolízisének sebességében. Ugyanez áll abban az esetben is, ha eltérő fajok vörösvértestjeinek glukolítikus sebességét vetjük össze.**

Jelenleg vizsgálataink az egyes fajok vörösvértestjeinek ouabain függő és ouabaintól független ATP-áz aktivitásainak meghatározására irányulnak. Ugyanígy vizsgáljuk a K-Rb kicserélődés sebességeit, valamint a glukolítikus aktivitás változásait. ^{3}H-ouabainnal tanulmányozzuk a ouabain kötő helyek számát is. Mindezen vizsgálatok erdeményei már most is érdekesek, de mivel közlésre őket össze még nem foglaltam, ezt az anyagot a jelen tézisekben nem szerepeltetem.

Dr. I. L. Cameronnal kooperációban vizsgáltuk individuális emberi vörösvértestek elektrolittartalmát röntgen sugár mikropróba analízis segítségével. A sejtek K, Na, Cl, Ca, Mg, és kén tartalmát vetettük össze. A **K és Cl tartalmak csökkennek, míg a Ca és kéntartalmak növekednek az öregebb, alacsonyabb víztartalmú vörösvértestekben, de a foszfor, Na és Mg tartalmak tekintetében nem lehetett értékelhető összefüggést találni** (III./1 ábra és III/1. táblázat).

A K és Cl tartalmak csökkenése egyértelműen a vörösvértestek vízvesztésével, zsugorodádásával társultak.

Mellékleletként került elő, de leíró szempontból igen érdekesnek bizonyult a szamarakban található vörösvértest K-Na polimorfizmus. A jelenség annyiban új, hogy kérődzőkön kívül nem volt ismeretes K és Na polimorfizmus. III./1-es ábránkról kitűnik, hogy az LK típusú állatok vörösvértest K tartalma nem olyan alcsony, s Na tartalma nem olyan magas, mint például a juhokban szokásos értékek. Egy meglehetősen diszkrét, általunk intermedier K tartalmunak nevezett csoport is azonosítható. Meglehet az LK/HK vörösvértest tulajdonság domináns/recesszív öröklésmenetű a juhokban, hasonló jelenség ott is ismert, a heterozigóta állatok esetében. Az LK tulajdonságot hordozó gén inkomplett dominanciája folytán szokás ezt inkomplett domináns-recesszív öröklésmenetnek nevezni. **Az általunk elérhető származási adatok lényegében ugyanerre utalnak a szamarak esetében is.** Némi -de nem túl nagy meglepetésünkre- nincs az országban szamár törzstenyészet, ezért kénytelenek voltunk a vérmintákat a dél Dunántúl több megyéjéből begyűjteni, s egyéni gazdák sokszor bizonytalan adataira támaszkodni.

A nem-ionos detergensek élettani hatásának tanulmányozása a különböző sejtek ion equilibrációjára laboratóriumunkban komoly múltra tekint vissza, ami elsősorban Dr. Kellermayer Miklós és Dr. Ludány Andrea működéséhez kapcsolt. Magam elsősorban Dr. Bogner Péter munkatársammal együtt ezen élettani hatások hátterében álló fiziko-kémiai mechanizmusokkal foglalkozom. Az itt bemutatott közlemények sejtmodelljei szintén vörösvértestek, szervmodellként a szemlencsét használtuk.

Mint az a IV. közleményünk 3. ábrájából kitűnik, a nagyon hasonló kémiai szerkezetű Triton X-100 és Brij 58 detergensek hatása CMC feletti koncentrációban alkalmazva őket, igen eltérő. Míg az előbbi csaknem azonnali ion-equilibrációt okoz, a Brij 58 hatása elhúzódóbb. Ezzel lényegében egyidejüleg fehérje kiszabadulást figyelhetünk meg mindkét esetben. A jelenség legfőbb érdekessége, hogy a Brij 58 esetében az elhúzódó K rilíz annak ellenére jön létre, hogy a plazma membrán már minden sejtben biztosan kinyílott. Figyelembe véve a K diffúziós hányadosát ($1.99cm^2/s$), s a sejt méretét, ez a sejt és környezete közötti azonnali ion-equilibrációt kellene hogy eredményezzen, amennyiben a citoplazma híg vizes oldatokra jellemző

módon viselkedik. Más szavakkal a citoplazmának erőteljes ion retenciós hatásának kell lennie, különben a jelenség nehezen értelmezhető. Mégis, mivel morfológiai bizonyíték csak korlátozottan használható egy fiziko-kémiai jelenség kontrolljaként, vizsgáltuk a plazma membránból történő lipid rilízt, s normálisan az élő sejtbe be nem jutó (szupravitális festék, tripánkék) felvételét is. Kimutatható, hogy a Brij 58 lipid mobilizáló hatása valóban jelentős. Ugyanígy, minden egyes sejt felveszi már rövid idejű Brij 58 kezelés után is a tripánkéket. Mindezek az adatok együtt azokkal a megfigyelésekkel, melyeket szintén Dr. Kellermayer Miklós eredeti megfigyelései ihlettek, jól igazolható, hogy: 1. A plazma membrán detergens mediált eltávolítása után is egy ideig fennmarad a sejtek magas K tartalma. 2. A fehérje rilízzel együtt jön létre a K vesztés. 3. Dr. I. L. Cameron és J. B. Clegg adatai alapján is nyivánvaló, hogy az equilibráció az egyéni sejtek számára nem folyamatos, hanem egy hirtelen létrejövő denzitás (fehérje) vesztéssel együtt alakul ki (52). A folyamatos jelleg csak azért alakul ki, mert általában viszonylag nagy sejtpopulációkat, -melyek életkor és sérülékenység tekinteteiben erősen eltérnek-, vizsgálunk.

A továbbiakban magunk elsősorban a detergens molekulák oldaláról közelítettünk a detergens hatásmechanizmusok felé. Mivel a CMC felett a detergensek általában erősen hemolítikusak, a CMC alatti detergens hatásokkal kezdtük vizsgálatainkat. Megjegyzendő ugyanakkor, hogy a detergensek kumulálódni is képesek a sejtben, ezért pl. viszonylag kis térfogatú CMC alatti koncentrációjú detergenssel kezelve a sejteket először azok nem lizálódnak, de ismételt mosás hatására igen.Elsősorban a Brij szériájú detergenseket vizsgáltuk, melyek általános képlete a következő:

$$\mathbf{R\text{-}O\text{-}(CH_2\text{-}CH_2\text{-}O)_nCH_3}$$

(R, zsírsav)

Főbb eredményeinket abban foglalhatjuk össze, hogy az **oxyethylén egységek számának a növelése egyideig (n 20-ig) fokozott ionkicserélődést okoz, majd meglepően hirtelen hatáscsökkenés következik be akkor, ha n-21** (V./2 ábra). Érdekes módon **az apoláros zsírsav farokrész hossza relatíve indifferens. Ugyanakkor a telítetlen olajsav sokkal effektívebben facilitálja az ion kicserélődést (azonos poláros hossz mellett), mint a telített sztearinsav,** V./3. ábra).

Ezeken az eredményeken túl megállapítottuk, hogy különböző fajok vörösvértestjeinek detergens rezisztenciája eltérő (adatokat itt nem mutatunk be). Ez a megfigyelés ugyan önmagában nem meglepő, de az egyes fajok membrán lipid és fehérjeösszetételének eltéréseit összevetve észleleteinkkel, fontos járulékos információkat adhatnak a vörösvértest membránok stabilitásával kapcsolatosan.

Ezekkel a megfigyelésekkel párhuzamosan végeztük azokat a kísérleteinket, melyekben a detergens hatás reverzibilitását vizsgáltuk in vivo. Mint az a VI./2. ábrából kitünik, az alacsony koncentrációjú Brij 58 detergens jelenlétében 0°C-on inkubált vörösvértestek jelentős mértékben veszítik a káliumot. Ez a hanyatlás azonban **visszafordítható abban az esetben, ha a vörösvértesteket reinkubáljuk plazmában vagy szérumban**. A továbbiakban választ adunk arra, hogy elsősorban a szérum **albumin** felelős a detergens molekulák sejt membránból történő eltávolításáért.
Érdekes módon a K tartalom nem annyira aktív transzport, hanem a sejt vízvesztési mechanizmusa révén áll helyre.

Szemlencse kísérleteinket 1988-ban kezdtük. Szándékunkban állt inkább egy tömegesebb szervet, mint egy sejtet vagy sejtcsoportot vizsgálni. A legtöbb szövet azonban alkalmatlan erre, mert a sejtrétegek egymást lefedően vannak jelen, s a kinetikai jellegű vizsgálatok az egyes rétegek konszektív megnyílása miatt lehetetlenek. Ugyancsak megnehezíti az értelmezést, hogy legtöbb szövet nem egynemű sejtekből áll. A szemlencse szerkezete, amely valmelyest a hagymáéra emlékeztet (2. ábra), lehetővé teszi, hogy az összes sejtet egyszerre nyissuk meg. A szemlencse kivételes abban a tekintetben is, hogy egynemű sejtekből áll, nincsenek benne véredények. Eltérő módszerekkel jutottunk arra a következtetésre, hogy a szemlencsében levő víz a korábban feltételzettnél intimebb viszonyban van a lencse fehérjékkel (ábrák). NMR titrációs eljárással, az ozmotikusan inaktív szemlencse vízfrakció meghatározásával, utracentrifugálással, s festékkizárási teszttel igazoltuk, **hogy a szemlencse víz 77-100%-a van kötött állapotban.** Egy igen érdekes, a detergensekkel kapcsolt kutatásainkhoz tartozó megfigyelésünk volt, hogy tripánkék, nigrozin és metilénkék nem festik meg a szemlencsét, még akkor sem, ha azokat 1% Triton X-100 jelenlétében 24 óráig inkubáljuk. Ugyanakkor a szkléra és más szövetek kiválóan festődtek.

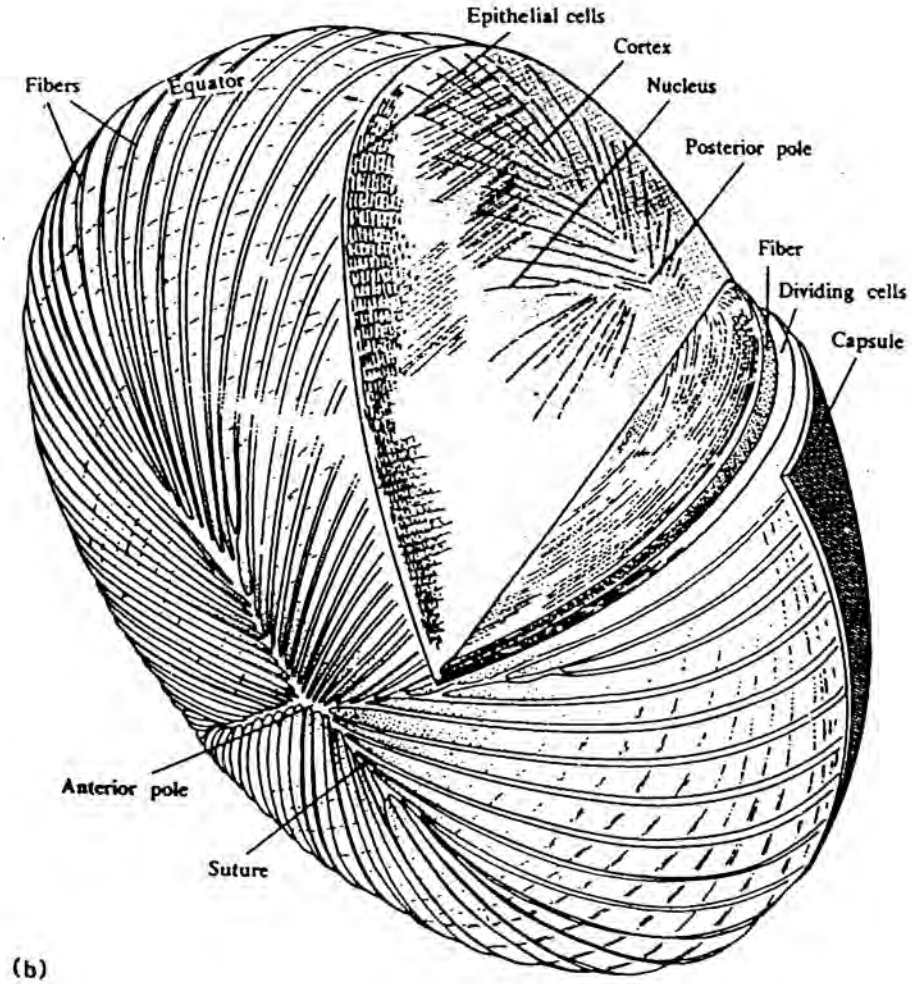
Epithelial cells
Cortex
Nucleus
Posterior pole
Fiber
Dividing cells
Capsule
Fibers
Equator
Anterior pole
Suture
(b)

FIG. 66b

Ennek a megfigyelésnek egyenes folytatása volt, az amikor a szaharóz bejutását és a K valamint Na ionok kijutását vizsgáltuk sertés szemlencséből detergenskezelés nélkül, s detergenskezeléssel együtt. Vizsgálataink fő konklúziója, hogy Triton X-100 kezelés mellett az **ionok kijutása arányos a szaharóz molekulák bejutásával** (IX/2. ábra). Mivel a szaharóz és a K-Na ionok között nincsen sem direkt, sem pedig indirekt kapcsolat, feltételeztük, hogy a szemlencse sejtek citoplazmájának a detergens mediált dekompozíciója az, ami elsősorban meghatározza az egyértékű kationok kiáramlását.
Ugyanez a folyamat az, melynek révén a szemlencse víz "jó oldószerré" alakul át a különben igen rosszul equilibrálódó szaharóz számára.

Arra, hogy játsznak-e szerepet az aktív transzporton kívüli tényezők a szemlencse ion-egyensúlyának alakításában, jó megközelítés a lencséket 0°C-on inkubálni.
Ilyenkor az energia metabolizmussal kapcsolt folyamatok nem működhetnek.
Eredményeink szerint az eltérő mennyiségű nátriummal komplementált szérumban inkubált sertés lencsék ugyan veszítenek káliumot és nyernek nátriumot, **de mindvégig (15-20 órán át) sokszorosan magasabb K koncentrációt tartottak fenn, mint a környező médium** (X./1 ábra). Igen érdekes, hogy a szemlencsék nem duzzadnak, ami a membrán-pumpa elmélettel nem egyezik. (Pumpamüködés hiányában a sejtek duzzadnak). A térfogat regulációval kapcsolatos problémáról itt csak megemlékezünk, de komplexitása miatt nem diszkutáljuk. Bővebb információért az olvasónak William Negendank összefoglalóját ajánljuk. **Becslésünk szerint a szemlencse kálium 35-40%-kötött állapotban van.**

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Human and Dog Erythrocytes: Relationship between Cellular ATP Levels, ATP Consumption and Potassium Concentrations

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Abstract: The intracellular K^+/Na^+ ratio of various mammalian cell types are known to differ remarkably. Particularly noteworthy is the fact that erythrocytes of different mammalian species contain entirely different potassium and sodium concentrations (1). The human erythrocyte is an example of the supposedly "normal" high potassium cell, while the dog erythrocyte contains ten times more sodium than potassium ions (Table 1). Furthermore, this difference is sustained despite the plasma sodium and potassium concentrations being almost identical in both species (high Na^+ and low K^+). In spite of these inorganic ion differences, both human and dog erythrocytes contain 33% dry material (mostly Hb) and 67% water.

Conventional cell theory would couple cellular volume regulation with Na^+ and K^+ dependent ATPase activity which is believed to control intracellular Na^+/K^+ concentrations. Since the high Na^+ and low K^+ contents of dog erythrocytes are believed to be due to the lack of the postulated Na/K-ATPase enzyme (3,11,15,17,18,19), they must presumably have an alternative mechanism of volume regulation, otherwise current ideas of membrane ATPase activity coupled volume regulation need serious reconsideration.

The object of our investigation was to explore the relationship between ATPase activity, ATP levels and the Na^+/K^+ concentrations in human and dog erythrocytes. Our results indicate that the intracellular ATP level in erythrocytes correspond with their K^+, Na^+ content. They are discussed in relation to conventional membrane transport theory and also to Ling's "association-induction hypothesis", the latter proving to be a more useful basis on which to interpret results.

THE RATIO OF INTRACELLULAR POTASSIUM and sodium ions varies within a broad range in different cell types. Nevertheless the intracellular potassium content is an order of magnitude higher than the intracellular sodium content in most cell types. The dog erythrocyte is an exception to this generalization, since the dog erythrocyte contains over ten times more sodium than potassium ions (Table I and refs 1,11,18,21).

The generally held "membrane-solute hypothesis" of living cells explains cellular volume regulation by the help of the ubiquitous Na^+/K^+ transport ATPase, which apparently does not function in the membranes of dog red blood cells (3,11,17,18,19,20,21). Yet the water content of dog erythrocytes is 2.03g water/g dry material, which agrees well with the water content of human erythrocytes (Table I).

This simple fact raises the question as to whether the dog erythrocyte developed a new mechanism of volume regulation simultaneously with the loss of the transport ATPase, and if not, whether the popular idea which explains volume regulation by work of the Na^+/K^+-ATPase must be corrected.

In the present paper experimental data supporting the idea that volume regulation in dog and human erythrocytes is not bound to the postulated Na^+/K^+-ATPase will be presented. Our observations support the idea that a simple relationship exists between the ATP level and potassium concentration of erythrocytes.

Materials and Methods

Blood from healthy human individuals and from dogs (provided by the Department of Experimental Surgery) was collected into heparinized vials and transferred to ice. Fresh bovine, sheep and pig blood was collected into heparinized vials at the local slaughterhouse. The blood of golden hamster was drawn from the arteria carotic and collected into heparinized vials. Blood samples were kept on ice until the start of the experiment. The processing of samples for experiments started within the shortest possible time, usually within one hour.

ATP measurements

ATP measurements were carried out with the firefly luciferin/luciferase ATP measuring kit (Boehringer-Mannheim). We used a Biolumat LB 9505 (Berthold) chemiluminometer. Blood samples were centrifuged on a tabletop (Hettich) centrifuge at 3000g at room temperature for 15 min. Five microliter aliquots were drawn into a Hamilton pipette and resuspended in 10mM TRIS-HCl containing physiological saline (151mM NaCl solution), pH 7.4 (solution A), or 5mM KCl complemented solution A (solution B), or 10mM TRIS-HCl containing isosmotic KCl solution, pH 7.4 (solution C) at 1:1000 vol/vol ratio. The samples were incubated at 37°C for 6 hours and 50 microliter test samples (duplicates) drawn at different time intervals. Since erythrocytes sink to the bottom of the test tube, they had to be carefully resuspended before drawing test samples. Test samples were lysed in 1ml of 0.1% Triton X-100 containing measuring buffer (10) and 50 microliter luciferin/luciferase solution added directly to the measuring cuvettes. Serial dilutions of an ATP solution of known concentration served as a standard curve. ATP concentration was calculated after the extrapolation of observed cpm results to the standard curve. Results are also expressed in cpm or in relative cpm units (see the legend of Figure 1).

Molecular/ionic species	Human erythrocyte	Dog erythrocyte	Ratio human/dog erythrocyte	Human plasma	Dog plasma
K mmol/l water	138 ± 3.5 n = 18	9.0 ± 1.1 n = 6	15.33	4.9 ± 0.3 n = 18	4.8 ± 0.2 n = 6
Na mmol/l water	18,0 ± 1.9 n = 18	150 ± 6.2 n = 6	0.12	150 ± 4.2 n = 18	148 ± 3.9 n = 6
ATP mmol/l RBC	1.65 ± 0.26 n = 70	0.80 ± 0.10 n = 4	2.06	——	——
H_2O water/dry mass	2,03 ± 0.10 n = 5	2.03 ± 0.17 n = 4	1.00	——	——

TABLE I. The intracellular potassium and sodium contents of human and dog erythrocytes differ remarkably. This difference is sustained despite the plasma potassium and sodium concentrations being similar. In spite of the difference in the intracellular K^+/Na^+ ratios between the erythrocytes of the two species, their erythrocyte water content is identical.

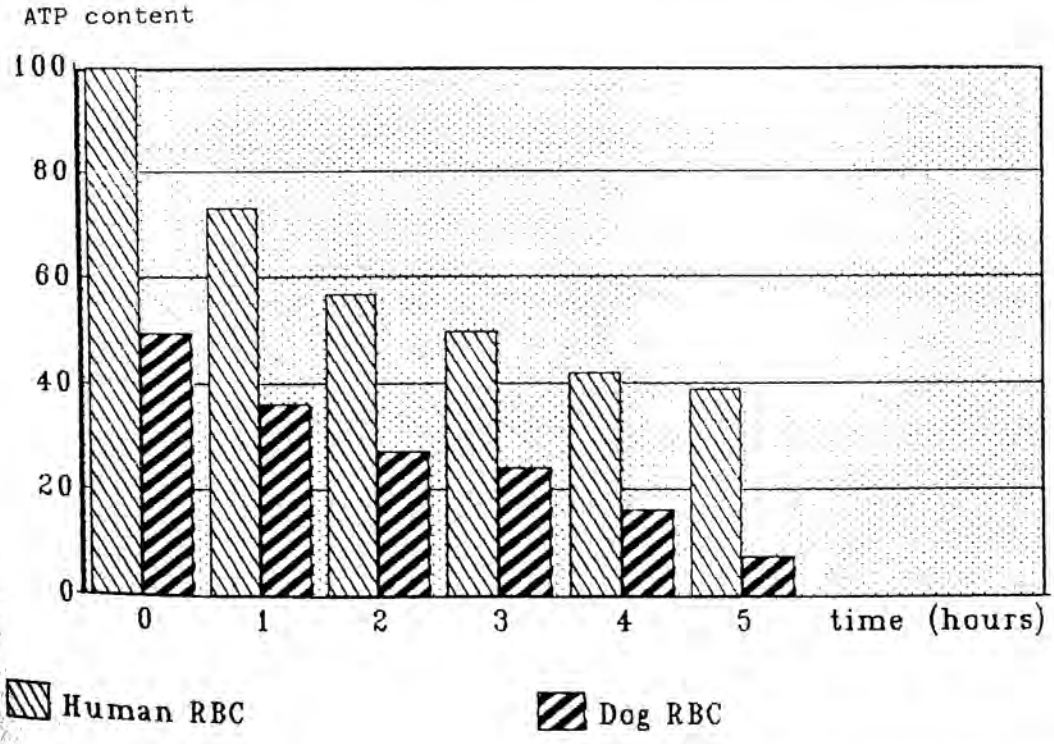

FIGURE 1. Dog and human erythrocytes were incubated in solution B (5mmol/l KCl complemented physiological salt solution, pH 7.4), at a 1:1000 cell:solution vol/vol ratio at 37°C. ATP content is plotted against the time of incubation. ATP contents have been normalized. The measured cpm values were divided by the cpm value of the *unincubated human erythrocyte* and multiplied by 100. Although the ATP content of the unincubated dog erythrocytes is about half of that of the human erythrocytes (Table I) the ATP consumption is only moderately faster in human erythrocytes.

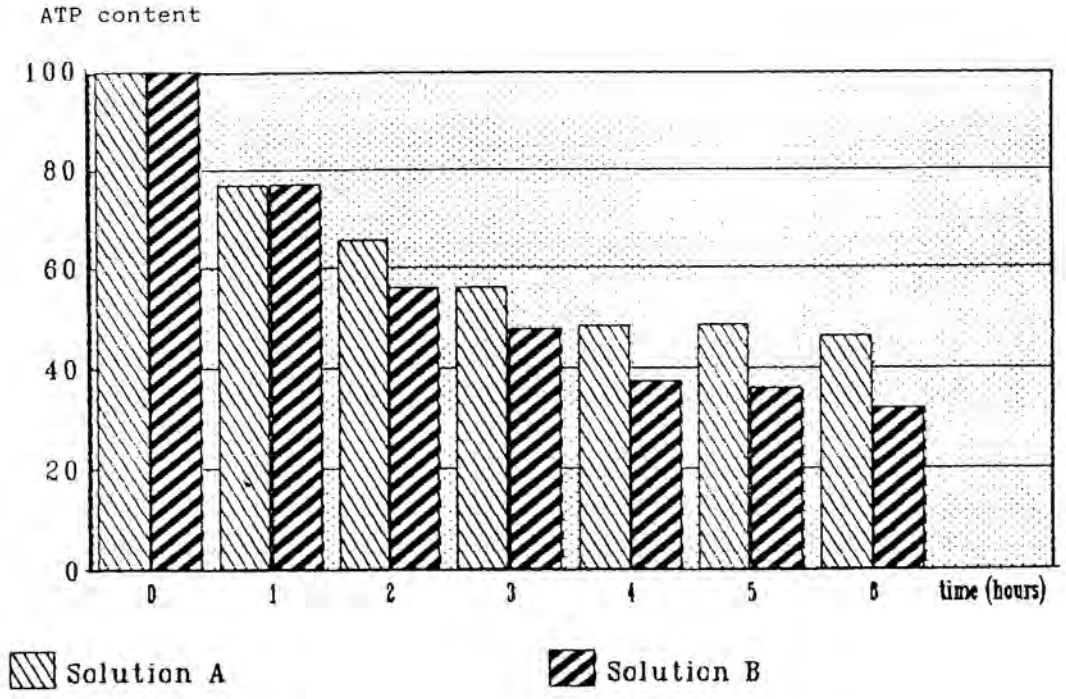

FIGURE 2. Human erythrocytes were incubated in solution A (physiological salt solution, pH 7.4), and B (5mmol/l KCl complemented physiological salt solution, pH 7.4). Relative ATP contents (see the legend of Figure 1.) of erythrocytes are plotted against the incubation time. Columns represent the average of 3 experiments. There is no striking difference between the ATP consumption of the solution A, and B incubated erythrocytes. The ATP content of solution B incubated erythrocytes decreases faster, especially in the second 3 hours of the experiment.

K^+, Na^+ and water measurements

Human or dog blood samples were centrifuged at 15,000g for 20 min, and the supernatant and buffy coats carefully removed. Duplicates of 50 microliter erythrocyte samples were drawn with an automatic dilutor (Braun Melsungen) with 5ml of bidistilled water added. K^+ and Na^+ were measured by an OMSZOV Digital (Hungary) flame photometer.

For the determination of erythrocyte water content, the weight of 15,000g erythrocyte pellets was measured gravimetrically before and after drying the samples at 104°C for 48 h.

Results and Discussion

Both human and dog erythrocytes contain 67% water and 33% of dry material (2.03g water/g dry mass). The latter is predominantly hemoglobin (Hb).

In spite of the obvious similarities between the two cell types their K^+/Na^+ ratio differs remarkably (Table I). While human cells similarly to many other cell types contain more potassium than sodium, dog erythrocytes contain more than ten times more sodium than potassium.

The popular membrane solute hypothesis explains cellular volume regulation in a way which is linked to the unequal ion distribution between the cell and its environment. Ac-

cording to the "membrane-solute" hypothesis, the intracellular space *behaves as a dilute aqueous solution*. The obvious *nonequilibrium* between the cellular and extracellular concentrations of potassium and sodium ions is maintained by the *Na^+/K^+-ATPase*. The enzyme pumps K^+ into the cell, and Na^+ out of the cell, consequently counterbalancing the constant outward leakage of K^+ and inward leakage of Na^+ ions (20). Water transport and cell volume regulation are in accordance with this ion traffic. The inhibition (for example ouabain inhibition) of the "pump" results in cellular water uptake and swelling due to the decreased intracellular K^+/Na^+ ratio. While the membrane solute hypothesis fails to give an adequate explanation for the observation that a high intracellular K^+/Na^+ ratio can be maintained in the absence of an intact lipid membrane (2,6,8,9,13,16), little attention has been paid to alternative theories.

Ling recommends that: *"The low Na^+ and high K^+ concentrations are not due to a non-equilibrium state which can be tapped for energy but in fact represent different facets of an equilibrium state."* (12)

There is little doubt that the 20–30% protein containing intracellular space is not an ideal dilute aqueous solution (2,4,12,24). According to Ling the role of intracellular proteins in cellular volume regulation is complex: (i) Proteins decrease the activity of water and other small molecules and ions by slowing their motion, or by adsorbing them. This causes a decrease in the number of osmotically active small molecules which is counterbalanced by the increased hydration of proteins. (ii) The association of water, K^+, Na^+, Cl^-, and other ions and molecules with intracellular proteins is dynamically regulated by the association of the energizing action of certain small molecular or ionic species (eg. ATP, Ca^{++}), called "cardinal adsorbents". The selection of lateral and terminal carboxyl groups of intracellular proteins between K^+ and Na^+ depends on the effect of cardinal adsorbents. Low ATP level causes an increase of the electron density at the adsorption sites (terminal and lateral carboxyl groups of amino acids), and the affinity for Na^+ increases relative to the affinity for K^+ (12). On the basis of "the membrane solute" hypothesis and Ling's "AI" hypothesis, two predictions can be formulated.

Prediction 1. According to the membrane solute hypothesis, it is generally estimated that 50% of the total energy available in human erythrocytes is consumed by Na^+/K^+ transporter ATPase (5). Since dog erythrocytes do not have the functional Na^+/K^+ transporter-ATPase molecule (3,11,17,18,19,20,21), they would be expected to have a higher ATP content and a lower ATPase activity than human erythrocytes.

Prediction 2. According to the AI hypothesis, preferential Na^+ binding over K^+ binding occurs at high electron density. Such "high electron density" and consequently high "C-value" takes place at low ATP levels (12). Therefore dog erythrocytes must have significantly less ATP than human erythrocytes. There may not be a difference in the total ATPase activity between human and dog erythrocytes due to the lack of the Na^+/K^+-ATPase. This does not exclude the possibility that there are differences in the ATP consumption due to other divergence factors between the two types of erythrocytes.

ATP consumption in human and dog erythrocytes and erythrocyte lysates

Dog erythrocytes have significantly lower ATP content than human erythrocytes (Table I). The net consumptions of ATP by human and dog erythrocytes were similar in a 5mM KCl containing, pH 7.4 physiological salt solution at 37°C (Figure 1), and therefore dog erythrocyte ATP should be exhausted sooner than human erythrocyte.

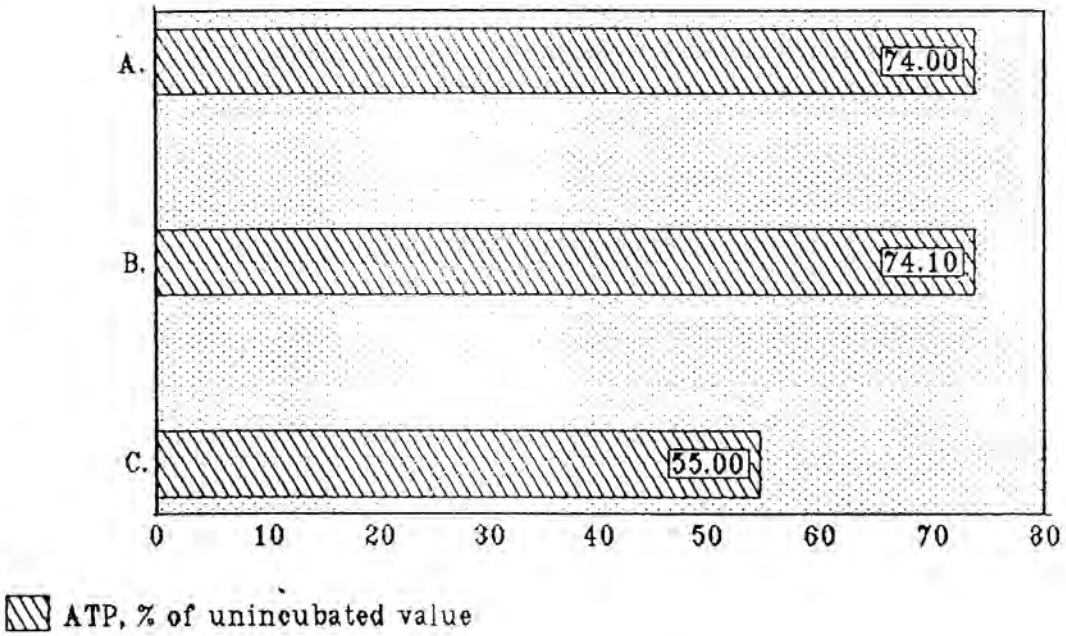

FIGURE 3. Human erythrocytes were lysed in sterile distilled water at an 1:5 vol/vol ratio. Twenty microliters of lysates were incubated in solutions A (physiological salt solution, pH 7.4), B (5mM KCl containing physiological salt solution, pH 7.4), and C (isosmotic KCl solution, pH 7.4), 5–5ml each for 5 h at 37°C. There is a higher ATPase activity and consequently faster ATP decrease in the isosmotic KCl solution.

Human erythrocyte consumed ATP at a faster rate in a pH 7.4 5mM KCl supplemented physiological salt (B solution) solution than in a pH 7.4 physiological salt solution (A solution) at 37°C (Figure 2). Nevertheless the difference in the amount of ATP used was only 14–15% after 6 h incubation. We should note, however, that the transport ATPase may not work in solution A, because solution A does not contain potassium ions. Dog red blood cells consume equivalent amounts of ATP in both A and B solutions (data not shown). This observation at first sight appears to support the existence of a Na^+ and K^+-dependent ATPase in human cells and its absence in dog erythrocytes.

When human erythrocytes were lysed in double distilled water at an 1:5 vol/vol ratio and incubated in A, B, and C solutions (the last one being a 151mM KCl solution, pH 7.4) the decrease of ATP content was similar in A and B solutions, but faster in solution C (Figure 3). This observation indicates the presence of an ATPase activity which is linked to high intracellular potassium level.

When intact human erythrocytes were incubated in physiological salt solution (solution A) (Figure 2), they had released 20–25% of their potassium contents within 1h. Insignificant decrease in the cellular potassium content was measured when human erythrocytes were incubated in solutions B and C for 1 h (data not shown). The decrease of the K^+ concentration and/or the increase of the Na^+ concentration within the cell is proposed to be the reason of the moderately decreased ATP consumption in solution A (Figure 2). This idea is further supported by the observation, that there is no significant difference between the decrease of cellular ATP levels in the first hour of the incubation in solution A and B (Figure 2).

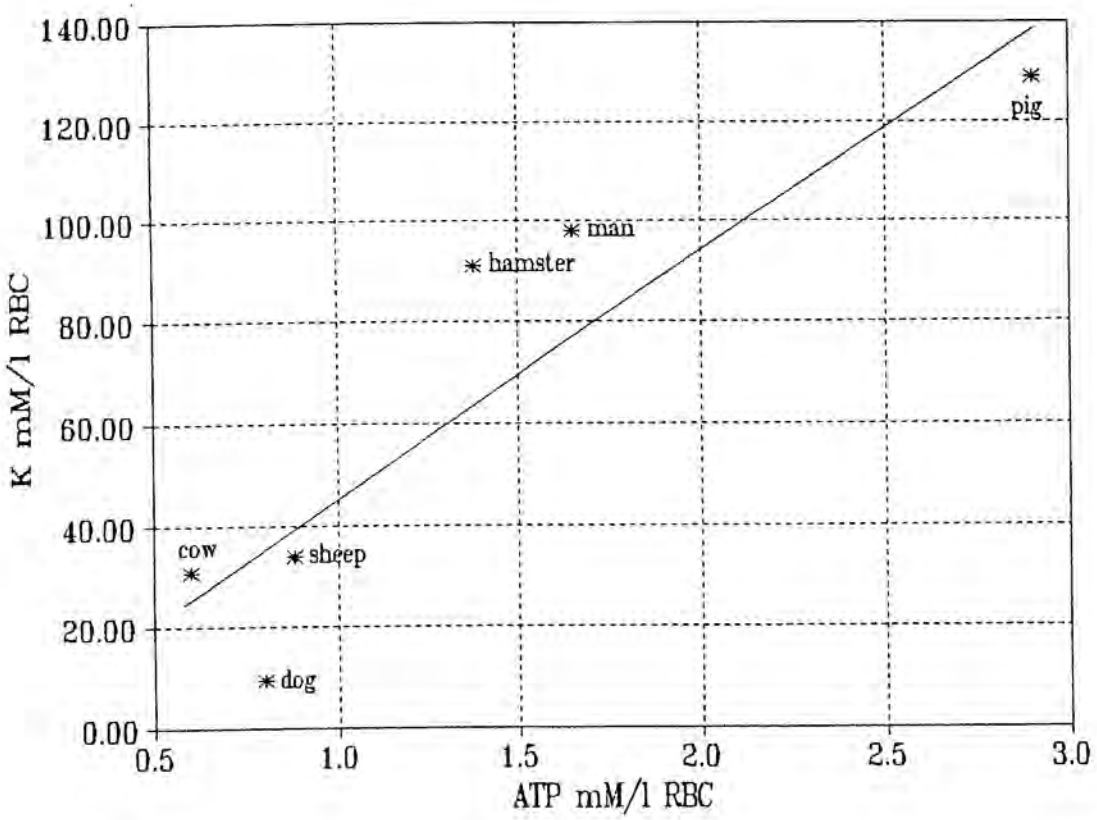

FIGURE 4. ATP and K^+ contents of human, pig, golden hamster, sheep, bovine, and dog erythrocytes. All units are in mmol/l RBC. Intracellular ATP corresponds with the cellular potassium content. Linear correlation coefficient R = 0.91.

When dog erythrocytes were incubated in solutions A and B, there were no differences in their K^+ and Na^+ contents other than that their initial 9.0mM K^+ content decreased to 7mM value during the 5 hour incubation period. This apparently does not induce any change in their ATPase activity.

ATP content and intracellular K^+ concentration in the erythrocytes of six mammalian species

Among the six species listed in Figure 4 the pig has the highest intracellular ATP content followed by man and hamster. Dog, sheep and bovine red cells contain about 36–53% of the ATP of the human erythrocyte level. There is a good correlation between the ATP content of different erythrocytes and their K^+ levels. The calculated linear correlation coefficient is R = 0.91. The pig RBC has the highest intracellular potassium content followed by the human and hamster erythrocyte. Bovine, sheep, and especially dog erythrocytes have much lower potassium concentrations than pig or human erythrocytes.

In conclusion, **our results are in line with prediction 2 and do not support the idea that a significant portion of the available ATP content in human erythrocytes is used for K^+/Na^+ transport.** The close relationship between the erythrocyte K^+ and ATP contents of different mammalian species is particularly noteworthy since the concentrations of

cardinal adsorbents other than ATP have not been measured. Also, the amino acid sequence and consequently the ATP binding of hemoglobin molecules of the species listed may differ. Data on the ATP and potassium contents of erythrocytes of several species are available, but has been determined by various authors and by different methods (25). Also, different individuals of the sheep and bovine may have entirely different K^+, and Na^+ concentrations (25). Such data therefore has limited value from the point of an analysis carried out by the authors.

Dogs must have the genes for a membrane protein reported as Na^+/K^+-ATPase, otherwise other cell types of this species would not have the more usual high K^+/Na^+ ratios similar to the cells of most other species. The loss of function of the membrane transporting ATPase presumably occurs at some late stage in cellular differentiation in the myeloid series of the bone marrow.

A more difficult problem is that cellular volume regulation is generally coupled with the Na^+/K^+-ATPase. Dog erythrocytes must have developed mechanisms to substitute this function simultaneously with the loss of their Na^+/K^+-ATPase, which is a highly unlikely evolutionary event. It is noteworthy that an alternative mechanism of volume regulation in dog red cells has been suggested by Parker and his coworkers (18,19). Their idea is based on the Ca^{++} induced shrinkage and Na^+ extrusion of dog erythrocytes preincubated in hypertonic saline solution. The shrinkage of osmotically swollen cells in the presence of Ca^{++} may be due to the contraction of cytoskeletal proteins. Chan described the lack of the Na^+ plus K^+ stimulated ATPase activity of dog red cells, as well as the ouabain resistance of their basal ATPase activity (3). It is noteworthy, however that his test system indicated quite different K^+ and Na^+ dependent ATPase activations in human and rat erythrocytes both having equally high intracellular K^+/Na^+ ratios. The ouabain-dependent reduction of ATPase activity was also different. Ouabain sensitive ATPase activity was measured by Meade and Inaba (15) in "normal" low potassium dog erythrocytes. Finally, the K^+, Na^+ concentrations used in the test (10mM KCl and 50mM NaCl) were remote from both intracellular and extracellular K^+, Na^+ concentrations.

Inescapably it is necessary to deal briefly with reports on the high potassium containing or HK erythrocytes of some dogs. They were studied and compared with the normal low potassium LK dog erythrocytes by Meade and Inaba (14,15). According to them the Na^+/K^+-ATPase activity of HK erythrocyte membranes is three times higher than that of the human erythrocyte membranes (14). There was no K^+, Na^+, Mg^{++} dependent ouabain sensitive ATPase in the membrane fraction of LK erythrocytes. Surprisingly they reported ouabain sensitive decrease in the ATP consumption in both HK and LK dog erythrocytes later (15). The ATP concentrations of both cell types appear to be similar, although the standard deviations of their results are very high (15). The increased ATP consumption due to pumping in HK erythrocytes is supposedly more than counterbalanced by a more active glycolysis and ATP production. However, ouabain intoxication does not increase the amount of ATP in HK cells (ref. 15. Table 4). Ouabain intoxication does not decrease the synthesis of glycolytic intermediaries in HK cells to the level of LK dog erythrocytes (15). The latter may be answered by the increased synthesis of reduced glutathione (15). Surprisingly, the *dog erythrocytes with Na^+/K^+-ATPase are swollen* as the mean corpuscular hemoglobin concentration is reported to be 28.3% in HK erythrocytes against the 34% Hb concentration of LK erythrocytes (14). The evaluation of results is further complicated by the fact, that the half life time of HK erythrocytes is less than the half of the 29.6 days (^{51}Cr

$T^1/_2$) half life time of normal LK dog erythrocytes (15). It is well known that the metabolic activity in younger erythrocytes is higher than in older ones (25).

Exceptions to the "rule" of transmembrane ionic distribution have redirected the attention to the fundamental problem of the basic theory on several separate occasions during the last fifty years. Dog erythrocytes are fully "competent" in their function despite the lack of "Na^+/K^+-ATPase". Their K^+/Na^+ ratio is similar to that of dying cells yet they clearly do not have volume regulation problems and are not swollen.

While the advocates of the "membrane solute hypothesis" lack a mechanism by which these transmembrane ionic distributions can be rationally explained, Ling's association-induction hypothesis can account for the behavior of dog erythrocytes. Erythrocytes of different mammalian species have been shown to have remarkably different activities of several metabolic enzymes (23). Their ATP levels are different. Even though evolution conserved the hemoglobin molecule among mammalian species, the affinity of Hb for K^+, and Na^+ clearly differs quite remarkably because of the different metabolism and the resulting different ATP levels of erythrocytes. **Their K^+/Na^+ ratio depends on their ATP level rather than on their ATPase activity.**

The authors thank Dr. Andrea Ludány and Dr. Tamas Kôszegi for the ATP and K data in pig and bovine erythrocytes. We are grateful to Ágnes Stocker and Éva Fábian for their excellent technical aid.

This work was supported by the grant of the Hungarian Academy of Sciences (OTKA No: 3108) and by the joint grant of the National Academy of Sciences U.S.A.–Hungarian Academy of Sciences No: 30.07032/86.

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Received August 30, 1991;
accepted October 3, 1991.

Biochimica et Biophysica Acta, 1175 (1993) 133–139

BBAMCR 13292

Relationship between cellular ATP, potassium, sodium and magnesium concentrations in mammalian and avian erythrocytes

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(Received 29 April 1992)
(Revised manuscript received 13 August 1992)

Key words: ATP; Potassium; Sodium; Magnesium; K^+/Na^+ ratio; (Mammalian erthrocyte); (Avian erythrocyte)

Intracellular K^+/Na^+ ratios of erythrocytes of various mammalian species are known to differ markedly. We have measured ATP, K^+, Na^+, Mg^{2+}, H_2O contents of erythrocytes of twelve mammalian and three avian species. Our results indicate that the intracellular ATP concentration in erythrocytes of different species is in close positive correlation with the K^+/Na^+ ratios (linear correlation coefficient, $r = 0.852$). Furthermore, ATP levels in erythrocytes of individual sheep with different potassium concentrations correspond with their K^+/Na^+ ratios ($r = 0.747$). Intracellular magnesium concentrations also correlate with ATP concentrations in erythrocytes of different species ($r = 0.629$) and in different sheep ($r = 0.549$).

Introduction

High intracellular K^+ and low intracellular Na^+ concentration is almost universal throughout the living world. Exceptions do occur and include the erythrocytes of carnivores and some ruminants (i.e., dogs, cats, sheep, goats, cattle) which contain low intracellular K^+ and high Na^+ concentrations [1]. Furthermore, these differences are sustained despite the low plasma K^+ and the high Na^+ concentrations encountered consistently in these species [1]. (Plasma K^+ in the 4.2–5.9 mmol/l plasma water, Na^+ in the 147–159 mmol/l plasma water range for each species examined in the present study.) Subsequent studies revealed that in addition to interspecies differences, significant intraspecies difference also exist in the K^+ and Na^+ contents of sheep, goats and cattle erythrocytes [2–5].

In spite of the large number of data on different chemical constituents of erythrocytes of different species, including cations [5] and organic phosphates [6], no systematic study was carried out on the relationship between these cellular constituents in the past 30 years. It must be noted, however that Kerr recommended a relationship between the erythrocyte potassium concentrations and 'acid-soluble phosphorus fractions' as early as 1937 [7].

Such analysis is important since ATP is believed to play a central role in the regulation of cation concentrations within erythrocytes [5]. Generations of new data on the K^+, Na^+, Mg^{2+}, H_2O and ATP on the erythrocytes of different species were necessary because of the scattered nature of available literature data, differences in measurement techniques, and because of the frequent lack of adequate references on the corresponding organic phosphate (ATP) or monovalent cation concentrations.

The erythrocyte ATP concentrations appeared to be in the 10^{-4}–$4 \cdot 10^{-3}$ mmol/kg RBC (mmol/kg packed cells) range for each species. The erythrocyte K^+ contents varied between 6 and 110 mmol/kg RBC, turkeys having the highest and dogs with the lowest erythrocyte K^+ contents. High K^+ (HK) and low K^+ (LK) -type erythrocytes containing subgroups of ruminants and donkeys are shown separately. Interspecies and intraspecies differences in the erythrocyte ATP and K^+, Na^+, Mg^{2+}, and water concentrations have been correlated with each other in this study.

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Materials and Methods

Heparinized human blood (125 IU heparin/ml blood) was obtained from the cubital vein of healthy volunteers.

Heparinized blood (125 IU heparin/ml blood) was obtained from the jugular or other suitable veins of various mammals and avians. The blood sample holders were immediately shifted to wet ice and the blood used for experimental purposes within the shortest possible time, usually within 2 h.

Measurements of erythrocyte ATP, K^+, Na^+, Mg^{2+} and water concentrations

All manipulations were carried out at 4°C if not indicated otherwise.

Erythrocyte ATP concentrations were determined by the chemiluminescent firefly luciferin/luciferase system (ATP bioluminescence kit CLS, Boehringer-Mannheim, Mannheim) according to the method developed in our laboratory [8]. Three aliquots of the same blood sample were measured and averaged in each case.

The erythrocyte K^+, Na^+, concentrations were measured by a flame photometer (Omszöv, Hungary). The heparinized blood samples were pelleted first by a table top centrifuge (Hettich, EBA 3S) at 4000 rpm, 10 min, the supernatants and buffy coats carefully removed, and 1.5-ml aliquots of the pelleted erythrocytes transferred into microcentrifuge tubes. The samples were pelleted at 12000 rpm 12 min, and the supernatants removed. Two 0.05-ml aliquots of the pelleted erythrocyte samples were taken by an automated dilutor (Braun, Melsungen), and used for flame photometric measurement. After correction by the erythrocyte water contents K^+ and Na^+ concentrations are given as mmol/kg RBC (mmol/kg packed cells).

The rest of the pelleted erythrocyte samples were transferred into Ni sample holders of known weight and their weights measured gravimetrically by an analytical balance (OWA Labor, Germany). After drying the samples at 104°C for 48 h their weights were

TABLE I

Erythrocyte ATP, K^+, Na^+, Mg^{2+}, H_2O concentrations (mean values)

The common names of animals are indexed. Subgroups of ruminants and donkeys with different characteristic K^+/Na^+ ratios are separated. (Goat C is cameroonian pygmy goat; sheep S and B are Suffolk-type and Booroola-type sheep, respectively. LK and HK labels indicate characteristic low-potassium and high-potassium-type subgroups of a species.)

Species	n	ATP mM [a]	K mM [a]	Na mM [a]	K/Na	Mg (mM) [b]	H_2O (%)
Man	64	1.65	93.1	15.3	6.08	3.78	66.41
Pig	5	2.22	88.3	14.1	6.26	2.13	64.05
Goat C	10	0.40	61.1	25.1	2.43	1.92	63.17
Goat	3	0.71	67.4	31.1	2.17	3.10	63.34
Sheep BLK	20	0.35	11.0	111.1	0.099	2.76	64.05
Sheep SLK	10	0.85	14.5	83.9	0.17	2.56	64.48
Sheep SHK	9	1.36	70.4	25.4	2.77	2.98	63.74
Cow	5	0.31	26.8	72.4	0.36	3.18	64.47
Dog	6	0.59	6.9	103.0	0.067	3.88	65.21
Horse	5	0.12	67.8	15.4	4.40	5.25	63.47
Donkey LK	1	0.23	45.7	55.5	0.93	4.23	65.57
Donkey HK	1	0.36	86.5	26.0	3.33	3.93	64.16
Deer	5	0.38	67.1	24.4	2.75	8.11	65.76
Hamster	5	1.23	92.0	17.2	5.35	4.33	64.62
Rat	5	1.26	96.9	17.2	5.62	5.23	65.56
Rabbit	5	3.10	100.3	14.5	6.90	6.22	67.85
Chicken	5	2.16	103.8	13.6	7.63	10.28	62.16
Turkey	5	3.71	108.2	10.6	10.7	7.81	63.06
Pigeon	3	3.86	95.8	11.6	8.25	13.51	62.55

Correlation matrix:

	ATP	K	Na	K/Na	Mg	H_2O
ATP	1	0.659 *	−0.504	0.852 **	0.629 *	−0.106
K	0.659 *	1	−0.923 **	0.901 **	0.484	−0.101
Na	−0.504	−0.923 **	1	−0.794 **	−0.418	0.102
K/Na	0.852 **	0.901 **	−0.794 **	1	0.632 *	−0.590
Mg	0.629 *	0.484	−0.418	0.632 *	1	−0.240
H_2O	−0.106	−0.101	0.102	−0.590	−0.240	1

[a] mmol/l RBC (packed cells); [b] mmol/l cell water; * significant at $P = 0.01$; ** significant at $P = 0.001$.

measured again. The water contents were calculated, and expressed as a percentage of the total erythrocyte mass.

For the determination of erythrocyte magnesium concentrations dried erythrocyte pellets were exposed to 3 ml 1 N HCl for 24h. The magnesium concentrations were measured by an atomic absorption spectrophotometer (Jarrel ASH, USA).

Correlation analysis has been carried out by the 'Quattro Pro, 3.01' (Borland International) datamanager program.

Erythrocyte glucose utilization

Heparinized full blood was incubated in sterile test tubes at 37°C and the glucose levels determined before and after 3 h incubation. Alternatively, erythrocytes were pelleted by centrifugation, the plasma and buffy coats removed, and the erythrocyte pellet resuspended in 5 mmol glucose containing Hanks' solution. Similar results were obtained (data not shown). Glucose levels were determined by the glucose oxidase–peroxidase method [9] (Reanal, Hungary). Blood hemoglobin concentrations were determined photometrically by a Coulter CBC5 machine and the glucose consumption expressed as mmol/kg Hb per h.

Results

Correlations among the ATP, K^+, Na^+, K^+/Na^+, and Mg^{2+} concentrations in erythrocytes of different species

Erythrocyte ATP concentrations correlates strongly and positively with cellular K^+/Na^+ ratios in different species (Table I and Fig. 1). A correlation coefficient of $r = 0.852$ was obtained between erythrocyte ATP concentrations and the K^+/Na^+ ratios, even when the horse, an animal with high erythrocyte K^+ but low ATP concentrations is included (if omitted the correlation rises to $r = 0.886$). Our correlation matrix between erythrocyte ATP and K^+, Na^+ concentrations suggests positive or negative correlations – albeit not as strongly as between ATP and K^+/Na^+ ratios – respectively.

It is noteworthy that the relationship is valid for the nucleated erythrocytes of the three domestic birds listed. A strong positive correlation was found between erythrocyte ATP and Mg^{2+} concentrations $r = 0.629$.

Correlations among the K^+, Na^+, K^+/Na^+, and Mg^{2+} concentrations in erythrocytes of different sheep

The intraspecies relationship between the erythrocyte K^+/Na^+ ratios and ATP contents have been studied on Booroola- and Suffolk-type sheep (Table II). Individual sheep are known to contain different

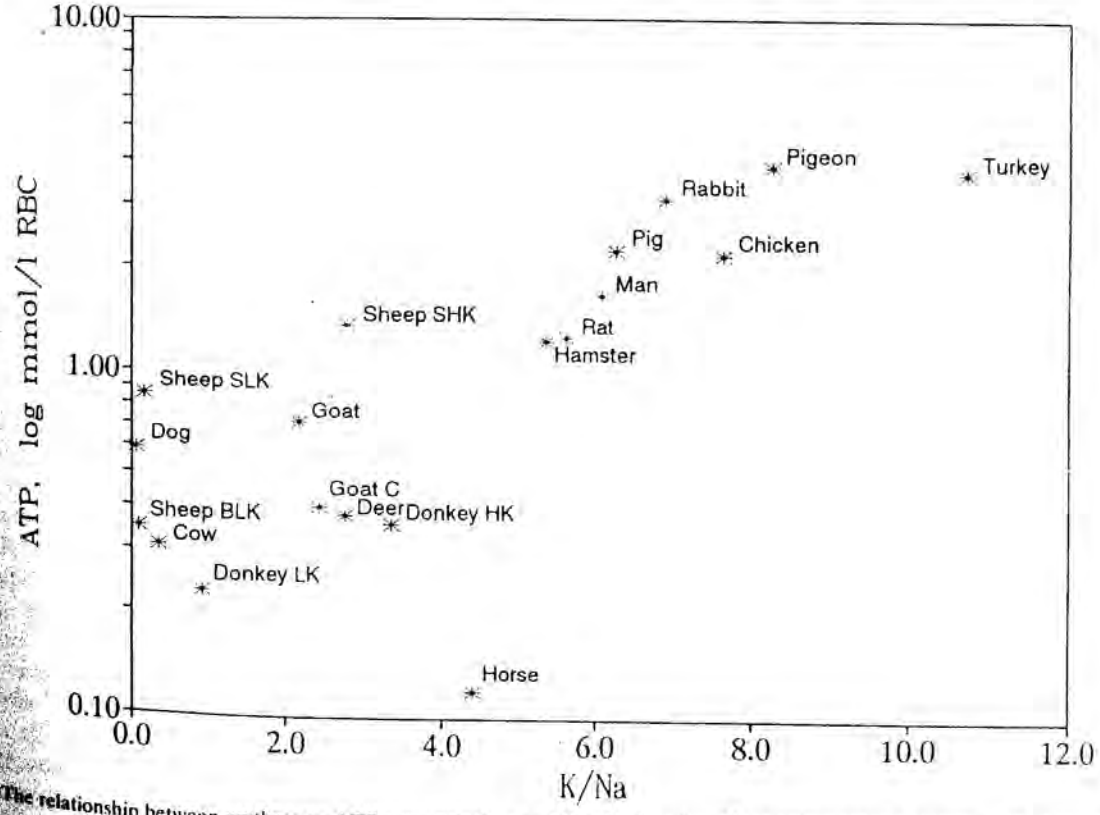

Fig. 1. The relationship between erythrocyte ATP concentrations and K^+/Na^+ ratios. Mean values extracted from Table I are displayed.

TABLE II

The erythrocyte ATP, K^+, Na^+, Mg^{2+} H_2O concentrations of 19 Suffolk-type sheep

Mean values for 20 Booroola-type sheep are indicated in the bottom row of the table. Sample handling and data analysis was carried out as described in the legend of Table I.

Animal no.	ATP mM [a]	K mM [a]	Na mM [a]	K/Na	Mg mM [b]	H_2O (%)
1	1.74	72.8	24.0	3.03	2.50	63.55
2	1.15	15.8	86.0	0.18	2.70	64.99
3	0.93	13.0	89.0	0.15	3.20	63.84
4	0.98	17.5	79.0	0.22	2.92	62.31
5	1.18	74.7	22.0	3.39	2.93	64.30
6	1.09	70.6	27.0	2.61	3.54	64.82
7	0.94	18.9	81.0	0.23	2.76	65.92
8	0.68	11.6	87.0	0.13	2.46	65.06
9	0.89	16.8	82.0	0.20	1.99	64.64
10	1.17	70.9	23.0	3.08	3.01	61.73
11	1.67	66.6	29.0	2.30	3.37	65.16
12	1.50	69.1	22.0	3.14	3.01	61.87
13	0.89	18.8	83.0	0.23	2.41	63.05
14	0.83	17.6	85.0	0.21	2.66	66.09
15	1.47	68.6	28.0	2.45	3.12	62.04
16	0.49	13.5	83.0	0.16	2.31	64.64
17	0.67	15.3	84.0	0.18	2.16	64.29
18	1.15	73.3	22.0	3.33	2.37	62.99
19	1.23	67.0	32.0	2.09	3.03	64.05
Booroola	0.35	11.0	111.1	0.099	2.77	64.05

Correlation matrix:

	ATP	K	Na	K/Na	Mg	H_2O
ATP	1	0.775 *	−0.770	0.747 *	0.549	−0.319
K	0.775 *	1	−0.997 **	0.985 **	0.498	0.101
Na	−0.770	−0.997 **	1	−0.988 **	−0.487	0.355
K/Na	0.747 *	0.985 **	−0.988 **	1	0.434	−0.397
Mg	0.549	0.498	−0.487	0.434	1	−0.076
H_2O	−0.319	−0.101	0.355	−0.397	−0.076	1

[a] mmol/l RBC (packed cells); [b] mmol/l cell water; * significant at $P = 0.01$; ** significant at $P = 0.001$.

K^+/Na^+ ratios and this is linked to inherited blood group characteristics [2,5,7,10]. The blood samples of 19 Suffolk-type and 20 Booroola-type sheep were analyzed. 9 out of the 19 Suffolk sheep were HK type, while the remaining 10 animals contained LK-type erythrocytes. All Booroola sheep turned out to be LK type, with lower K^+/Na^+ ratios than LK-type Suffolk sheep. This also correlated with their lower intracellular ATP concentrations.

Erythrocytes of the 9 HK-type Suffolk sheep contained K^+ levels between 67 and 75 mmol/kg RBC. Their ATP level was 1.36 ± 0.23 mmol/kg RBC (mean ± S.D.). The erythrocytes of LK-type Suffolk sheep contained 11–20 mmol/kg RBC K^+ and their ATP content was 0.85 ± 0.18 mmol/kg RBC, significantly lower than in erythrocytes of HK-type sheep. A correlation coefficient of $r = 0.747$ was calculated between the erythrocyte ATP concentrations and K^+/Na^+ ratios in Suffolk sheep. When the data analysis includes the data of 20 LK-type Booroola sheep (summary data shown in Table I only) r becomes 0.820.

We must note, however, that these animals could be separated into two or three (if Booroola sheep are included) distinct subgroups with regards their erythrocyte K^+ and Na^+ contents. Therefore we analyzed the relationships between the parameters within each of the subgroups.

The K^+/Na^+ ratios of LK-type sheep show a wide variance $K^+/Na^+ = 0.129 \pm 0.050$ (values include the data of 10 Suffolk-type and 20 Booroola-type sheep). A correlation coefficient of $r = 0.782$ was calculated between cellular ATP concentrations and K^+/Na^+ ratios in the subgroup of LK-type sheep (Fig. 2), which is remarkable if the small differences in the K^+ concentrations and the standard errors of both sample handling and K^+ and ATP measurements are taken into account. $r = 0.404$ in the Suffolk, and $r = 0.520$ in the Booroola subgroups. We found no significant correla-

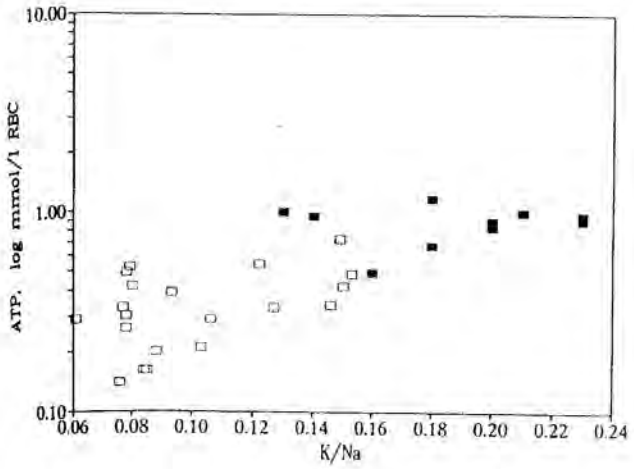

Fig. 2. The relationship between erythrocyte ATP concentrations and K^+/Na^+ ratios in LK-type Suffolk and in Booroola sheep. (■) Suffolk sheep; (□) Booroola sheep.

tion between the erythrocyte ATP concentrations and K^+/Na^+ ratios of HK-type sheep or other animals. This is probably due to the fact that erythrocyte K^+/Na^+ ratios show little variance in these animals and in man.

Erythrocyte Mg^{2+} concentrations correlate positively with ATP concentrations in sheep $r = 0.549$.

Glucose utilization in HK and LK-type erythrocytes

The glucose consumption of HK- and LK-type erythrocytes of Suffolk type sheep, man and dogs have been determined by measuring the decrease of glucose levels in the plasma (medium). We either used complete heparinized blood or washed erythrocytes (data not shown because similar results were obtained).

There was no significant difference between the glucose consumption of HK- and LK-type sheep erythrocytes.

Human red blood cells consume somewhat more glucose than sheep erythrocytes, but the glucose consumption of dog erythrocytes is higher. Note that significant differences in the glucose consumption were measured between different individuals of the same species or animal subgroup.

Discussion

We demonstrate a simple relationship between erythrocyte ATP concentrations, K^+/Na^+ ratios among different mammalian and avian species, and also within different individual sheep. Erythrocyte Mg^{2+} and ATP concentrations also show a positive correlation. The latter is likely to be due to the presence of Mg-ATP complexes in erythrocytes.

Any data have to be consistent with any model put forward to explain intracellular ion distribution, especially with regard to the usual converse relationship between intracellular K^+/Na^+ compared with extracellular K^+/Na^+ ratios. They must also fit with theories on the central role of ATP in the regulation of the intracellular environment.

It is widely accepted that cellular K^+, and Na^+ concentrations are maintained against concentration gradients by an active 'ion pumping' activity involving a K^+/Na^+-dependent ATPase [5,11], i.e., the constant outwardly directed 'leak' of K^+ and inwardly directed leak of Na^+ ions is counterbalanced by the work of the K^+/Na^+-transport ATPase. This enzyme uses the energy locked into the high-energy phosphate bonds of ATP and transports K^+ and Na^+ ions against their concentration gradients [5,11]. The transport mechanism of the enzyme is still not clearly understood. The energy cost of maintaining the non-equilibrium situation between the intra/extracellular spaces for K^+ and Na^+ has been estimated at as much as 50% of the total erythrocyte ATP used in human red blood cells [12].

The low intracellular K^+/Na^+ ratio in the erythrocytes of carnivores and some ruminants is explained by the decrease or lack of K^+/Na^+-ATPase activity for transporting these ions [5,13]. If such mechanisms are operational, erythrocytes containing high K^+/Na^+ ratios must use more energy (ATP) and must be expected to have lower ATP contents than those having low K^+/Na^+ ratios nearer to equilibrium with the extracellular solutes. This does not exclude the possibility that a significant difference in the erythrocyte ATP content may exist due to other factors such as: a difference in glucose metabolism, or to non K^+/Na^+-ATPase dependent mechanisms of ATP catabolism.

ATP in erythrocytes is being used (i) for the regeneration of reduced glutathione, which plays an important role in the stabilization of hemoglobin, and instable SH groups of enzymes, (ii) for maintaining cell shape and size (iii) and for the maintenance of the non-equilibrium situation for small molecules between the cell and its environment [14]. It has been reported however, that the LK type erythrocytes of ruminants, dogs, and cats have considerably lower or virtually no K^+/Na^+ transport ATPase activity [5,13]. This suggests that LK-type erythrocytes would have higher intracellular ATP contents (considering a similarly active metabolism). As we have demonstrated this is not only invalid, but erythrocyte ATP concentration shows a strong positive correlation with intracellular K^+ contents and K^+/Na^+ ratios.

Alternatively, it is also possible that the loss of the K^+/Na^+-transport ATPase function and the resulting decrease of the intracellular K^+/Na^+ ratio decreases the activity of glycolytic enzymes, and consequently decreases the generation of ATP in LK-type erythrocytes. There is evidence, that the only known K^+ stimulated glycolytic enzyme the pyruvate kinase (this

TABLE III

Glucose consumption of LK- and HK-type Suffolk sheep, dog and human erythrocytes

Mean values and standard errors of the mean are indicated in case of HK- and LK-type sheep erythrocytes.

Animal:	No.	Glucose mM [a]	Hb g/l plasma	Glucose consumption mM/kg Hb per hour
Sheep HK	1	2.96	90	2.44
	2	2.84	102	3.92
	3	2.93	110	4.02
	4	3.14	104	1.39
	5	3.00	109	3.35
	6	4.15	120	2.03
	7	3.42	91	3.01
				2.95 ± 1.08
Sheep LK	1	2.73	113	1.21
	2	2.92	94	3.78
	3	2.87	97	3.51
	4	2.52	103	2.48
	5	3.49	93	3.83
	6	4.49	126	3.71
	7	4.29	102	2.53
				3.01 ± 0.98
Dog	1	3.79	161	4.33
	2	3.60	177	3.54

[a] mmol/l plasma.

enzyme is not K^+, Na^+ sensitive in dog erythrocytes [15]) may not be rate limiting in regards to the generation of ATP molecules via the Embden-Meyerhof pathway in sheep erythrocytes [16]. The Embden-Meyerhof pathway is the singular source of ATP in mammalian erythrocytes. Glucose consumption may indicate the amount of energy which is used by different HK and LK-type erythrocytes.

However, we found no difference in the glucose consumption of HK and LK-type sheep erythrocytes (Table III). Also, the LK-type dog erythrocytes consume more glucose than the HK-type human erythrocytes. Since no evidence is provided that HK-type erythrocytes must use more energy (glucose) to maintain a much steeper ionic gradient than their LK-type counterparts, a more careful study is required to measure the generation and consumption of ATP and levels of ADP + P_i in different erythrocytes. This may explore the correlation between the extra/intracellular ionic gradients and the rate of ATP synthesis.

We can say that the relationship between ATP levels and K^+/Na^+ ratios could not be explained in any obvious or logical way, and that an alternative hypothesis might also have to be considered.

Ling's 'association-induction' (AI) hypothesis [17] suggests the relationship between cellular ATP concentration and K^+/Na^+ levels with remarkable accuracy. That theory predicts that intracellular K^+/Na^+ concentrations are directly linked to the concentrations of energizing small molecular species 'cardinal adsorbents', ATP being one of the most important among them. High intracellular ATP concentration is supposed to be associated with preferred potassium binding on the lateral and terminal carboxyl residues of proteins over sodium ions [17]. The underlying reason for this is a change of affinity of fixed anionic groups of proteins towards potassium and sodium ions [17]. A direct relationship between ATP concentrations and intracellular K^+ levels has been demonstrated on frog muscles [18] and on human erythrocytes [19] exposed to different metabolic poisons. Our data are also in agreement with our previous report on the relationship between ATP levels, ATP consumption and K^+ concentrations in human and dog erythrocytes [20]. We must add, that the erythrocytes of newborn or young animals or reticulocytes of anaemic animals have higher metabolic rates and consequently higher ATP contents [6] than the erythrocytes of mature animals, and that this corresponds with high erythrocyte K^+/Na^+ ratios even in LK-type animals [5]. Here the K^+/Na^+ ratio reverses during erythrocyte maturation [5].

In conclusion, the relationship between erythrocyte ATP levels and K^+, Na^+ concentrations appears to be statistically significant, and perhaps important. Further studies on the organic phosphates and on metabolism in erythrocytes may explore other important factors affecting erythrocyte K^+, and Na^+ concentrations.

Acknowledgments

The authors wish to thank the helpful comments of Drs. P. Dunham, G.N. Ling, B. Sümegi and E.M. Tucker. We are also grateful for the valuable technical assistance of Ms. Agnes Stocker, Mrs. István Takács, Gyula Nagy and István Rózsahegyi.

This project was supported by OTKA F 5197 and OTKA R 3108.

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Cell Biology International, Vol. 17, No. 1, 1993

CHANGES IN THE CONCENTRATION OF IONS DURING SENESCENCE OF THE HUMAN ERYTHROCYTE.

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ABSTRACT

Flash frozen samples of normal human blood were cryosectioned and cryodried for electron probe x-ray microanalysis of the concentration of ions and elements in individual erythrocytes (RBCs). The data (expressed in mM/kg dry weight) demonstrated a systematic pattern of variation between the concentration of ions and elements in the RBCs. Specifically as K^+ and Cl^- decreased in concentration, Ca^{2+} and sulfur increased in concentration. Phosphorous, Na^+ and Mg^{2+} did not demonstrate a significant pattern of change. These findings are related to the dehydration and to the volume decrease that accompanies senescence of the RBC.

INTRODUCTION

Various techniques have been used to obtain erythrocyte (RBC) populations of various ages. Most of the investigations of RBC aging have been based on the assumption of an age dependent difference in RBC density. Some authors have questioned that density is a good marker of age (Morrison et al., 1983; Dale and Norenbert, 1979; Luthra et al., 1979; Mueller et al., 1987; Beutler, 1988). Waugh et al. (1992) combined different techniques for the unambiguous isolation of aged RBCs and found that progressive dehydration and volume decrease are indeed characteristic features of human RBC aging. Waugh et al. also concluded that as RBCs age they do become more dense, but that this density change is a stochastic process such that not all RBCs increase density at the same rate. Waugh et al, conclude that the most dense RBCs are definitely older than the least dense RBCs.

What contributes to the senescence dependent decrease in RBC volume? One consistently reported factor is a significant net loss of potassium (K^+) (Prankerd, 1958; Bernstein, 1959; Borun, 1964; Cohen et al., 1976; Bartosz et al., 1981; Lee et al., 1984; Clark, 1985; Bartosz, 1991). This loss of K^+ (the major monovalent cation in the human RBC) is accompanied by water loss and RBC dehydration. Thus a progressive decrease in K^+ concentration (mM/kg dry mass) in the RBC can be used as a marker of senescence of the RBC.

RBCs in normal human blood are a heterogeneous population of different densities and degrees of senescence. Given that the K^+ concentration of an individual RBC can be used to mark the degree of senescence of the RBC and given that

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electron-probe x-ray microanalysis technique allows the quantitative analysis of multiple ions and elements in each RBC (Cameron et al., 1983; Tormey, 1981) then it becomes feasible to study systematic variations in the ionic concentration of the RBC during the senescence process. Such a study would circumvent the need for RBC separation procedures and should allow the collection of data needed to answer questions concerning the ionic events taking place during the senescent process of the human RBC. For example the study should allow a test of the hypothesis that RBC senescence is associated with an increase in calcium (Ca^{2+}) (La Celle et al., 1972a, 1972b; Clark, 1985) as well as allow an evaluation of the concentration of other ions (Na^+, Cl^-, Mg^{2+}) and elements (phosphorous and sulfur, abbreviated P and S respectively) associated with RBC senescence.

MATERIALS AND METHODS

For electron probe x-ray microanalysis a small drop of centrifugally packed erythrocytes (1200 xg for 5 min) from a single healthy human volunteer was placed on a brass pin. Centrifugation at higher g forces and longer times did not result in further packing of the erythrocytes. The specimens were frozen by immersion in liquid propane cooled in a liquid nitrogen bath and were then transferred to and stored in liquid nitrogen until the time of sectioning. Sectioning was done on the LKB Ultratome V equipped with a modified cryokit and cooled to a temperature of -100° C for both specimen and knife. A dry glass knife with a 40° angle and a sectioning speed of 0.5 mm/s was used for cutting. Ultrathin sections were obtained by advancing 0.1µm on the microfeed. The 0.1µm-thick sections were positioned on a film of formvar (0.25% in dioxane) spanning a 1.5mm hole in a 3mm carbon grid. To minimize curling or movement of the sections, carbon-coated formvar film on an aluminum ring was placed over the sections. The sandwiched specimen was dried within the LKB chamber at -100°C in a custom-made cryosorption apparatus by evacuation with a rotary pump for 12 hr. The sections were warmed to room temperature, vented with dry nitrogen gas, and stored in a desiccator. At the time of analysis the aluminum ring was removed, leaving a flat section sandwiched between two layers of formvar film. The sections were examined in a JEOL JSM35 scanning electron microscope under the following conditions: STEM mode, accelerating voltage 25 kV, specimen current 0.20 nA, raster size 0.27 μm^2, analysis time 100 s, takeoff angle 40°, and specimen-to-detector distance 15 mm. Analysis was done by a Si(Li) x-ray detector and Tracor Northern NS-880 x-ray analysis system.

Our quantification technique is based on the Hall mass fraction method (Hall 1971, 1973). Continuum counts, due to the formvar, were measured and were subtracted prior to the calculation of elemental peak-to-continuum values, which were converted to element content by comparison with a series of cryosectioned standards with known amounts of dried salts added to a 20% bovine serum albumin solution.

RESULTS

The results of the electron-probe x-ray microanalyses for ion and element concentration in individual RBCs in a sample of human blood are plotted in figure 1A and B. The data plotted in figure 1A and B were subjected to least squares linear regression analyses to determine if significant relationships exist between the concentration of K^+ and the concentration of each of the other ions and elements measured. A summary of results of the regression analyses are reported in Table 1. Chlorine concentration (presumably Cl^-) decreased ($p < 0.05$) as K^+ concentration decreased. Conversely Ca^{2+} increased ($p < 0.01$) as K^+ decreased.

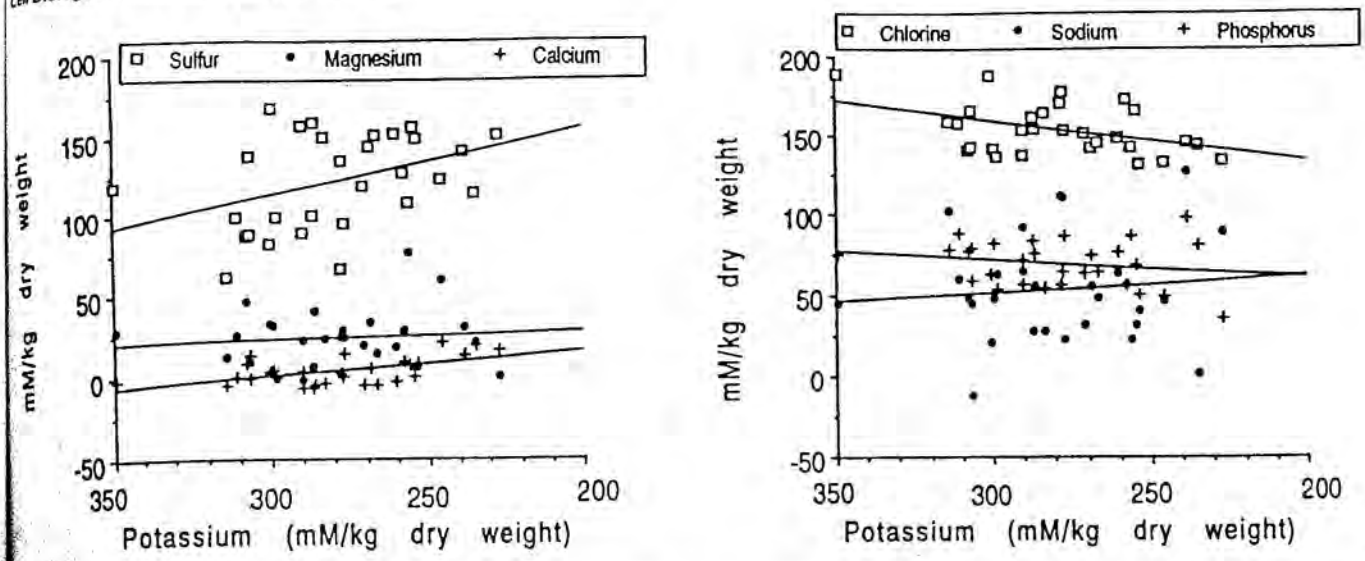

Figure 1A and B. Relationships between the concentration of K^+ and the concentration of other ions and elements in 29 randomly selected human erythrocytes. The concentration of K^+ is a marker of erythrocyte senescence (see text). The results of linear regression analyses of the data in this figure is summarized in Table 1.

The other significant concentration change was S which increased ($p < 0.05$) as K^+ decreased. Phosphorus (P), Na^+ and Mg^{2+} did not change to a significant extent as K^+ decreased.

Table 1 Summary of linear regression analyses of the relationships between the concentration of K^+ and the concentration of other intra-erythrocyte ions and elements in a random sample of human RBCs from blood (n = 29 RBCs).

Ion or element	Slope	Correlation Coefficient	Significance of slope (p value)
Chlorine	-0.251	.443	< 0.05
Phosphorus	-0.106	.207	N.S.*
Sodium	+0.102	.084	N.S.
Sulfur	+0.420	.383	< 0.05
Magnesium	+0.047	.077	N.S.
Calcium	+0.147	.513	< 0.01

* N.S. = not significant

DISCUSSION

A major problem associated with examination of erythrocyte senescence has been the difficulty of isolating RBCs of known age. Waugh et al. (1992) have shown that RBCs that are chronologically older do exhibit an increase in density and a decrease in volume. However the linkage between chronological age of the RBC and physical changes was less than perfect (Waugh et al., 1992). Given that RBCs lose volume and become more dense during senescence and that these changes are not tightly linked to chronological age of an RBC suggests that RBC senescence is a stochastic process and that physical changes in the RBCs provide reasonable and useable markers of the state of senescence of individual RBCs. Accepting that senescence of human RBCs is characterized by loss of cell volume and is accompanied by a loss of K^+, regardless of the way the K^+ concentration data are expressed (Prankerd, 1958; Bernstein, 1959; Borun, 1964; Cohen et al., 1976; Bartosz et al., 1981; Lee et al., 1984; Clark, 1985; Bartosz, 1991), indicates that the concentration of K^+ in individual RBC can be used as an indicator of the stage of senescence of that RBC.

Thus given that K^+ concentration of an individual RBC is an indicator of the stage of senescence of that RBC and knowing that electron probe x-ray microanalysis offers the ability of measuring the concentration of several ions and elements simultaneously in individual RBCs within a heterogeneous population of RBCs then an assessment of the ionic and the elemental changes that occur during the senescence process becomes possible. In this regard, the data in Figure 1A and B and Table 1 reveal the following significant ion and element changes associated with RBC senescence. Chlorine (Cl^-) decreases 33% in concentration during senescence. Given that the water content decreases during RBC senescence it follows that water loss is accompanied by a net loss of K^+ plus Cl^- from the RBC. The presence of S in the RBC can be accounted for mainly by S in the amino acids of hemoglobin and other RBC proteins and to a lesser extent by sulfur-containing molecules such as glutathione. It seems doubtful that any significant amount of S exists as ionic sulfate within the RBC. Thus the significant (66%) increase in S during RBC senescence can be accounted for by the preferential loss of other elements contributing to the dry mass fraction of the RBC.

Much evidence links increased cellular Ca^{2+} to cell senescence and death (Schanne et al., 1979). La Celle et al. (1972) proposed an increase in RBC Ca^{2+} which accompanies an increase in RBC density (senescence), but this increase has apparently not been adequately documented in prior studies (Clark, 1985; Bookchin, et al., 1985). The data in Figure 1 and Table 1 indicate a significant and marked (450%) increase in RBC Ca^{2+} concentration during senescence. This finding of a significant increase in Ca^{2+} documents that a change in Ca^{2+} does occur but this fact does not tell if the increase in Ca^{2+} is causal or simply a companion to the senescent process.

The data presented do demonstrate a systematic pattern of variation between ions and elements in human RBC's in freshly drawn blood. Specifically as K^+ and Cl^- decrease in concentration, Ca^{2+} and S increase in concentration. Phosphorous, Na^+ and Mg^{2+} did not demonstrate a significant pattern of change. These findings are in keeping with the past observations and suggestions reported above, mainly that RBC senescence is accompanied by: decreased water, decreased K^+ and decreased volume, in addition to increased density and increased Ca^{2+}.

What are the mechanisms responsible for these changes? A possible failure of membrane "pumps" comes to mind. Although

the failure of the membrane Na^+, K^+ ATPase pump would account for the loss of K^+ from the RBC, it would not account for the fact that Na^+ did not increase. Likewise the increase in RBC level of Ca^{2+} is expected if the membrane localized Ca^{2+} ATPase pump failed. However the measured increase in Ca^{2+} level within the RBC was so profound (450%) that the calculated intraerythrocyte concentration of Ca^{2+} of some RBCs reached levels higher than the known Ca^{2+} level in the extracellular blood plasma. Such an observation can best be explained by the binding of Ca^{2+} to intracellular sites. The measured magnitude of Ca^{2+} concentration change suggests either that Ca^{2+} plays a causal role in RBC senescence or that the Ca^{2+} change is simply a secondary reflection of the senescence state of RBCs. If the Ca^{2+} increase is not causally related to RBC senescence then it is at least a usable marker of RBC senescence.

The idea that RBC volume change is achieved and maintained by balancing osmotic pressure by loss or gain of inorganic electrolytes and compatible solutes between the intracellular and the extracellular water compartments, both of which are assumed to have the properties of bulk water and free ions, has been challenged (Cameron et al. 1988 ab, 1990, 1991, Miseta et al., 1992). These reports indicate: that a large fraction of RBC water has motional properties different from bulk water (Cameron et al., 1988b, 1991) that K^+ within the RBC is not free to diffuse (Cameron et al., 1988a, 1990), and that ATP within the RBC is used to maintain the relatively immobile state of K^+ not for the functional maintenance of RBC K^+ due to the membrane localized ATP hydrolyzing ions transport pumps (Miseta et al., 1992). RBC volume regulation is proposed to involve changes in the osmotic properties of RBC and ions interacting with intracytoplasmic proteins. Accordingly the positive relationship between K^+ concentration and level in RBCs (Miseta et al., 1992) has been attributed to the role the ATP plays in maintaining the conformational state of protein needed to "order or structure" intracellular water and to decrease the mobility of K^+ and Cl^- not to the operation of membrane pumps.

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Paper received 20.07.92. Revised paper accepted 02.11.92.

Comp. Biochem. Physiol. Vol. 106A, No. 3, pp. 479–482, 1993
Printed in Great Britain

0300-9629/93 $6.00 + 0.00

ERYTHROCYTE POTASSIUM AND SODIUM POLYMORPHISMS IN DONKEY (*EQUUS ASINUS*)

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(*Received* 3 *December* 1992; *accepted* 5 *February* 1993)

Abstract—1. Erythrocyte potassium polymorphism in donkeys (*Equus asinus*) is reported.

2. The erythrocyte K^+/Na^+ ratio is 6.51–13.6 in the high potassium and 1.19–1.67 in the low potassium donkey subgroups.

3. There are some animals, however, that have intermediate K^+/Na^+ ratios of 2.21–3.91 in addition to these two distinct subgroups.

4. The low potassium type erythrocytes appeared to be smaller than the high potassium type ones.

5. Data supporting an incomplete recessive/dominant inheritance pattern of this erythrocyte potassium polymorphism is also provided.

INTRODUCTION

Intracellular K^+ and Na^+ levels vary within a wide range in erythrocytes of different species (Bernstein, 1954; Ellory and Tucker, 1983; Miseta *et al.*, 1992b), in strict contrast to other cell types within different species. The erythrocytes of most mammals, including man, contain three to 15 times more potassium than sodium ions [high potassium (HK) type erythrocytes of 80–120 mmol/l RBC, mmol/l of packed cells]. At the other extreme, erythrocytes of carnivores contain low K^+ levels (6–15 mmol/l RBC), (Bernstein, 1954; Chan, 1964; Romualdez *et al.*, 1972; Parker, 1973; Ellory and Tucker, 1983; Miseta *et al.*, 1992a). Consequently, erythrocytes of carnivores are often referred to as low potassium (LK) type or high sodium (HNa) type erythrocytes. Exceptions do however, exist. For example Meade *et al.* (1983) described an inherited strain of mongrel dogs containing HK type erythrocytes.

Distinct erythrocyte potassium polymorphisms were described in the erythrocytes of ruminants including cattle (Christinaz and Schatzmann, 1972), goats (Evans and King, 1955; Erkoc *et al.*, 1987) and in many different breeds of sheep (Evans, 1954; Agar *et al.*, 1972; Tucker *et al.*, 1973; Ellory and Tucker, 1983; Clarke *et al.*, 1989). These studies revealed that the erythrocytes of ruminants may be typical HK or LK type, although in most cases the erythrocyte potassium and sodium levels are less extreme than in the erythrocytes of carnivores (Ellory and Tucker, 1984). For example, both in the HK and LK type erythrocytes of different breeds of sheep various K^+/Na^+ ratios have been reported (Ellory and Tucker, 1983). The mechanism of inheritance of erythrocyte potassium contents in sheep is recessive/dominant (Evans and Phillipson, 1957). The LK gene appears to be incompletely dominant over the HK gene in sheep. Therefore, the erythrocytes of heterozygous animals contain somewhat higher potassium and lower sodium levels than the erythrocytes of homozygous LK sheep (Ellory and Tucker, 1983).

The low erythrocyte potassium and high erythrocyte sodium concentration in the erythrocytes of members of carnivorous and ruminant species is traditionally explained either by the lack or decrease of "pumping" activity of the membrane K^+/Na^+ stimulated ATPase, or by an increase in leakiness of the plasma membrane for potassium ions (Chan, 1964; Romualdez *et al.*, 1972; Ellory and Tucker, 1983).

Recently, we reported a close positive relationship between the erythrocyte K^+/Na^+ ratios and ATP concentrations in different species and in different individuals of the same species of sheep (Miseta *et al.*, 1992a,b). We also identified erythrocyte K^+/Na^+ polymorphism in donkey (*Equus asinus*) in this study.

In the present report, we provide information on the nature of this K^+/Na^+ polymorphism, hematological and physicochemical properties of donkey erythrocytes, and inheritance patterns of their respective potassium and sodium contents.

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Table 1. The K/Na ratios and the results of hematological analysis of the three subgroups of donkeys

	HK	IK	LK
K/Na	6.51–13.6	2.21–3.91	1.19–1.67
N	10	11	7
RBC (l/pl)	6.14 ± 0.90	5.98 ± 0.83	5.98 ± 0.95
Hb (g/dl)	12.05 ± 1.49	11.73 ± 1.39	10.96 ± 1.53
Hk (%)	35.59 ± 5.58	35.42 ± 6.12	31.83 ± 5.29
MCV (fl)	58.32 ± 7.13	59.10 ± 6.03	53.50 ± 6.61*
MCH (pg)	19.80 ± 1.75	19.68 ± 1.29	18.53 ± 2.18
MCHC (g/dl)	34.13 ± 2.28	33.55 ± 2.45	34.70 ± 1.91

*$P = 0.1$.

MATERIALS AND METHODS

Sample taking and the analysis of hematological parameters

Heparinized blood (approximately 125 IU heparin/ml blood) was obtained from a suitable vein of donkeys (*Equus asinus*). The blood sample holders were immediately shifted to wet ice and the blood used for experimental purposes within the shortest possible time, usually within 2 hr.

Analysis of hematological parameters (Table 1), including erythrocyte numbers (RBC), plasma hemoglobin concentrations (Hb), plasma hematocrit (Hk), mean erythrocyte volume (MCV), mean erythrocyte hemoglobin content (MCH), and mean erythrocyte haemoglobin concentration (MCHC) were measured by a Coulter Model S Plus counter.

Measurements of erythrocyte K^+, Na^+, and Mg^{2+} concentrations

Erythrocyte K^+ and Na^+ concentrations were measured by a flame photometer (OMSZÖV, Hungary). The heparinized blood samples were pelleted first by a table top centrifuge (Hettich, EBA 3S) at 4000 rpm for 10 min, the supernatants and buffy coats carefully removed and 1.5 ml aliquots of the pelleted erythrocytes transferred into microcentrifuge tubes. The samples were pelleted at 12,000 rpm for 12 min, and the supernatants removed. Two 0.05 ml aliquots of the pelleted erythrocyte samples were taken by an automated dilutor (Braun, Melsungen), and used for flame photometric measurement. K^+ and Na^+ concentrations are given as mmol/kg RBC (mmol/kg packed cells).

The rest of the pelleted erythrocyte samples were transferred into Ni sample holders of known weight and their weights measured gravimetrically by an analytical balance (OWA Labor, Germany). After drying the samples at 104°C for 48 hr their weights were measured again. The water contents were calculated, and expressed as a percentage of the total erythrocyte mass.

For the determination of erythrocyte magnesium concentrations dried erythrocyte pellets were exposed to 3 ml 1 N HCl for 24 hr. Magnesium concentrations were measured by an atomic absorption spectrophotometer (Jarrel ASH, U.S.A).

ATP measurements

Erythrocyte ATP concentrations were determined by the chemiluminescent firefly luciferin/luciferase system (ATP bioluminescence kit CLS, Boehringer, Mannheim) according to the method developed in our laboratory (Köszegi, 1988). Hemoglobin-free erythrocyte ghosts were prepared according to Dodge's method (Dodge *et al.*, 1963), incubated as described by Chan (1964) and their ATPase consumption measured by the above chemiluminescent method. Three aliquots of the same blood or erythrocyte ghost samples were measured and averaged in each case.

RESULTS

Erythrocyte potassium and sodium polymorphism in donkeys

Figure 1 illustrates the respective erythrocyte K^+, Na^+ and total monovalent cation ($K^+ + Na^+$) concentrations in 30 donkeys. These animals do not represent a selected breed, and sometimes different individual donkeys had no records of ancestry.

While the total erythrocyte monovalent cation concentrations ($K^+ + Na^+$) of different donkeys fell within a narrow range (100–114.2 mmol/l RBC), significant differences were recorded between K^+ and Na^+ concentration (and consequently in the K^+/Na^+) among different donkeys. Eight animals had erythrocytes with only moderately higher intracellular K^+ than Na^+ concentrations, with ratios between 1.19 and 1.67. Ten animals contained typical HK type erythrocytes with K^+/Na^+ ratios equal or higher than 6.51.

Another 12 animals contained erythrocytes with intermediate K^+ and Na^+ levels (IK erythrocytes), i.e. ratios between 2.21 and 3.91.

It must be noted that although most animals were well separated on the basis of their erythrocyte K^+ and Na^+ contents, and therefore ordered into charac-

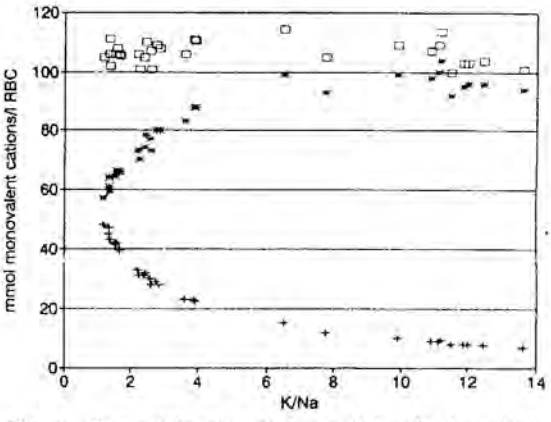

Fig. 1. The relationship between K^+, (*) Na^+ (+), $K^+ + Na^+$ (□) concentrations and K^+/Na^+ ratios in donkeys (*Equus asinus*).

teristic subgroups, two animals in the high potassium and three animals in the intermediate potassium subgroups contained significantly less or more potassium respectively than most other individual animals in the relevant subgroups. Therefore, our classification remains arbitrary to a certain extent.

The limited information value extracted from the records of ancestry of related donkeys show that: (i) LK type parents have LK type descendants, (ii) HK type parents may have either HK or IK and sometimes LK type descendant, (iii) HK and LK type ancestors may have LK or IK type descendants.

Hematological parameters in donkeys with different erythrocyte K^+ and Na^+ concentrations

High potassium type (HK) and intermediate potassium type (IK) donkey erythrocytes have similar hematological parameters. Only the mean corpuscular volume (MCV) of low potassium type erythrocytes was significantly smaller than the MCV values of either the HK and IK type erythrocytes. The mean values of the blood hemoglobin (Hb), hematocrit (Hk) and mean corpuscular hemoglobin content (MCH) of LK type erythrocytes appear to be smaller than the Hb, Hk or MCH of other erythrocyte groups, but these are statistically not significant differences. There is no significant difference in the mean corpuscular hemoglobin concentration (MCHC) of the three erythrocyte subgroups.

In an osmotic lysis experiment, HK and LK type erythrocytes were exposed to serial dilutions of a 0.89% NaCl solution at room temperature for 2 hr. The start of hemolysis occurred in 0.46% NaCl solutions, and the hemolysis was complete in 0.40% NaCl solutions in the case of either erythrocyte type.

ATP concentrations and ATPase activities in donkey erythrocytes and erythrocyte ghosts

The erythrocyte ATP levels in donkeys were low, (0.133 ± 0.029 mmol/l RBC) when compared to human erythrocytes or to the erythrocytes of most other mammalian species (Bernstein, 1954; Miseta *et al.*, 1992b). No significant differences in the ATP or Mg^{2+} concentrations could be detected among the three erythrocyte subgroups classified according to their K^+/Na^+ ratios (Table 2).

However, there were significant differences in the ouabain dependent ATPase activities in intact erythrocytes (data not shown) and in hemoglobin free ghosts of LK and HK type erythrocytes. HK type erythrocytes or erythrocyte ghost prepared according to Dodge's method (Dodge *et al.*, 1963) possessed a larger ouabain (0.2 mmol/l ouabain) inhibitable ATPase fraction than their LK type counterparts (Table 2).

Table 2. ATP and Mg^{2+} concentrations and ATP consumption of the different subgroups

Subgroup	ATP (mmol l RBC)	Mg^{2+} (mmol kg water)	ATP consumption μM g^{-1} protein hr^{-1}	
			Control	Ouabain
HK	0.124 ± 0.030	4.18 ± 0.42	182 ± 31	85 ± 11
IK	0.129 ± 0.033	4.76 ± 0.86	—	—
LK	0.145 ± 0.026	4.63 ± 0.35	158 ± 21	145 ± 13

DISCUSSION

Polymorphism in the erythrocyte K^+/Na^+ content of ruminants is well known (Evans, 1954; Agar *et al.*, 1972; Tucker *et al.*, 1973; Erkoc *et al.*, 1987; Clarke *et al.*, 1989; Ellory and Tucker, 1983; Miseta *et al.*, 1992b). According to our knowledge, no such polymorphisms were noted in other mammalian species including the members of the equine genus. In the present report we describe erythrocyte K^+ and Na^+ polymorphisms in donkeys (*Equus asinus*).

Donkeys may be categorized into three subgroups based on their erythrocyte K^+ and Na^+ contents (Fig. 1). Approximately one-third of the total population investigated in the present study have erythrocyte potassium and sodium contents comparable to those of most mammalians including man (K^+/Na^+ is in the 6–15 range). Twenty other individual donkeys had significantly lower K^+/Na^+ ratios, and they may be categorized into two other subgroups. The subgroup with the lowest K^+/Na^+ ratios is named as LK (low potassium type) throughout the present report, while the other subgroup with somewhat higher K^+/Na^+ ratios was labelled as IK (intermediate potassium type). It must be noted that LK type erythrocytes of ruminants may contain significantly less potassium and more sodium than LK type donkey erythrocytes (Ellory and Tucker, 1983).

The phenomenon of erythrocyte K^+ and Na^+ polymorphism is usually explained on the traditional "pump and leak" model (Ellory and Tucker, 1983). Thus the low erythrocyte K^+/Na^+ ratios (LK type erythrocytes) are explained by either a decreased K^+/Na^+ pump function or by an increased outwardly directed leak of K^+ and inwardly directed leak of Na^+ (Chan, 1964; Romualdez *et al.*, 1972; Ellory and Tucker, 1983). We determined higher ouabain sensitive ATPase activities in HK type erythrocytes and in ghosts prepared from HK type erythrocytes than in their LK type counterparts (Table 2). Although a discussion of the problems associated with this simplified view of erythrocyte monovalent cation transport is beyond the scope of the present paper, we briefly refer to the fact that detergent-permeabilized chicken erythrocytes fail to realease their K^+ immediately (Cameron *et al.*, 1988). A growing body of evidence supports the hypothesis that the cytoplasmic binding of monovalent cations may play a significant role in the maintenance of high intracellular K levels (Kellermayer *et al.*, 1986; Ling and Ochsenfeld, 1991; Miseta *et al.*, 1991).

The mechanisms by which a maturing LK type canine or ruminant erythrocyte loses K^+ and gains Na^+ have been studied extensively (Kirk *et al.*, 1983) but the decrease of the erythrocyte K^+/Na^+ ratio

does not simply correlate to an erythrocyte maturation dependent decrease in the number of "membrane K^+ and Na^+ pump" molecules (Ellory and Tucker, 1983). The youngest donkey studied by us was 2-months-old, and had LK type erythrocytes with similar K^+ and Na^+ concentrations to the parent animals.

Recently we found an excellent correlation between erythrocyte K^+/Na^+ ratios and ATP concentrations, a phenomenon which remains to be explored, but nevertheless directs our attention to a link between erythrocyte metabolism and K^+, Na^+ concentrations other than supplying the plasma membrane K^+ and Na^+ activated ATPase (Miseta *et al.*, 1992a,b).

HK and LK type erythrocytes of the same species may differ in their hematological parameters. Although the LK type erythrocytes of donkeys appear to have lower hematocrit, MCV and MCH values than the HK or IK type erythrocytes, this is only seen as a tendency rather than a statistically significant difference.

The inheritance of HK and LK characteristics in donkey erythrocytes may be similar to those seen in sheep (Ellory and Tucker, 1983). An autosomal dominant/recessive inheritance pattern of the HK and LK characteristics where the LK character is dominant and HKLK (heterozygous) animals (IK group) would give somewhat higher K^+/Na^+ ratios than LK/LK (homozygous) animal, fit with available data. Unfortunately, we have been unable to detect a well-recorded donkey population here in Hungary. Therefore, other inheritance patterns cannot be entirely ruled out.

Acknowledgements—We wish to thank Dr E. M. Tucker for calling our attention to the importance of this subject. We thank the valuable technical assistance of Ms Agnes Stocker and Peter Rózsahegyi. The study has been supported by OTKA F-5197.

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JOURNAL OF CELLULAR PHYSIOLOGY 159:197–204 (1994)

Release of Potassium, Lipids, and Proteins From Nonionic Detergent Treated Chicken Red Blood Cells

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The plasma membrane of erythrocytes, as of other cells, is thought to act as the barrier responsible for maintaining intracellular gradients of most ions and small molecular species between the cell and its environment. Controlled application of the nonionic detergent Brij 58 effectively opened the erythrocyte plasma membrane, as judged by electron microscopy and lipid mobilization, but the cytoplasm maintained much of its integrity for about 30 min. Release of K^+ correlated well with release of protein into the surrounding medium. The results demonstrate that permeabilization of the erythrocyte plasma membrane does not result in an instantaneous equilibration of small ions, such as K^+, between the cell and its environment. A comparison was made between erythrocytes treated with Brij 58 and Triton X-100. The lipid and protein solubilizing actions of Triton X-100 were not as easily separable in time as those of Brij 58. The results of treatment of the erythrocytes with different types of nonionic detergents suggest that the membranolytic and cytoplasmic protein destabilizing actions of nonionic detergents correspond with their hydrophilic-lipophilic balance numbers (HLB values).

It is generally perceived that the plasma membrane plays the major role in maintaining the nonequal distribution for small ionic and molecular species between the cell and its environment. The results of classical studies dealing with intact erythrocytes (Post, 1989) accepted, and were based on the premise, that the plasma membrane of erythrocyte ghosts is capable of creating and maintaining molecular and ionic gradients (Freedman, 1976).

However, experiments involving the selective removal of membrane lipids while leaving the cytoplasm relatively intact are providing a growing body of evidence that the small molecules and ions in the cytoplasm of diverse cells and tissues such as frog sartorius muscle (Ling, 1978), detergent permeabilized thymus lymphocytes (Kellermayer et al., 1984), monolayer fibroblast cultures (Kellermayer et al., 1986), porcine lens (Miseta et al., 1991), and chicken erythrocytes (Cameron et al., 1988) do not equilibrate with the environment as rapidly as expected if they were freely diffusible. Dispersion of the cytoplasmic architecture which might be expected to occur almost immediately after the mechanical or chemical removal of the plasma membranes does not necessarily follow at the time of membrane poration. For example, the treatment of cells with the nonionic detergent Brij 58 allows this interval to be extended to 30 min or more. Previous studies indicate that dispersion of cytoplasmic architecture could be the rate-limiting step in the equilibration of small ions and molecules between the intra- and extracellular compartments rather than loss of integrity of the plasma membrane per se (Miseta et al., 1991; Cameron et al., 1988, 1991). Consequently, the general assumption that the aqueous cytoplasm behaves like a dilute solution and has no significant role in maintaining intracellular gradients for both small and large molecular species appears untenable. For example, evidence already exists that the supposedly "soluble" enzymes of the glycolytic pathway do not disperse as expected from permeabilized cells in the manner expected of molecules in true solution (Clegg, 1991).

We have previously reported that not a single chicken erythrocyte membrane was free from disruptions after 1 min exposure to 0.2% Brij 58 and that high cytoplasmic and nuclear K^+ concentrations relative to the extracellular concentration were determined by electron probe X-ray microanalysis at significantly

Received July 21, 1993; accepted November 12, 1993.

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longer exposure to the detergent (Cameron et al., 1988). In the present report, we provide further structural and biochemical evidence that the plasma membranes of chicken erythrocytes exposed to solutions containing the nonionic detergent Brij 58 are effectively opened and their lipid components appear in the medium, but their cytoplasmic contents are slowly mobilized. This slow mobilization of cytoplasmic contents allows a relatively high cytoplasmic/extracellular gradient of K^+ to be maintained for at least 30 min, the rate of equilibration being primarily determined by the rate at which the cytoplasmic macromolecules are disassembled and released.

The sequence of molecular events associated with the action of different nonionic detergents on the plasma membranes and on the cytoplasm is described in this report.

MATERIALS AND METHODS

Materials

Triton X-100 (iso-octylphenoxy-polyoxyethylene) and Brij 35 (polyoxyethylene-23-lauryl-ether) were purchased from Reanal (Budapest, Hungary); Brij 56 (polyoxyethylene-10-cetyl-ether) and Brij 58 (polyoxyethylene-20-cetyl-ether) were purchased from Serva (Heidelberg, Germany). Brij 78 (polyoxyethylene-20-stearyl-ether) and Brij 99 (polyoxyethylene-20-oleyl-ether) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were purchased from Reanal (Budapest, Hungary).

Preparation of washed erythrocytes

Blood was obtained from white Leghorn chickens by decapitation and exsanguination into a heparinized beaker. The hematocrit value was determined by capillary centrifugation. Heparinized chicken blood was suspended in 0.15 M NaCl at a 1:4 (vol:vol) ratio and pelleted at 2,000g for 10 min in a Sorvall RC5 centrifuge at room temperature (22–25°C). The supernatants were discarded and the sedimented cells resuspended and washed twice in 0.15 M NaCl solution at a 1:15 (vol:vol) ratio. Aliquots (5 ml) of this cell suspension were transferred into test tubes and centrifuged at 2,000g for 10 min, and the packed cells were used as experimental samples.

Detergent treatment

Erythrocyte pellets were resuspended in 5 ml of incubation medium (1:15 erythrocyte:solution ratio). The incubation media were (1) 0.15 M NaCl containing 0.2% Triton X-100, (2) 0.15 M NaCl containing 0.2% Brij 58, and (3) 0.15 M NaCl. In other experiments, the effects of equimolar (1.9 mmol/l) concentrations of Brij 56, Brij 58, Brij 78, and Brij 35 detergents were compared.

Incubations were carried out for 2, 5, 10, and 30 min at room temperature, with duplicate specimens being taken for morphological analysis. The remaining cell suspensions were immediately centrifuged at 3,000g for 5 min and the pellets and supernatants processed for further investigations. Pellets were washed twice in 5 ml 0.15 M saline solution and their K^+, protein, and lipid contents analyzed.

The time-dependent release kinetics of proteins, hemoglobin (Hb), potassium, and lipids into the supernatants were followed.

K measurements

Potassium released into the supernatants was measured with a digital flame photometer (OMSZÖV, Budapest, Hungary) and its concentration expressed as a percentage of the total K in the original (unincubated) cell suspension.

Protein release

The protein concentration of supernatants was measured by a modification of Lowry's method (Lowry et al., 1951). Ten percent SDS solution was added instead of water to the sample/reagent mixture prior to the Folin-Ciocalteu reagent. This modification eliminates the disturbing effect of nonionic detergents during photometry. The released (extracellular) proteins were expressed as a percentage of protein concentration obtained after 30 min of 0.2% Triton X-100 treatment.

Protein electrophoresis

Proteins in the supernatant fractions, and in the detergent-resistant and control pellets, were analyzed by the SDS-PAGE procedure. Samples were treated by the protocol of Carroll et al. (1982). Equivalent aliquots of the supernatant and pellet fractions were loaded onto 10% polyacrylamide-SDS slab gels and electrophoresed as described by Laemmli (1970). Sigma MW-SDS 200 standards were used as molecular weight markers. The gels were stained in 0.2% Coomassie Brilliant Blue R-250 in 45% methanol/10% acetic acid.

Lipid analysis

One milliliter samples from both the supernatants and the resuspended pellet were used. Lipids were extracted twice with 5 ml chloroform/methanol (2:1) according to the method of Folch et al. (1957). For separation of lipids, a three-step, one-dimensional thin-layer chromatographic procedure was used (Tompkins and King, 1974). Isolation was carried out on silica gel plates (Merck 60, Merck, Darmstadt, Germany). The total lipid extracts of the samples were evaporated by heating in a water bath at 70°C. The residues were dissolved in 0.5 ml chloroform/methanol (2:1), from which 0.15 ml aliquots were applied to the origin of the TLC plates. The plates (5 × 20 cm) were first placed into chloroform:methanol:water (65:25:4 volume ratios) and developed to a height of 8 cm in order to separate the phospholipid fractions. After air-drying for 60 sec, they were placed in hexane:diethylether:acetic acid (70:20:4 volume ratios) and developed to a height of 12 cm to separate monoglycerides, diglyceride fractions, and free cholesterol. The plates were air-dried for 60 sec and placed into a tank containing hexane:diethylether:acetic acid (85:15:1) to separate cholesterol esters and triglyceride fractions.

Spots were sprayed with a staining solution (Chedid et al., 1972) and the lipids visualized by heating the plates to 85°C for 20 min. The positions and intensities of spots of the equivalent aliquots of samples were visually compared.

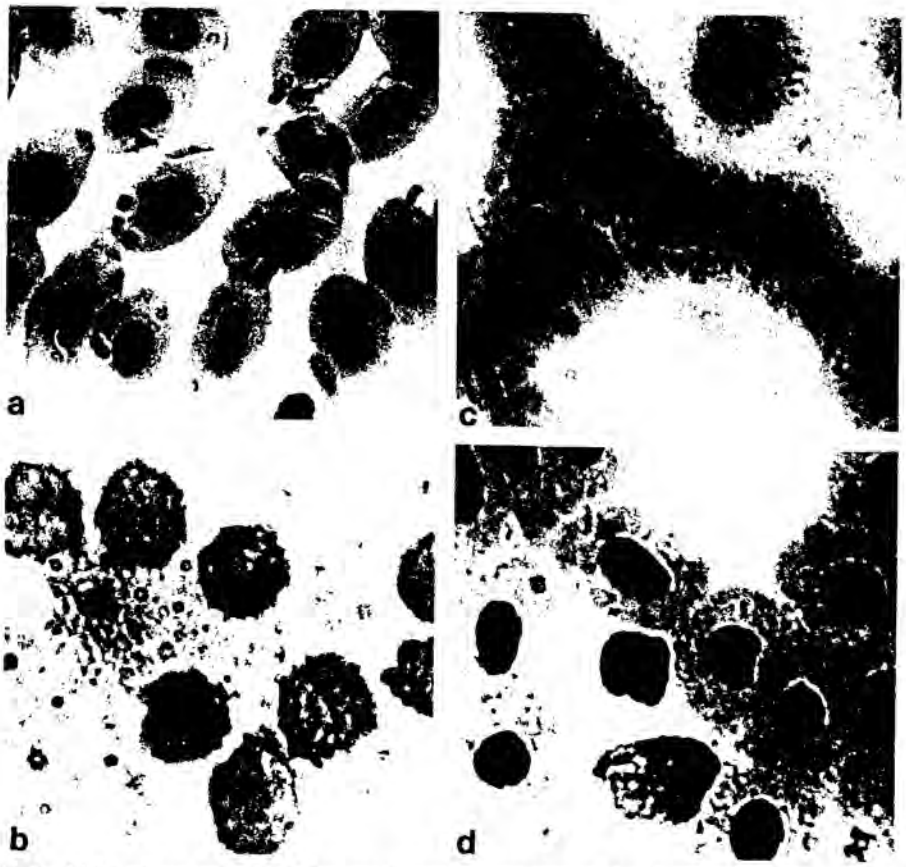

Fig. 1. Light micrographs of chicken erythrocytes incubated in the absence and in the presence of nonionic detergents. **a:** Control, no detergent. **b:** Erythrocytes exposed to 0.2% Triton X-100 for 2 min. **c:** Erythrocytes exposed to 0.2% Brij 58 for 2 min. **d:** Erythrocytes exposed to 0.2% Brij 58 for 10 min. Giemsa staining. ×2,800.

Light and electron microscopy

Samples prepared for light microscopy were air-dried, fixed in methanol, and stained with Giemsa solution. For electron microscopy, the control cells and the detergent-resistant cellular components were fixed in 2.5% glutaraldehyde and postfixed in 1% OsO_4. After fixation, the cells were embedded in Araldite. Sections were cut with a LKB ultramicrotome and stained with uranyl acetate and lead citrate before examination in a JEOL 100C electron microscope. Fifty to one hundred cells were checked in each section. The electron density of individual erythrocytes was visually estimated. A quantitative determination, based on electron probe X-ray microanalysis of sulphur contents, has previously been described (Cameron et al., 1988).

RESULTS

Morphology of detergent treated erythrocytes

The light microscopic appearance of chicken erythrocytes incubated in 0.15 M NaCl for 30 min was identical to that of intact erythrocytes (Fig. 1a).

Erythrocytes exposed to 0.2% Triton X-100 disintegrated quickly, and the cell boundary disappeared within 2 min (Fig. 1b). Only a residual fibrillar network of nuclear chromatin resisted the immediate solubilizing effect of the detergent.

The effect in Brij 58 was less dramatic than incubation in Triton X-100 but clearly recognizable in each of the cells (Fig. 1c). The cells were more spherical (swollen) than controls, and the sharp continuous cellular exterior boundary with the extracellular environment showed interruptions. The cytoplasm was nevertheless still rich in Hb. With passing time, the hemoglobin of the cytoplasm decreased, inhomogeneity developed, and further swelling occurred (Fig. 1d). Cell nuclei appeared to have an increased density of packed chromatin from the very beginning of the incubation, but their overall shape and location within the cell did not change. An increased aggregation of cells was noted.

The lytic effect of Brij 58 on the erythrocyte membranes was also visualized by electron microscopy. After 2 min incubation the integrity of plasma membrane of each erythrocyte was severely interrupted, a typical example being shown in Figure 2. The sharp distinction in density between the cell boundary and its environment decreased. The wide space between the nuclei and the plasma membrane, present in control cells, almost completely disappeared.

Despite of the moderate swelling of the Brij 58 treated erythrocytes, there was no visible electron microscopical density difference in the cytoplasm between control and most 0.2% Brij 58 exposed cells. However,

Fig. 2. Electron micrograph of chicken erythrocyte incubated in the presence of Brij 58 detergent for 2 min. Arrows point to major membrane lesions. ×60,000.

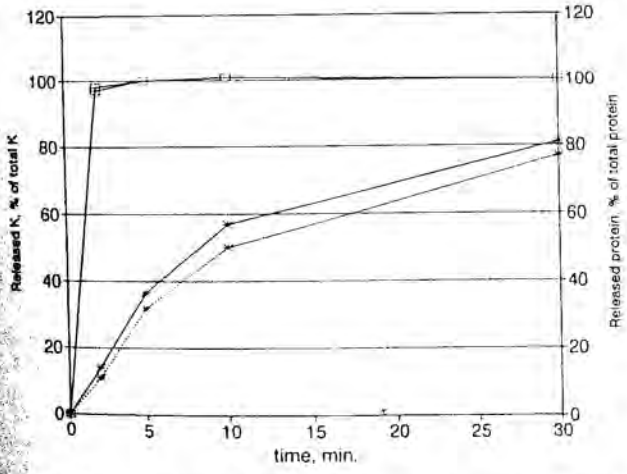

Fig. 3. Detergent-induced release of potassium (continuous line) and proteins (dashed line) from chicken erythrocytes exposed to 0.2% Triton X-100 (open squares) or 0.2% Brij 58 detergents (stars).

some erythrocytes lost their cytoplasmic electron density, and there was a gradual increase in their numbers the longer cells were exposed to the detergent.

Release kinetics of K^+ and proteins

No significant release of K^+ and proteins occurred in cells incubated in 0.15 M NaCl (control).

In contrast, the potassium concentration of the incubation media containing Triton X-100 or Brij 58 treated erythrocytes increased with incubation time. The released K^+ had reached its new equilibrium within the first time-point (2 min) in 0.2% Triton X-100 containing solution (Fig. 3)—that is, the K^+ concentrations of the supernatant and of the water of the residual pellet were identical (Table 1).

During exposure to 0.2% Brij 58, loss of K^+ was more gradual (Fig. 3). A 50% loss of the initial K^+ content took 8 min incubation and increased gradually with further incubation. Nevertheless, a fivefold excess of erythrocyte K^+ over extracellular K^+ was still present even after 30 min incubation (Table 1).

TABLE 1. Potassium concentration of incubation media and erythrocytes before and after Brij 58 or Triton X-100 treatment

Detergent	Time (min)	n	K mmol/kg cell water	K mmol/kg water	K in/K out
None		8	139.8 ± 2.6	—	—
Brij 58	2	8	119.7 ± 5.2	1.00 ± 0.23	125.6 ± 25.1
	5	8	75.5 ± 2.1	2.97 ± 0.10	25.4 ± 1.5
	10	8	62.1 ± 2.7	3.50 ± 0.12	17.8 ± 1.4
	30	8	27.2 ± 2.7	5.07 ± 0.13	5.4 ± 0.7
Triton X-100	2	8	6.4 ± 1.2	6.05 ± 0.06	1.1 ± 0.2

The release of proteins (mostly Hb) correlated well with the release of K^+ ions with each detergent (Fig. 3). As indicated in the following section, the obvious dissociation of K^+ loss, in terms of total release of K^+ into the supernatants compared to residual K^+ of pelleted cells, was due to pelleted erythrocytes at the later time-points including a greater proportion of more detergent-resistant population.

In some experiments chicken erythrocytes were incubated in 0.15 M NaCl solution in the absence or presence of 0.2% Brij 58, as described in Materials and Methods, but 0.01% trypan blue was added. After 2 min incubation the cells were pelleted at 3,000g for 2 min, the supernatants discarded, and the pellets resuspended in 0.15 M NaCl. The washing step was repeated twice and the resuspended erythrocytes checked in a Buerker chamber under a light microscope. Whereas all erythrocytes remained unstained in the absence of Brij 58, none were left unstained in its presence.

Comparison of K^+ release from Brij 35, Brij 58, and Brij 56 treated erythrocytes

The lipolytic character of detergents was most closely associated with their critical micellar concentrations (CMC). Equimolar concentrations (1.9 mmol/l; i.e., at least one order of magnitude above their respective CMCs) of Brij 35, Brij 58, and Brij 56 detergents in isosmotic NaCl solutions had different effects on the K^+ and protein release from chicken erythrocytes (Fig. 4). The effect of Brij 56 was similar to that of Triton X-100, resulting in a quick release of K^+ ions and proteins. In contrast, K^+ release from Brij 35 treated erythrocytes proved to be even more protracted than from Brij 58 treated erythrocytes, and protein release was significantly smaller than K^+ release. In each case, the amount of released K^+ ions correlated well with the release of proteins. This phenomenon showed the closest correlation with the HLB values of the detergents, since for Brij 56 HLB equaled 12.9, for Brij 58 HLB equaled 15.8, and for Brij 35 HLB equaled 16.9. The LB for Triton X-100 is 13.5. Results similar to those seen in Brij 58 treated erythrocyte samples were obtained with other Brij series detergents (Brij 78, Brij 99) which have almost identical HLB values (15.3) (data not shown).

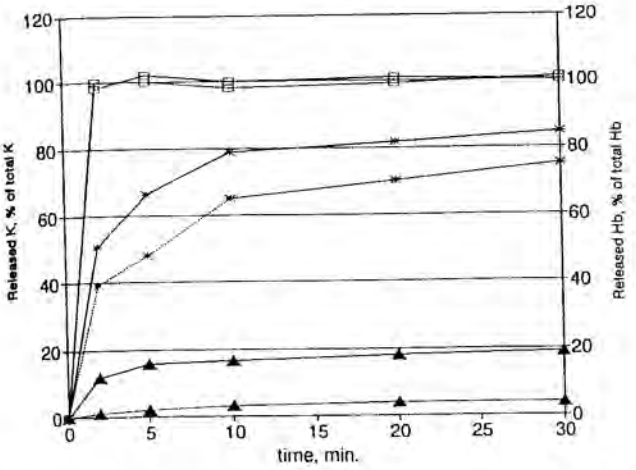

Fig. 4. Detergent-induced release of potassium (continuous line) and proteins (dashed line) from chicken erythrocytes exposed to 1.9 mmol/l Brij 35 (triangles), Brij 58 (stars), and Brij 56 (open squares).

The release of K^+ and proteins was not significantly affected by the addition of 0.2 mM ouabain to the incubation medium.

Polyacrylamide gel electrophoresis of detergent-released and detergent-resistant proteins

The detergent-soluble and -resistant proteins of chicken erythrocytes exposed to 0.2% Triton X-100 and 0.2% Brij 58 were analyzed by conventional SDS-PAGE procedures, with the sum of released and pelleted proteins being kept constant. Comparative electrophoresis revealed no significant differences between solubilized or residual (pelleted) protein populations of detergent treated cells incubated for 2, 5, 10, and 30 min in Triton X-100 (Fig. 5). This is probably due to the quick (within 2 min) protein mobilizing effect of Triton X-100.

The detergent-soluble proteins of Brij 58 treated cells became increasingly richer in low molecular weight protein species (Mw. < 35KD) as incubation time increased. The opposite was true for the Brij 58–resistant pellet, where the known detergent-resistant cytoskeletal components, as judged by reference to molecular weight (i.e., spectrin, actin), became increasingly more prominent as incubation time increased.

Consistent with the idea that proteins and K^+ are lost concurrently under these experimental conditions, the 30 min Brij 58–resistant pellet compared best with the Triton X-100–resistant fractions.

Lipid release from detergent treated cells

The TLC method of Tompkins and King (1974) proved suitable for the simultaneous development of major lipid fractions (Fig. 6 from bottom to top: phospholipid fractions, mono- and diglycerides, free cholesterol, triglycerides, and cholesterol-esters). Chromatograms of the lipid fractions extracted from whole chicken blood or from washed chicken erythrocytes and lipids present in the supernatant after 5 min Brij 58 treatment are shown in panel A. All lipid fractions present in erythrocytes were released into the medium

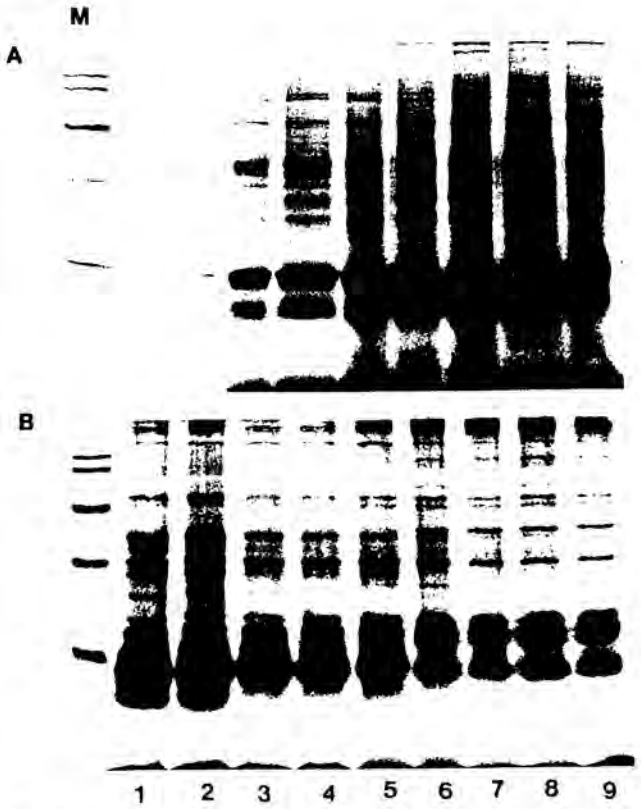

Fig. 5. SDS-PAGE electrophoretograms of proteins separated from control and detergent treated chicken erythrocytes. Proteins were stained with Coomassie Blue. **A:** Supernatant fractions. **B:** Control and detergent-resistant pellets. **Lane 1:** Control, no detergent. **Lanes 2–5:** Brij 58 treatments for 2, 5, 10, and 30 min, respectively. **Lanes 6–9:** Triton X-100 treatments for 2, 5, 10, and 30 min, respectively. **M:** Molecular weight markers: from top to bottom: myosin (206 KD), beta-galactosidase (116 KD), phosphorylase B (97 KD), bovine albumin (66 KD), egg albumin (45 KD), carbonic anhydrase (29 KD).

after 5 min exposure to 0.2% Brij 58. The medium of washed controls cells incubated in 0.15 M NaCl contained no detectable lipids.

We also compared the lipid mobilizing effects of Triton X-100 and Brij 58. The released and residual lipids of erythrocytes incubated for 5 and 30 min are shown in panel B of Figure 6. There was no significant difference in the lipid mobilizing capabilities of the two detergents. Furthermore, there was no significant increase in the amounts of lipids released between 5 and 30 min incubation in the presence of either Triton X-100 or Brij 58. Some nonmobilized lipids were detected in cell pellets after treatment with both of these detergents, but they appeared to be qualitatively similar. Detergent-resistant, strongly bound residual lipids were described by other authors (Traub et al., 1986).

DISCUSSION

Nonionic detergents are widely used in routine and experimental cell fractionation procedures. Among these, Triton X-100 is one of the most frequently used. The use of the less familiar Brij 58 is now becoming popular because it allows a gentler chemical "dissec-

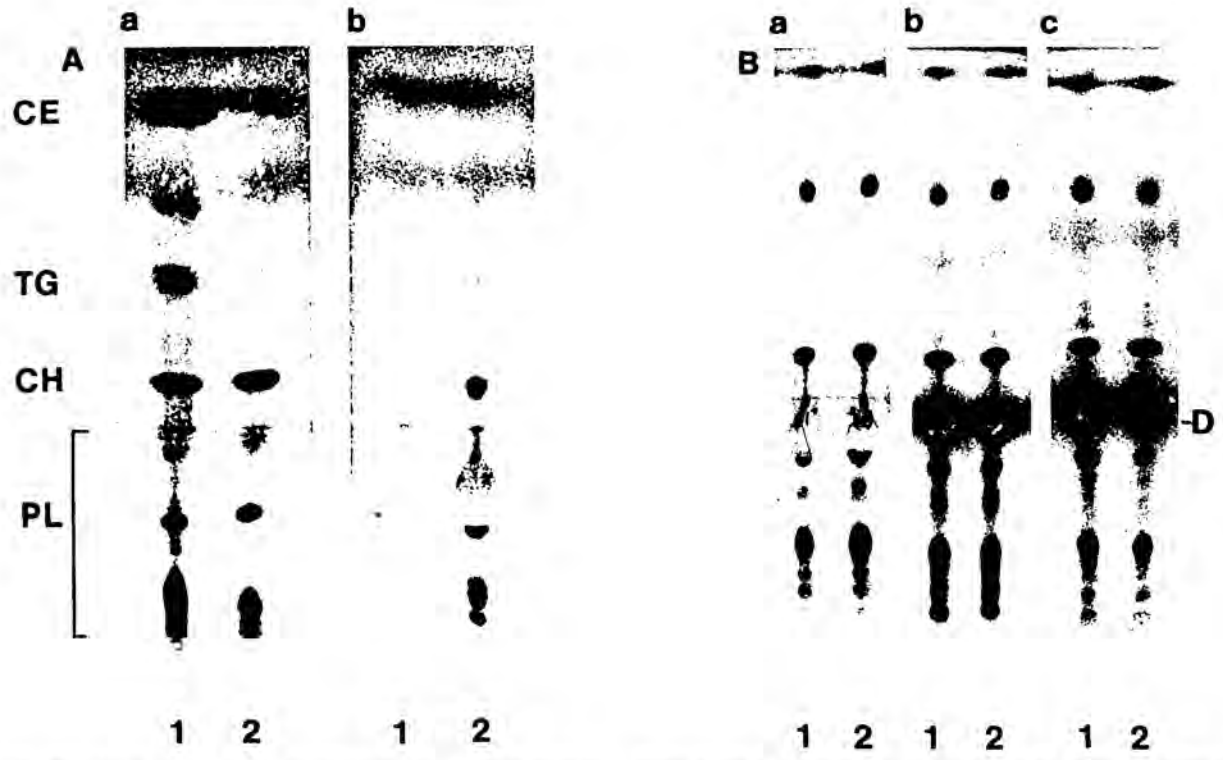

Fig. 6. Thin-layer chromatographic (TLC) pattern of total lipid in control chicken erythrocytes and lipids mobilized by Brij 58 detergent. **A: a/1:** Whole chicken blood. **a/2:** Washed erythrocytes (control erythrocytes). **b/1:** Supernatant fraction of control cells (no detergent). **b/2:** Supernatant fraction of cells treated with Brij 58 detergent for 5 min. Equivalent numbers of cells were compared. **B:** TLC separation of lipids released by detergents from chicken erythrocytes and of residual lipids in the detergent-resistant pellets. **a:** Brij 58 treatment for 5 (**lane 1**) and 30 min (**lane 2**). Supernatant fractions. **b:** Triton X-100 treatment for 5 (**lane 1**) and 30 min (**lane 2**). Supernatant fractions. **c:** Brij 58 (**lane 1**) and Triton X-100 (**lane 2**) treatments for 30 min. Detergent resistant pellets. Equivalent numbers of erythrocytes were compared. Lipid fractions (from bottom to top): PL, phospholipid fractions; CH, free cholesterol; TG, triglycerides; CE, cholesterol-esters; D, detergents.

tion" of more delicate intracellular protein assemblies (e.g., Schliwa et al., 1981). Evidence that the lipid solubilizing (plasma membrane disrupting) and the protein mobilizing (cytoplasm dispersing) capabilities of Brij 58 can be dissociated in time indicates that Brij 58 may be usefully exploited in comparing the role of the plasma membrane with that of the cytoplasm in maintaining intracellular gradients, as exemplified by the previous findings of Kellermayer et al. (1984, 1986), Cameron et al. (1988, 1991), Hazlewood and Kellermayer (1988), Miseta et al. (1991), and Ridsdale and Clegg (1991).

In agreement with the reports of Kellermayer et al. (1984, 1986) and Cameron et al. (1988), the morphological (Fig. 1) and chemical (Fig. 3) disintegration of Triton X-100 treated cells proceeded faster than that of the Brij 58 treated cells. In the case of the Triton X-100 incubated erythrocytes, light and electron microscopic observations indicate a quick loss of density (Hb and other proteins) with the result of a residual nuclear chromatin structure within 2 min (Fig. 1). These morphological events proceed more slowly in the case of the Brij 58 incubated erythrocytes, and even after 30 min incubation cells were less disassembled than after 2 min in the Triton X-100 incubated cells. These morphological findings correlate well with the release of Hb or proteins into the surrounding medium. Release of erythrocyte contents was much faster and could not be separated from the removal of lipids in the case of the Triton X-100 incubated erythrocytes but was relatively slow (i.e., delayed after the removal of lipids) in the case of the Brij 58 incubated erythrocytes. However, a more drastic effect like that of Triton X-100 could be achieved when Brij 58 (HLB = 15.8) was substituted by Brij 56, which has a HLB ratio of 12.9 comparable to that of the Triton X-100 (HLB = 13.5; see Fig. 4). In contrast, Brij detergents with higher HLB numbers than Brij 58 caused modest hemolysis and an even more prolonged release of erythrocyte K^+.

Thus, the HLB values of polyoxyethylene adduct detergents correlate well with their membranolytic and cytoplasm dispersing properties (Fig. 4). Although excellent studies relate the HLB values of nonionic detergents to their abilities to extract certain membrane-bound proteins (Umbreit and Strominger, 1973; Slinde and Flatmark, 1976), a thorough systematic morphological and biochemical study of erythrocytes exposed to nonionic detergents with different HLB ratios, especially the ones with higher values, could accelerate our understanding of cytoplasmic entrapment of ions and other small, charged molecular species.

We previously reported that chicken erythrocytes exposed to Brij 58 develop discontinuities in their plasma

membranes within about 1 min of incubation, as judged from electronmicrographs (Cameron et al., 1988). Earlier, similar observations were made on bovine lymphocytes (Kellermayer et al., 1984) and on H-50 cells (Kellermayer et al., 1986). The loss of electron density largely attributable to (Hb) occurs quickly in individual cells (Cameron et al., 1988). Similar results were obtained by Ridsdale and Clegg (1991) on cultured mouse L929 cells. Electron probe X-ray microanalysis revealed that K^+ and phosphorus contents of erythrocytes which had not lost electron density remained high, and the overall loss of K^+ was due to an increasing number of cells that had decreased electron density, with a correspondingly low cytoplasmic and nuclear K^+ levels during incubation (Cameron et al., 1988). It was surmised that the vulnerability of chicken erythrocytes to detergents depended upon erythrocyte age. Since older human erythrocytes often have decreased water contents and consequently increased densities (Cameron et al., 1993), we measured but were unable to detect significant differences in the release of K^+ and Hb from density fractionated chicken erythrocytes (unpublished observation).

One may question whether the membranes of Brij 58 treated erythrocytes are truly "permeabilized." The evidence to back this is that (1) the morphological appearance of the plasma membranes of Brij 58 treated chicken (Cameron et al., 1988; the present study) and human erythrocytes (Cameron et al., 1991) revealed that no stretches greater than 50 nm of the plasma membranes in any of the erythrocyte was free from disruptions, and (2) membrane lipids quickly appear (<5 min) in the medium (Fig. 6), and their level does not seem to increase significantly thereafter during incubation. The continuity of membranes in all cells is disrupted but the release of K^+ delayed. These observations indicate that the "permeabilization" of a cell might not be exclusively a membrane phenomena.

Since we have shown that the cytoplasm of Brij 58 treated chicken erythrocytes is in direct contact with the medium, it is clear that these events were not accompanied by an equally rapid loss of erythrocyte K^+. Considering the size of the chicken erythrocyte, if the intracellular K^+ were free, and had its expected diffusion constant in dilute aqueous solutions (1.99 cm^2/sec), it is predicted that an equilibrium between the cell and its environment would take place in a fraction of a second after opening the plasma membrane. Possible mechanisms that delay this equilibration need to be considered.

A very efficient pumping of K^+ into the cell could, to a certain extent, counterbalance the leak of K^+ caused by the membrane damaging action of Brij 58 but is extremely unlikely since the incubation solution does not contain K^+ (i.e., not until some has leaked out from the permeabilized erythrocytes). Secondly, the medium contains no source of energy needed to maintain a gradient under such unfavorable circumstances. Thirdly, the release of K^+ ions was not affected by the presence of 0.2 mmol/l ouabain in the incubation medium (unpublished observation). And finally, results of earlier experiments on Brij 58 treated monolayer fibroblast cultures (Kôszegi et al., 1987) and human erythrocytes (Kôszegi et al., 1988) showed that the kinetics of ATP release—similarly to K^+—follows an extended sigmoidal curve. The sharp decline of erythrocyte ATP levels in Brij 58 treated cells was due in part to the leak of ATP and an increased ATPase activity which is ouabain-insensitive (unpublished observation).

The existence of unequal subcellular ion distribution before and after the fenestration of plasma membranes may alter equilibration between the cell and its environment. Indeed, there are observations indicating a considerably higher concentration of K^+ in the nuclei of intact chicken erythrocytes than in the cytoplasm (Cameron et al., 1988). Nevertheless, neither nuclear nor cytoplasmic K^+ concentration decreased significantly during incubation in the presence of detergent until there was a loss of density of erythrocytes, following which both cytoplasmic and nuclear K^+ concentrations abruptly fell (Cameron et al., 1988). The K^+ release kinetics correlated well with the release kinetics of proteins. Since solubilized proteins, including Hb, are unlikely to bind potassium ions (Ling, 1984), it can be argued that the simultaneous release of K^+ and Hb (proteins) is not due to a direct physical association between the two components. However, that such an association existed before the release of these two components cannot be eliminated. The delayed equilibration of K^+ after the effective opening of cell membranes suggests an efficient protein entrapment or binding of intracellular K^+. This is not an unreasonable hypothesis, considering that there is strong evidence that a significant portion of the intracellular K^+ appears to be adsorbed to proteins or microcompartmented in an energy-dependent manner within the living cell (Ling and Cope, 1969; Hazlewood, 1979; Kellermayer and Hazlewood, 1979; Ling 1984, 1992; Edelmann, 1988; Kellermayer, 1991).

In studies on the eye, Miseta et al. (1991) demonstrated that the crystalline lens is surprisingly resistant to 1% Triton X-100 solution and releases small quantities of protein relative to K^+ release. However, the K^+ loss was proportional to the entry of tracer molecules (sucrose) from the incubation medium, suggesting that it is not the release of proteins per se that accounts for the release of K^+. Prior to the solubilization and release of proteins, the relationship of K^+ to them in situ presumably changes in the lens, and the bound K^+ molecules that lose their association begin to leave the cytoplasm. Since diffusion efficiently supports the equilibration of molecules over the dimension of an erythrocyte but is ineffectual in the porcine lens, proteins and K^+ will leave the erythrocyte almost simultaneously, while the release of the two components remains well separated in time in the case of the porcine lens.

Transition of Hb from a relatively immobile state during Brij 58 treatment has been demonstrated recently by water suppression NMR (Cameron et al., 1991). Whether this motional acceleration of Hb molecules is directly or indirectly accompanied by the release of K^+ remains to be explored.

ACKNOWLEDGMENTS

We thank Dr. Károly Trombitás for his excellent electron microscopic work on our samples. We are also grateful for the valuable technical assistance of Ms.

Agnes Stocker and Eva Fábián. This work was supported by OTKA R-3108 and OTKA F-5197.

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EFFECT OF NON-HEMOLYTIC CONCENTRATIONS OF BRIJ -SERIES NON-IONIC DETERGENTS ON THE METABOLISM-INDEPENDENT ION-PERMEABILITY PROPERTIES OF HUMAN ERYTHROCYTES

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Abbreviations: CMC, critical micellar concentration; HLB, hydrophile-lipophile balance; Htc, hematocrit

Key words: erythrocyte, detergents, ampiphiles, potassium, sodium, plasma membrane

ABSTRACT:

Sub-critical micellar concentrations (sub-CMC) of Brij series detergents alter ion movements between erythrocytes and their environment when metabolism has been slowed down by cold incubation. The effect of equimolar, non-hemolytic concentrations of detergents on the erythrocyte K^+ and Na^+ movements is described. Results indicate striking difference in inside-out and outside-in monovalent cation movements depending on the number of hydrophilic polyoxyethylene units (n). There is an increasing loss of K^+ and gain of Na^+ where n increases from 4 to 20. Where $n \geq 21$, ion movements are no significantly different from those found in control (untreated) erythrocytes. The length of the detergent fatty acid residue appeared to be relatively unimportant, but detergents with unsaturated (oleic acid) hydrophobic regions potentiated K^+ release and Na^+ uptake dramatically when compared to the corresponding saturated fatty acid (stearic acid).

INTRODUCTION:

Polyoxyethylene-type non-ionic detergents are widely used in membrane fractionation procedures. In general, detergents with hydrophile-lipophile balance (HLB) values of 12-20 are used for the isolation of functionally intact membrane proteins (1,2). Detergents in the lower HLB range are recommended for the isolation of integral membrane proteins, while those in the higher HLB range are more useful in solubilizing external membrane proteins.

Less is known about the direct action of detergent molecules on the ion-permeability properties of membranes. Beyond the critical micellar concentrations (CMC) many conventionally used non-ionic detergents are lipolytic and cause hemolysis (3). Hemolysis is a complex time-, temperature- and environment-dependent phenomenon (4,5). Not only are the detergent properties and the characteristics of the incubation important, but the nature of the blood sample and, in particular, the erythrocyte properties themselves have to be considered (6). For example, blood group-dependent individual differences in the detergent-resistance of human erythrocytes have been described (7). Detergent action on erythrocytes is considerably more complex than the simple removal of membrane lipids with the subsequent disintegration of membranes and lysis of the erythrocytes. We have previously demonstrated by electron microscopic fenestration of plasma membranes of bovine thymus cells, cultured fibroblasts, and chicken and human erythrocytes within one min. after treatment with Brij 58 (3, 8-10). In all cell types, the cytoplasm maintained high K^+ and low Na^+ concentrations for much longer periods of time than would theoretically

be expected for total equilibration of an aqueous compartment the size of the cell with its environment. The loss of K^+ from individual chicken erythrocytes was investigated using electron probe X-ray microanalysis by Cameron et al. (10), who found that it was not apparently a continuous process, but was associated with a quick transition of erythrocytes from a high to a low density type, due to a co-operative loss of proteins (*cf.* Ridsdale and Clegg ; 11) and K^+.

Although it was noted that detergents facilitate K^+ loss and Na^+ uptake in sub-CMC, non-hemolytic concentrations, only a limited number of studies, and in part with different aims were carried out on detergent properties which are involved in this process (4,5,12-14). One of the simplest ways to investigate detergent action is in the absence of energy-dependent transport mechanisms. In the present report we describe the dependency of detergent-mediated ion permeability changes on (a) the size of the hydrophilic polyoxyethylene chain, and (b) the nature of the hydrophobic fatty acid residue (Fig. 1.).

MATERIALS AND METHODS:

Materials: Heparinized blood was obtained from healthy volunteers, samples being shifted to wet-ice immediately after collection.

Polyoxyethylene 4-stearyl ether, polyoxyethylene 10-stearyl ether (Brij 76), polyoxyethylene 20-stearyl ether (Brij 78), polyoxyethylene 21-stearyl ether (Brij 721),

polyoxyethylene 100-stearyl ether (Brij 700), and polyoxyethylene 10-oleyl ether (Brij 96) were purchased from Sigma (St. Louis, USA). All other chemicals were purchased from Reanal (Hungary) .

Measurements of erythrocyte K^+, Na^+, Rb^+ and water levels: Blood samples were centrifuged at 3 000g for 10 min, and the plasma and buffy-coat carefully removed. The erythrocyte pellets were resuspended in ice-cold 0.15M NaCl solution, centrifuged as described above, and the supernatant removed. The washing procedure was repeated twice. For experimental purposes, erythrocytes were resuspended at a hematocrit of 4% in ice-cold TBSR solution (0.01M TRIS-HCl, pH 7.4, 148 mM NaCl and 2 mM RbCl.). This solution served as control. In other cases, TBSR was complemented with one of the above described detergents. The final concentration was 50 μM, at which none of the above mentioned detergents proved hemolytic. 10 ml samples were taken immediately after the start of incubation, and at required time-points up to 4 h. The samples were centrifuged as described above, and the supernatants removed. The erythrocytes were transferred into microcentrifuge tubes of known weights, centrifuged at 16 000g for 10 min. The supernatants were removed, and residual pellets weighed gravimetrically. before being freeze-dried in a Savant speed vacuum (SC-110) system. Dry weights were subsequently measured. 0.5-1 ml of 1M HCl was added to each sample, and incubated at room temperature on a rocker table for not less than 24 h.

K^+ and Na^+ levels were measured in a flame photometer (OMSZOV, Hungary), and Rb^+ levels in a Varian AA-20 atomic absorption photometer (Varian Techtron, Australia). Actual ion concentrations were calculated after correction for dilution factors and were based on sample weight data. Since the quantity of blood available from a single volunteer was only about 14 ml, the number of parallel sample parameters which could be taken were limited, unless the number of time-points were reduced. We therefore repeated each experiment at least 3 times. Results of representative experiments are presented in this report.

RESULTS:

Human erythrocytes were incubated in the presence of 50 μM of each detergent at a hematocrit (Ht) of 4% at 0°C for maximum 4 h. None of the detergents induced hemolysis at these concentrations (always < 2% at the end of treatment.) Figure 2 demonstrates an experiment with polyoxyethylene (n)-stearyl ethers where the number (n) of oxyethylene units varied from 4 to 100. The results confirm that polyoxytheylene 4-stearyl ether has little effect on K^+ release and Na^+ uptake of erythrocytes. K^+ release and Na^+ uptake appears to increase with increasing oxyethylene chain length up until $n = 20$. Particularly striking was the fact that polyoxyethylene 21-stearyl ether fell into an entirely different category. The changes of erythrocyte K^+ and Na^+ concentrations it induced resembled those seen in the case of the polyoxyethylene 4-stearyl ether, or in control (untreated) erythrocytes. Further increase of n does not cause significant difference. Similar results were obtained when polyoxythylene (n)-lauryl ethers were used.

Erythrocyte water content remained remarkably constant, being similar to that of normal untreated (control) human erythrocyte (67 - 69%) following incubation.

In a similar set of experiments, the number polyoxytheylene residues were kept constant (n = 10). Polyoxyethylene 10-stearyl ether caused a modest acceleration of K^+ release and Na^+ uptake when compared to untreated erythrocytes (Figures 2 and 3). The results clearly demonstrate that the chain length of saturated fatty acids has little effect on electrolyte movements (Figure 2). Stearic (C = 18), cetyl (C = 17) or lauric ethers (C = 12) caused similar effects. Striking difference occurred where polyoxyethylene (10)-oleyl ether (C = 18) was used. The loss of K^+ and the gain in Na^+ and Rb^+ ions was dramatically accelerated.

Erythrocyte water contents also remained remarkably constant at close to those of untreated (control) human erythrocytes under these conditions.

DISCUSSION:

Polyoxyethylene fatty acid ethers (Brij series detergents) were added to normal human erythrocytes in non-hemolytic concentrations and the loss of K^+ compared with the uptake of Na^+ and Rb^+ studied during incubation in a simple TBSR (2 mM RbCl complemented TBS medium) at 0°C for a maximum of 4 h. The number of polyoxyethylene units (n) appeared to be critical in the equilibration process. Small detergent molecules (n = 2 - 4) with low HLB values (HLB ≅ 5.4 - 10 respectively) did not significantly facilitate K^+ loss and Na^+ uptake. Increasing oxyethylene unit numbers are associated with increased ion

permeabilities, but there is a sharp reversal of the effect of $n \geq 21$. Earlier, similar observation was made with Brij 58 ($n = 20$) and Brij 35 ($n = 23$) detergents (unpublished observation). Since the physico-chemical properties of the 20- and 21 stearyl ethers are very similar, it is likely that specific molecular interactions between the detergents and erythrocyte plasma membranes are involved into permeability changes.

A further interesting observation is that the length of the fatty acid residue appears to be relatively unimportant in ion movements in the size range investigated here. Stearic acid (C = 18) ethers behave in a similar manner to lauric acid ethers (C = 12) if the number of oxyethylene units is constant (Figure 2). The effect of oleic acid ethers (C = 18) is significantly different. Ion traffic appears to be accelerated in both directions, and the outside⇒inside Na^+ and Rb^+ movements are similar. It is likely that the hydrophobic regions of the two detergents interact differently with the membranes. To explore these possibilities, electron spin resonance investigations on the state of the erythrocyte plasma membranes have begun (results to be reported elsewhere).

Tragner and Csordas described how a structurally similar Triton series of oxyethylene adduct non-ionic detergents stabilized erythrocytes against osmotic lysis in low concentrations (5). One possible explanation is a direct expansion of the erythrocyte plasma membranes due to the incorporation of detergent molecules (12,13). This would appear to be supported by the fact that the non-hemolytic Triton X-45 ($n = 5$) stabilizes erythrocytes in a similar manner to the hemolytic Triton series detergents. Another

possibility is that an accelerated equilibration between the detergent-treated erythrocytes and their environment could be important. Fast compensatory movements of hydrated ions and small molecules presumably work against swelling induced by low osmolarity solutions, where fast net water uptake can itself cause disintegration of the plasma membrane and lysis of the cell. If this latter theory holds true, the stabilizing effect of Brij series detergents will correlate with our results. Unfortunately a direct comparison between the findings of Tragner and Csordas (5) and our data is not possible, because they carried out their experiments at higher temperatures, and their detergents, while similar to ours, were not identical. A critical testing of the above hypotheses is currently in progress.

Acknowledgments: We thank the excellent technical assistance of Pappné Bácskai Sarolta. The work was supported by Hungarian National Science Foundation Grants OTKA F-5197 and OTKA R-3108.

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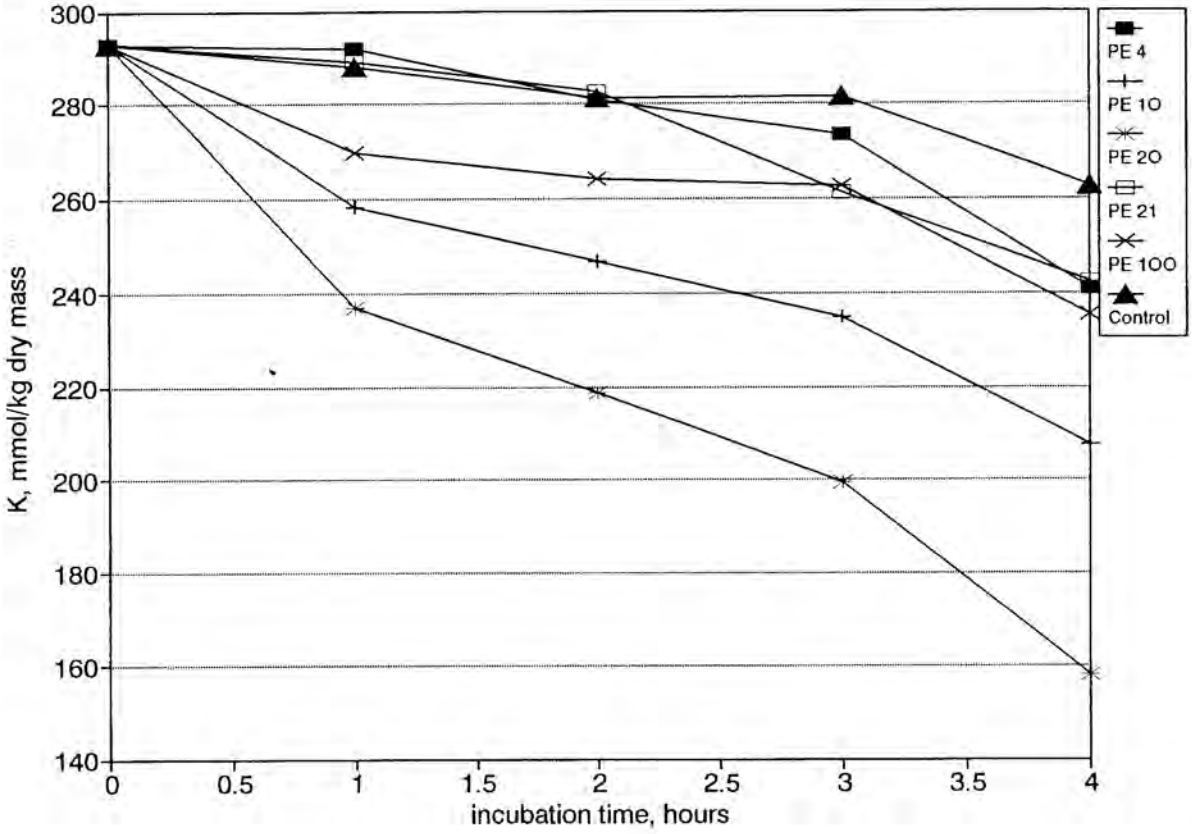
FIGURE LEGENDS:

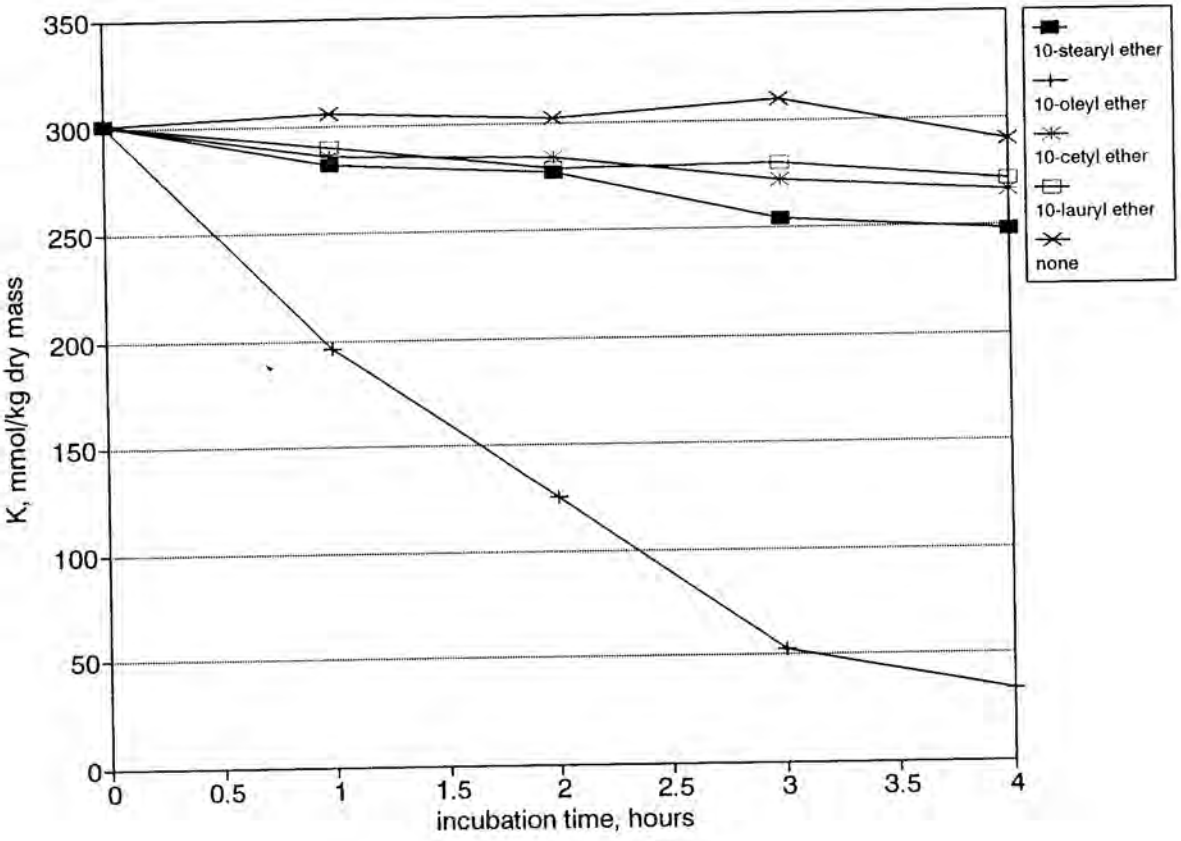
Fig. 1. The chemical structure of Brij series polyoxyethylene adduct non-ionic detergents. "R" represents a fatty acid residue. "*n*" is the number of oxyethylene repeats.

Fig. 2. Incubation of human erythrocytes in the presence of n-stearyl ethers. Variations in the number of polyoxyethylene residues (*n*) in stearic-acid-ether type Brij series detergents alter the release of potassium ions from human erythrocytes incubated on wet ice. Equimolar 50 μM concentrations of detergents were used at a Htc of 4%. Increasing potassium losses were observed if n increased from 4 to 20. A sudden reversal of the effect took place if *n* was $n \geq 21$.

Fig. 3. Incubation of human erythrocytes in the presence of 10-fatty-acyl ethers. The size of the saturated fatty acid residue did not play a significant role in the loss of potassium ions (Fig. 3a), or in the uptake of sodium or rubidium ions (Fig. 3 b and c) in various Brij series detergents, if the number of polyoxyethylene repeats was $n = 10$. Nevertheless, the substitution of the saturated stearic acid residue with an unsaturated oleic acid residue resulted in significant acceleration of the equilibration processes.

$$R\text{-}O\text{-}(CH_2\text{-}CH_2\text{-}O)_nH$$


300
280
260
240
220
200
180
160
140
K, mmol/kg dry mass
0
0.5
1
1.5
2
2.5
3
3.5
4
incubation time, hours
PE 4
PE 10
PE 20
PE 21
PE 100
Control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K, mmol/kg dry mass
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incubation time, hours
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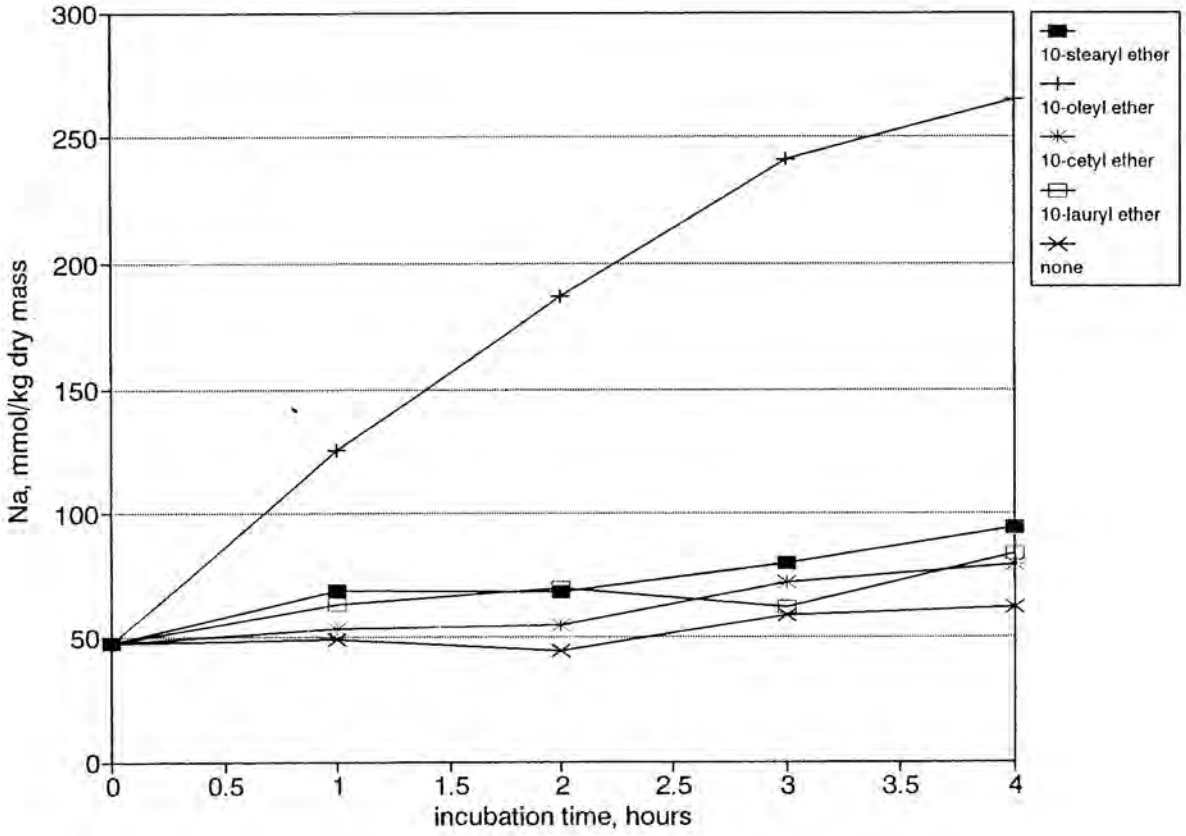
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Na, mmol/kg dry mass
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0.5
1
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incubation time, hours
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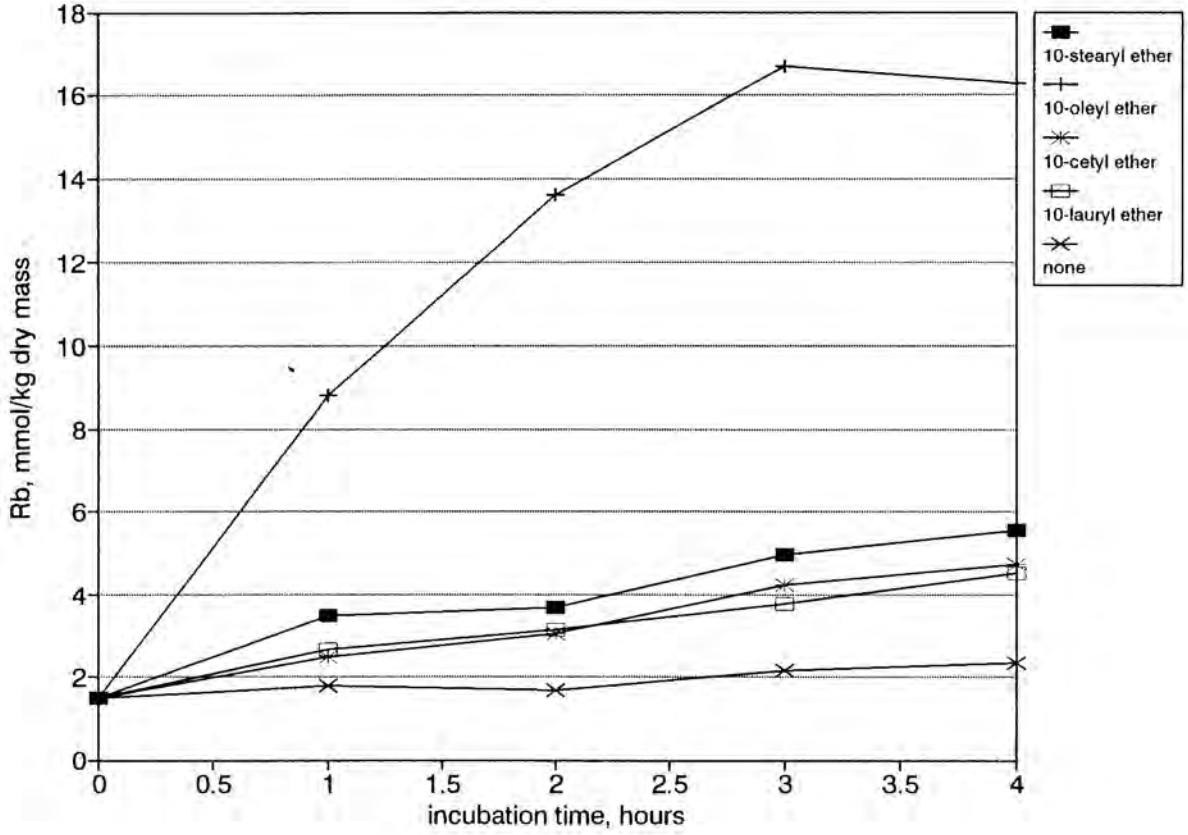
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6
4
2
0
0
0.5
1
1.5
2
2.5
3
3.5
4
incubation time, hours
10-stearyl ether
10-oleyl ether
10-cetyl ether
10-lauryl ether
none

K^+ in Serum-Incubated Human Erythrocytes Treated with Non-Ionic Detergent of the Brij Series

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Running title: Bogner *et al.*: K^+ in detergent-treated erythrocytes

Keywords: Human erythrocytes - Brij - non-ionic detergents - serum - temperature - inhibitors

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Abstract:

Exchange of erythrocyte intracellular (i/c) K^+ for extracellular (e/c) Na^+ in human erythrocytes treated with sub-CMC concentrations of Brij series non-ionic detergents and reincubated in serum is described. Progressive equilibration of the K^+ contents of detergent-treated human erythrocytes with the incubation medium was reversed by an albumin-mediated withdrawal of detergent molecules from the cell. Re-establishment of near normal [K^+] in terms of K^+/l RBC and K^+/kg water proceeds in two ways: (i) a metabolism-dependent net accumulation of K^+ ions, and (ii), more significantly, a metabolism-independent shrinkage of erythrocytes.

Introduction:

The polyoxyethylene adduct non-ionic detergent, Brij 58, has recently been used in different cell systems, with effective permeabilisation occurring in bovine thymus lymphocytes (Kellermayer *et al.*, 1984), cultured mouse fibroblasts (Kellermayer *et al.*, 1986), L 929 cells (Ridsdale and Clegg, 1991), porcine lens epithelium (Miseta *et al.*, 1991), human erythrocytes (Cameron *et al.*, 1991) and chicken erythrocytes (Cameron *et al.*, 1988; Kellermayer *et al.*, 1993). That is, *provided* the detergent concentration exceeded the critical micellar concentration (CMC), as judged by EM evidence of plasma membranes fenestration (Kellermayer *et al.*, 1986; 1994; Cameron *et al.*, 1988; 1991), removal of plasma membrane lipids (Kellermayer *et al.*, 1994), and uptake of dyes (Ridsdale and Clegg, 1991; Kellermayer *et al.*, 1994). All cell types maintained high i/c $[K^+]$ and low $[Na^+]$ for much longer periods of time than would theoretically be required for equilibration of a truly free aqueous compartment the size of the cell with its environment. Loss of K^+ from individual chicken erythrocytes - investigated by electron probe X-ray microanalysis (Cameron *et al.*,1988) - is not apparently a continuous process, but appears to be associated with a quick transition of erythrocytes from a high to a low density type due to a co-operative loss of proteins (*cf.* Ridsdale and Clegg, 1991) and K^+.

Although the lipolytic action of detergents is known to be associated with their critical micellar concentration (CMC) values, non-ionic detergents facilitate an accelerated K^+/Na^+ exchange between erythrocytes and the incubation medium at concentrations lower than their CMC values (Isomaa and Hagerstrand 1988; Bogner *et al.*, 1989). Recently, we reported that the sub-CMC effect of Brij-series detergents is primarily dependent on the size of hydrophilic polyoxyethylene chain, and on the nature (unsaturated versus saturated) of the hydrophobic fatty acid portion of the molecule (Miseta et al., submitted). Apart from corroborating and extending these findings, in our further studies described below, we will show a partial reversibility of the detergent-mediated exchange of erythrocyte K^+

with extracellular Na^+ in human erythrocytes treated with sub-CMC concentrations of the Brij series of detergents.

Materials and Methods

Materials and blood specimens:

Brij 35 (polyoxyethylene-23-lauryl-ether) was purchased from Reanal (Hungary). Brij 58 (polyoxyethylene-20-cetyl-ether), Brij 78(polyoxyethylene-20-stearyl-ether), Brij 99 (polyoxyethylene- 20-oleyl-ether), iodoacetamide and ouabain were purchased from Sigma (U.S.A.). All other chemicals were the products of Reanal (Hungary). Heparinized blood samples (ca. 125 U heparin/ml blood) from healthy young volunteers were used immediately after drawing the blood.

Blood samples were centrifuged at 3 000 g at 4 ^{o}C for 10 min. The plasma was removed for storage, and the erythrocytes resuspended in ice-cold 10 mM Tris-HCl buffered 0.89% NaCl solution (TBS) at pH 7.2. Samples were centrifuged 3 times as described above and the supernatants were discarded before the pelleted erythrocytes were used for experimental purposes

Heterologous plasma or serum samples were collected from stored blood specimens. The pH of the collected serum was alkaline, pH 8.1 - 8.4, due to equilibration of the bicarbonate buffer with the low pCO_2 of air. Unless otherwise indicated, we did not use additional buffers or adjust the pH, but checked the pH of serum prior to experiments.

The quantity of blood obtained from each volunteer was usually 15-20 ml, which limited us to no more than two samples at each of the 4-5 time-points collected within any experiment. Nevertheless, the results were highly reproducible. Where experiments were repeated using blood samples obtained from different volunteers, variability in net electrolyte losses and gains was considerably higher, but the trends and tendencies remained the same.

However, this additional problem, coupled with other factors such as blood group characteristics, which affect detergent action (Pazous-Sanou and Mata-Segrada 1985), take us beyond the scope of the present experiments and will not concern us here.

Permeabilisation and reincubation of erythrocytes:

Erythrocytes were resuspended in ice-cold TBS solution at a 2.5% hematocrit ratio. Brij 58 was added from a 0.2% freshly prepared stock solution, giving a final concentration of 0.004%, gently mixed, and incubated on ice for 1 h, unless otherwise indicated. Following incubation, samples were centrifuged at 3 000 g for 10 min, and the supernatants removed. Erythrocyte samples were reincubated in heterologous or autologous plasma, otherwise in Hank's solution at a 2.5% hematocrit ratio with or without the addition of 0.5 mM ouabain, 1 mM iodoacetamide, 10-20 mM NaF, 10-20 mM Na arsenate, 10 mM Na dithionite, or 10 mM methylene blue.

Analysis of erythrocyte Na^+, K^+, Cl^- and water contents:

10 ml samples were centrifuged at 3000 g for 10 min. The supernatants were removed and stored at 4 ^{o}C. Erythrocyte pellets were transferred to 1.5 ml microcentrifuge tubes of known weights, and centrifuged at 15 000 g for 10 min, the supernatants being discarded. 50 μl aliquots of erythrocyte pellets were taken up in a Braun automatic dilutor into 5 ml water purified by ion exchange-chromatography and reverse osmosis. K^+ and Na^+ concentrations were measured with an OMSZOV (Hungary) flame photometer. The residue of each sample was used for the determination of erythrocyte water content. After weighing tightly capped microcentrifuge tubes containing the samples, they were dried in a Savant SC 110 freeze-drier on "high" setting for 3 h before the tubes were reweighed. Water contents were calculated as units water/unit dry mass. 0.2-0.4 ml 4% perchloric acid (PCA) was added, depending on the size of the dried pellets, and

the capped tubes shaken vigorously for 30 min and centrifuged at 15 000 g for 2 min. Cl^- levels were measured in the PCA supernatant by the popular mercuric thyocanate method of Hamilton (1966).

Analysis of serum (plasma) glucose, lactate and erythrocyte ATP concentrations:
0.1 ml aliquots of serum or incubation medium samples were taken before and after the incubation of samples, suspended in 1 ml ice-cold 3% trichloroacetic acid (TCA) solution, and glucose levels were determined with the aid of a glucose oxidase/peroxidase kit (Reanal, Hungary). For the determination of lactate levels, 1 ml aliquots were taken and suspended in 3 ml ice-cold 4% PCA. Samples were centrifuged, and 2 ml supernatants removed and neutralised with 0.1ml 69% K_2CO_3. Potassium perchlorate was pelleted by centrifugation, and the supernatants used for lactate determination. We used 0.2 ml sample, or lactate standard aliquots. 2 ml glycine-hydrazine buffer (75 g glycine, 52 g hydrazine-sulphate, and 2 g EDTA was dissolved in distilled water, 510 ml 2 M NaOH was added, and the final volume adjusted to 1 litre by distilled water, pH 9.0), 0.04 ml freshly prepared 30 mmol/l NAD solution, and 4-6 IU lactate dehydrogenase (Reanal) were added. Lactate levels were determined after incubation at 37 ^{0}C for 15 min. Serial dilutions of glucose and lactate samples of known concentrations were treated in exactly the same way as the serum or plasma samples, to provide standards. Photometric determinations of the end-products of the above reactions were carried out with a Perkin-Elmer Lambda 2 UV/VIS spectrophotometer at 514 and 365 nm respectively.

Hemoglobin (Hb) concentrations of samples were measured photometrically, and the glucose consumption or lactate production expressed as millimoles of glucose or lactate / kg Hb / h.

50 μl aliquots of samples were taken directly from the suspended erythrocyte specimens for determination of ATP concentrations, made possible by the fact that there were no detectable ATP in the centrifuged supernatants of incubation

samples. Erythrocyte ATP concentrations were determined by a modified chemiluminescent firefly luciferin / luciferase method (Kôszegi, 1988).

Results:

Release of monovalent cations from detergent-incubated erythrocytes at 0-4 °C:

Intact human erythrocytes released small quantities of K^+ and took up similarly insignificant quantities of Na^+ when incubated in iso-osmotic NaCl solution at 4 °C for 4 h (Fig. 1). More than 25% of the original i/c K^+ was found in the incubation medium after 24 h "incubation".

Addition of 0.004% Brij 58 detergent promoted the release of K^+ ions from the erythrocytes, which was replaced by Na^+ taken up from the medium. The phenomenon was dependent upon detergent concentration. Higher concentrations of Brij 58 caused not only accelerated K^+ / Na^+ exchange i/c ⇔ e/c, but increased hemolysis (Fig. 2).

Comparable results were obtained with other Brij series detergents with similar physico-chemical properties such as Brij 78, and Brij 99. Many other Brij-series detergents with considerably shorter or longer hydrophobic or hydrophilic residues were also studied, and behaved differently. Results were reported elsewhere (). The reasons behind choosing Brij 58 were that it caused significant changes in the electrolyte concentrations of erythrocytes within a relatively short period of time, and that it was relatively easy to achieve this effect at non-haemolytic concentrations.

Detergent-treated cells accumulation of K^+ and release of Na^+ on reincubation in human plasma or serum:

Reincubation of 0.004% Brij 58 treated human erythrocytes in detergent-free serum or plasma at 37°C resulted in an increased i/c [K^+] and a decreased [Na^+]

content, albeit to a lesser extent (Fig. 3). The combined K^+ + Na^+ content appeared to be higher than in the control (unincubated) erythrocytes, since erythrocyte water content decreased during the incubation in the presence of detergent, and particularly during the first 30 min of the reincubation period. The changes in erythrocyte monovalent cation content were expressed as monovalent cations/unit water or monovalent cations/unit dry mass (essentially Hb) (Fig. 3a, and b). When reincubated in plasma, erythrocytes re-established their initial i/c $[K^+]$ concentration in terms of cell water, but since this had decreased, the absolute $[K^+]$ remained significantly lower than in unincubated erythrocytes (Fig. 3b). The uptake of Na^+, compensated for the loss of K^+ when the cells were incubated in the presence of detergent, but there was only a modest transient decrease of erythrocyte Na^+ levels when cells were reincubated in plasma, as a result of which the total monovalent cation concentration in cell water increased significantly. However, the total monovalent cation content expressed in absolute terms was re-established (Fig. 3b), and prolonged incubation in the presence of detergents resulted in a decline in the ability of erythrocytes to re-accumulate K^+ during the reincubation period in the presence of serum.

Effect of exposure to albumin-containing solutions on the detergent-induced disintegration of erythrocytes:

Incubation of detergent treated erythrocytes in the presence of human serum or plasma resulted in a net K^+ uptake and a modest exclusion of Na^+. The effect diminished when cells were reincubated in serum gradually diluted by Hank's solution (Table 1). A filtrate of plasma containing molecules of Mr $<$ 50 KD was ineffective, the release of K^+ from erythrocytes persisting. Similarly, the release of monovalent cations and the disintegration of detergent incubated human erythrocytes continued when the cells were reincubated in Hank's solutions with or without glucose. The addition of bovine albumin to Hank's solution prevented the further loss of K^+. Similarly, human serum dialysed against distilled water

stopped the disintegration of erythrocytes, but there was no elevation of K^+ during incubation. In contrast, detergent-treated erythrocytes incubated in serum dialysed against glucose-free Hank's solution were capable of accumulating K^+. In a separate experiment, the missing components of the Hank's solution were restored one by one to a serum dialysed against 5 mM KCl-complemented Tris-buffered saline (TBSK). Calcium ions restored the reaccumulation process, while other compounds (magnesium, bicarbonate, and inorganic phosphate) failed to do so.

When detergent-incubated erythrocytes were briefly exposed to Hank's solution containing serum or albumin, their tendency to equilibrate with the environment when reincubated in protein-free Hank's solution was diminished, indicating that the detergent molecules responsible for the progressive equilibration of erythrocytes with the medium in a protein-free medium had indeed been effectively removed.

Energy metabolism and the increase of erythrocyte K^+ levels: the role of energy-dependent net accumulation and shrinkage:

There was a significant decrease of i/c [ATP] during exposure to detergent (Fig. 4). A transient increase and a subsequent gradual decrease of ATP occurred when erythrocytes were reincubated in serum at 37°C.

Brij 35 or Brij 58 treated erythrocytes incubated in serum consumed glucose with a rate of 6.06 (SD 0.11) and 5.36 (0.09) mM / kg Hb / h, about twice the rate of glucose consumption of control erythrocytes (2.56, SD 0.12). Similarly, Brij 35 and Brij 58 incubated erythrocytes produced lactate with rates of 12.75 and 11.81 mM / kg Hb / h respectively, about twice as fast as normal plasma incubated erythrocytes (5.01 mM / kg Hb / h). While this evidence supports the existence of an ATP consuming process fuelled by glucose to re-establish approximately the initial i/c $[K^+]$ levels during reincubation in serum or plasma, we consistently encountered elevation of K^+ levels at temperatures of 0-4 °C (Table 2). Also,

erythrocytes incubated in serum which was dialysed against glucose-free Hank's solution had elevated K^+ concentrations (Table 1). Erythrocytes incubated on ice were unable to utilise measurable quantities of glucose, and produced no detectable lactate. Neither inhibition of enolase by the addition of 20 mM NaF nor net ATP generation by 10 mM arsenate prevented the elevation of K^+ at 37 ^{o}C, just as at 0 ^{o}C. In contrast, addition of 1 mM iodoacetamide resulted in a rapid loss of K^+ and disintegration of cells at 37 ^{o}C, but *not* at 0 ^{o}C. Nor did addition of 0.5 mM ouabain prevent the elevation of K^+. However, addition of 10 mM Na dithionite or methylene blue prevented the reaccumulation of K^+ during reincubation in serum.

While incubation of human erythrocytes without an energy source in cold or in the presence of metabolic or Na^+ / K^+-ATPase inhibitors failed to prevent elevation of K^+ levels, net reaccumulation of K^+ was diminished in terms of mM of K^+ / kg dry mass.

Discussion:

Although the critical micellar concentration (CMC) of detergents is best associated with their lipolytic characteristics, they can be haemolytic at low levels (Tragner and Csordas, 1987); however, at both non-haemolytic and haemolytic sub-CMC detergent concentrations, the loss of K^+ from erythrocytes is facilitated (Isomaa and Hagerstrand, 1988; Bogner *et al.*, 1989). We reported recently that the size of the polyoxyethylene chain, and the level of saturation of the fatty acid residues of detergent molecules determine the rate of exchange of monovalent cations(Miseta et al., submitted).

In the present report, we demonstrate that human erythrocytes release significant quantities of K^+ and take up similar quantities of Na^+ from the environment when incubated in the presence of, mildly haemolytic sub-CMC

concentrations of Brij 58, or in the essentially non-haemolytic Brij 35 (Fig. 1). Reincubation of detergent treated erythrocytes in detergent-free Hank's solution results in a further quick loss of K^+, swelling (Table 1) and hemolysis. Similar results were obtained when the detergent-treated erythrocytes were reincubated in different media which did not contain proteins, or in serum filtrates containing molecules of smaller than 50 KD (Table 1). This process was quickly stopped and partially reversed by the reincubation of erythrocytes in human serum or plasma (Fig. 3 and Table 1).

A biphasic effect of Triton series non-ionic detergents on the lysis of human erythrocytes was noted by Tragner and Csordas (1987). Low detergent concentrations comparable to those applied in the present study stabilised erythrocytes against osmotic lysis, but no clear explanation was provided. These results, along with the evidence of Isomaa and Hagerstrand (1988) and our own findings presented above, suggest that increased permeability of erythrocytes to K^+ accelerates the loss of this osmotically active ions in a hyposmotic environment, and consequently decreases the fast net rate of accumulation of water in the erythrocytes, resulting in slower swelling and hemolysis.

Polyoxyethylene adduct detergents are known to interact with lipids (Tragner and Csordas, 1987), and bind to the hydrophobic domains of membrane and other proteins. It is well established that the addition of albumin, a protein noted for its capacity to bind different hydrophobic molecular species, effectively and competitively removes these detergent molecules and prevents the further breakdown of erythrocytes. A protective effect of plasma lipids is also conceivable, since most plasma lipids are integral parts of high molecular weight lipoprotein particles such as chylomicrons, VLDL, LDL, IDL, or HDL. However, in Hank's solution containing 40-60 g/l bovine serum albumin prevented the equilibration of monovalent cations between the erythrocytes and the medium. Indeed, a brief exposure of detergent-treated erythrocytes to serum or to albumin

containing Hank's solution stabilises erythrocytes during subsequent incubation in protein-free environment.

Our observations indicate that the apparent elevation of i/c K^+ levels in serum involves two components. One is associated with the loss of erythrocyte water and a relative increase of K^+ levels in the decreased aqueous compartment; and the other is an active component responsible for the net accumulation of K^+ ions (Fig. 3) and for the decrease of the $[K^+]$ in the supernatants (data not shown) seen at 37°C. The loss of water from the erythrocytes was closely associated with a decrease in the concentration of its mobile monovalent ions, primarily due to a decreased $[Cl^-]$.

One notable feature of erythrocyte shrinkage was its independence of energy metabolism, occurring in the presence of inhibitors of the Embden-Meyerhof pathway, ouabain, and/or in cold-incubated erythrocytes (Table 2). This is a particularly important observation, since an increased negative charge to Hb at higher pH leading to a decreased i/c $[Cl^-]$ is a known phenomenon. Among the inorganic components of serum or Hank's solution, Ca^{2+} is probably the most important. In the absence of calcium-sequestering organelles, its total i/c concentration is low in human erythrocytes (albeit the free Ca^{2+} level is similar to that measured in other cell types), whereas Mg^{2+} is in the millimolar range (Miseta *et al.*, 1993). The i/c Ca^{2+} level appears to be higher in the denser (i.e. supposedly older) erythrocytes (Cameron *et al.*, 1993), which could be responsible for some differences in the behaviour of individual erythrocytes.

The second mechanism by which erythrocytes increased their $[K^+]$ was by a net uptake of ions at 37 °C (Fig. 3b). It is widely held that erythrocytes, like other cells, maintain their high $[K^+]$ by using energy generated by the hydrolysis of ATP and converted to the counter movement of K^+ and Na^+ in the plasma membrane "pump", the Na^+/K^+-dependent ATPase (Post 1989). Unlike most other cell types, the sole source of ATP in human erythrocytes comes from the Embden-Meyerhof pathway (Agar and Board, 1983). Indeed, a fall of ATP level

has been demonstrated (Fig. 4), as was an accelerated consumption of plasma glucose associated with a proportional increase of lactate production in detergent-treated erythrocytes reincubated in serum. Addition of known inhibitors of the Embden-Meyerhof pathway, such as NaF or arsenate, prevented the consumption of glucose and the production of lactate, but nevertheless the increase of i/c $[K^+]$ in terms of K^+/ l RBC or K^+/ kg water did not diminish (Table 2). However, there was no net accumulation of K^+. Similar results were obtained when erythrocytes were incubated in serum in the presence of ouabain, or at 0 ^oC. Neither erythrocyte metabolism nor the membrane "pump" is active at this temperature (Agar and Board, 1983). The drastic effect of iodoacetamide may be the result of a non-specific alkylation of SH groups of several proteins. Indeed, an accelerated exchange of K^+ to Na^+ occurred at 0 ^oC, and there was also an increased hemolysis at 37 ^oC. The fact that the elimination of energy-requiring K^+ and Na^+ transport did not result in a significantly lower K^+ levels renders this mechanism secondary to shrinkage in the re-establishment of near normal erythrocyte K^+ levels after detergent treatment. The re-establishment of higher erythrocyte $[K^+]$ probably involves two processes, the first and major contributing factor being a shrinkage which occurs independently of metabolism and active transport, but is affected by pH and Ca^{2+} ions for clearly understandable reasons. And the second contributing factor is probably metabolism-dependent, and plays a secondary role.

Thus, the mechanism by which human erythrocytes release K^+ at sub-CMC detergent concentrations is probably different from that seen at higher than CMC detergent concentrations (Cameron *et al.*, 1988; 1991; Kellermayer *et al.*, 1993). Above the CMC value of Brij 58, a co-operative loss of proteins and K^+ is observed, whereas at sub-CMC detergent concentrations, an accelerated ionophore-like exchange of K^+ for Na^+ takes place.

The search for detergents which are effective solubilizers of biological membranes, but do not functionally destroy the isolated proteins, links the

physico-chemical properties of detergents, especially the HLB numbers, to this process (Slinde and Flatmark, 1976; Umbreit and Strominger, 1973). The HLB numbers (hydrophobic-lipophobic balance) of detergents employed in the present studies were high (15.6 for Brij 58, 78 and 99, and 16.8 for Brij 35). The behaviour of detergents also appeared to be very similar, except for Brij 35 which is less haemolytic at equimolar concentrations.

Finally it is necessary to emphasise that the extent to which human erythrocytes release K^+ during incubation in the presence of detergents, and the extent of the increase of the K^+ concentration during reincubation in serum differed from blood sample to blood sample, albeit the tendencies were always the same. Data is available that the detergent-sensitivity of erythrocytes carrying various blood group antigens is different (Pazos-Sanou and Mata-Segrada, 1985). Here again, further work is required to establish proper quantitative standardisation.

Acknowledgements:

We are grateful to Mrs. Pappné Bácskai Sarolta, Mr. Csaba Borbély and Norbert Süle for their excellent technical assistance. This work was supported by OTKA F-5197 and R-3108.

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Figure legends:

Figure 1.

Changes of monovalent cation concentrations of erythrocytes incubated in TBS, and in 0.004% Brij 58 complemented TBS during incubation at 4 °C. First columns: potassium levels in TBS incubated erythrocytes. Second columns: sodium levels in TBS incubated erythrocytes. Third columns: potassium levels in 0.004% Brij 58 complemented TBS incubated erythrocytes. Fourth columns: sodium levels in 0.004% Brij 58 complemented TBS incubated erythrocytes.

Figure 2.

Percentage hemolysis of human erythrocytes incubated in the presence of various concentrations of Brij 58 for 3 h. Stars; 37 °C: squares; 0 °C.

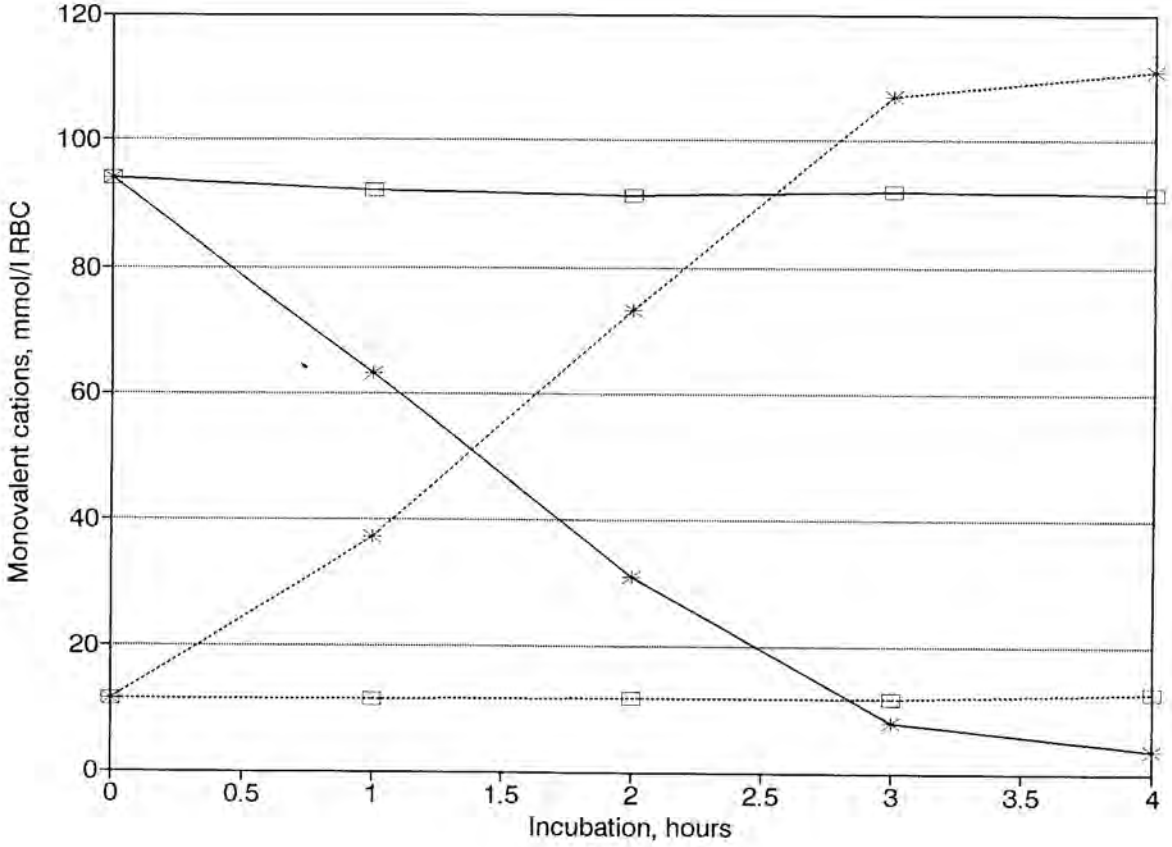
Figure 3.

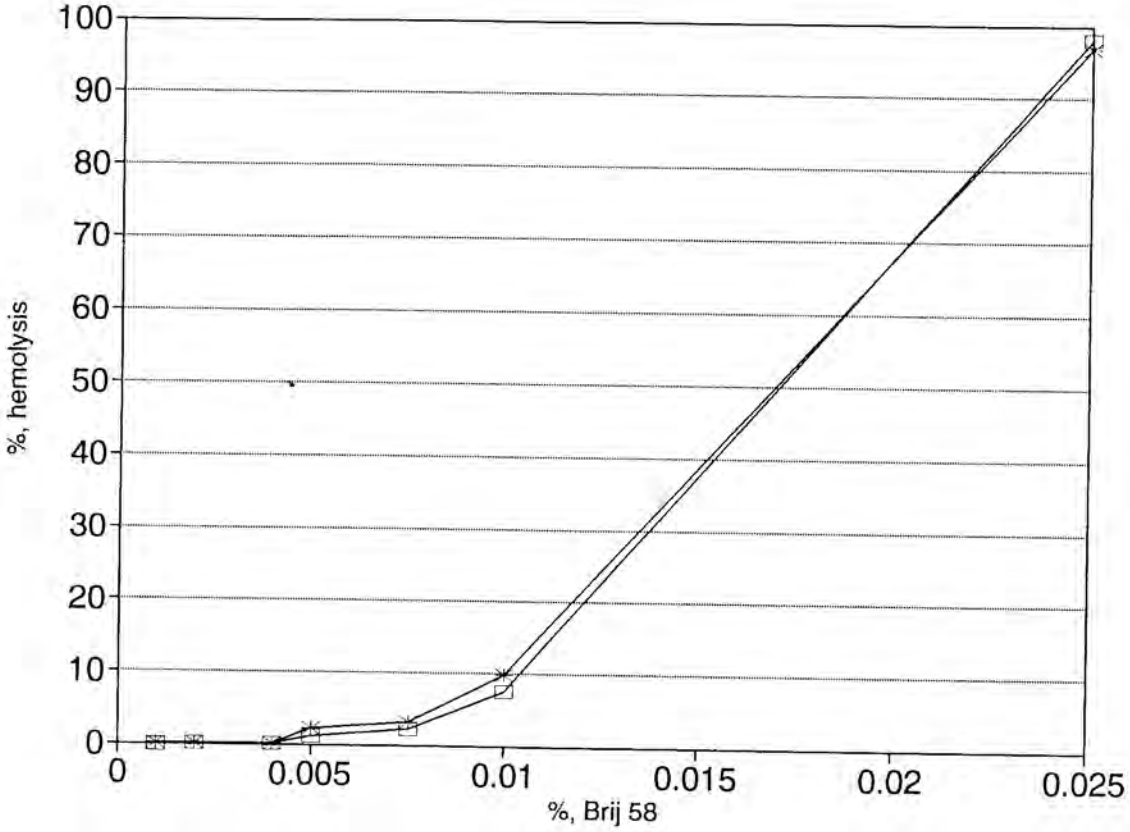
Changes in monovalent cation concentrations (primary Y axis) and water contents (secondary Y axis) of erythrocytes incubated in 0.004% Brij 58 complemented TBS at 4°C for 1 h, and re-incubated in serum at 37 °C for 3 h. (Vertical line separates incubation in the presence of detergent from re-incubation in serum.) Crosses; K^+, squares; Na^+, stars; $K^+ + Na^+$, hourglass; water. Monovalent cation concentrations are shown in mmol/l RBC water (Fig. 1a.), or mmol/l dry mass (Fig.1b) units (see text for explanation). Each point represent the mean value of three measurements.

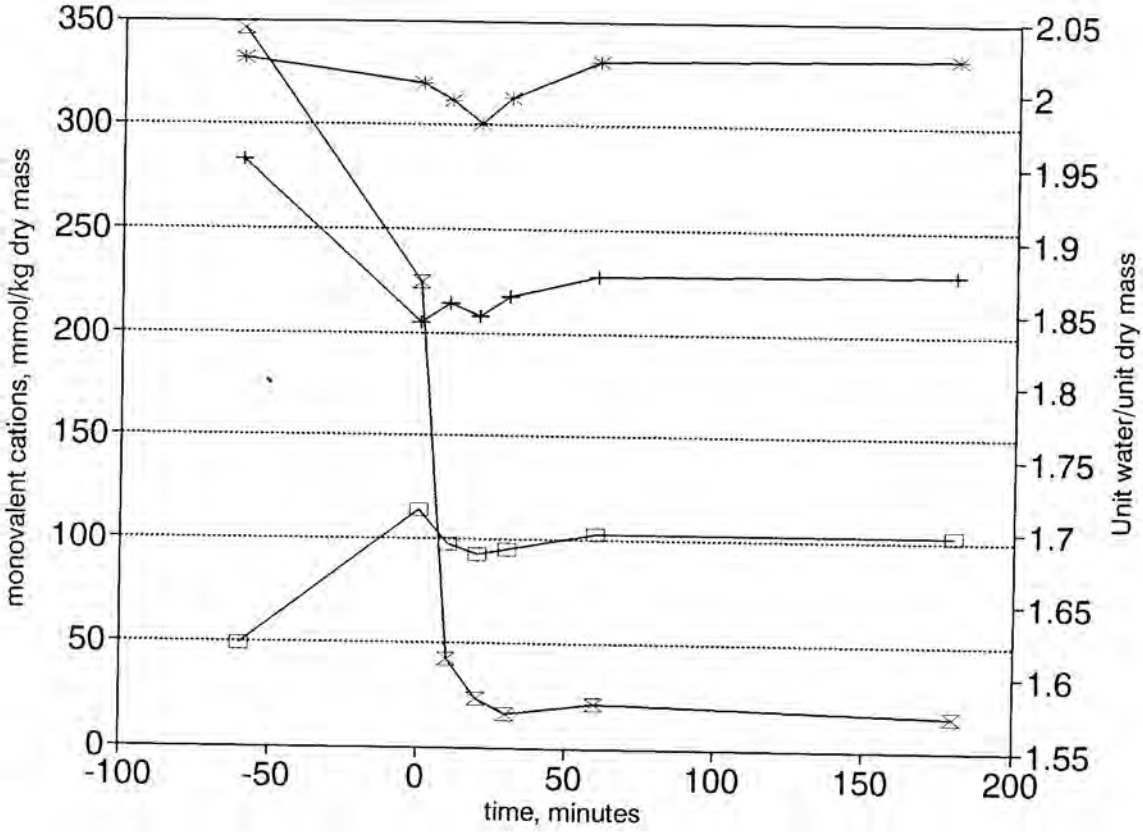
Figure 4.

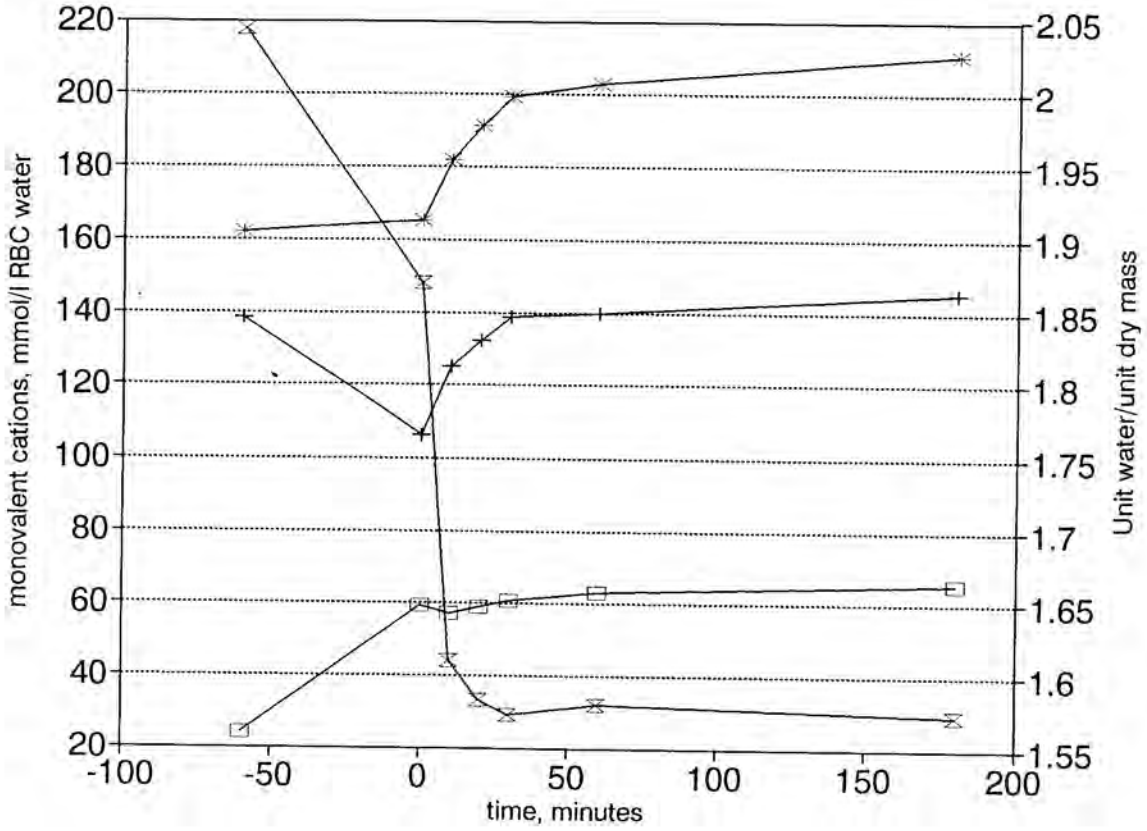
Changes in monovalent cation concentrations (primary Y axis) and ATP levels (secondary Y axis) of erythrocytes incubated in 0.004% Brij 58 complemented TBS at 4 °C for 1 h, and re incubated in serum at 37°C for 3 h. (Vertical lane

separates incubation in the presence of detergent from re incubation in serum.) Crosses; K^+, squares; Na^+, stars; $K^+ + Na^+$, triangles; ATP. Each point represents the mean value of three measurements.


120
100
80
60
40
20
0
Monovalent cations, mmol/l RBC
0
0.5
1
1.5
2
2.5
3
3.5
4
Incubation, hours


100
90
80
70
60
50
40
30
20
10
0
%, hemolysis
0
0.005
0.01
0.015
0.02
0.025
%, Brij 58


350
300
250
200
150
100
50
0
monovalent cations, mmol/kg dry mass
2.05
2
1.95
1.9
1.85
1.8
1.75
1.7
1.65
1.6
1.55
Unit water/unit dry mass
-100
-50
0
50
100
150
200
time, minutes


220
200
180
160
140
120
100
80
60
40
20
monovalent cations, mmol/l RBC water
2.05
2
1.95
1.9
1.85
1.8
1.75
1.7
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1.6
1.55
Unit water/unit dry mass
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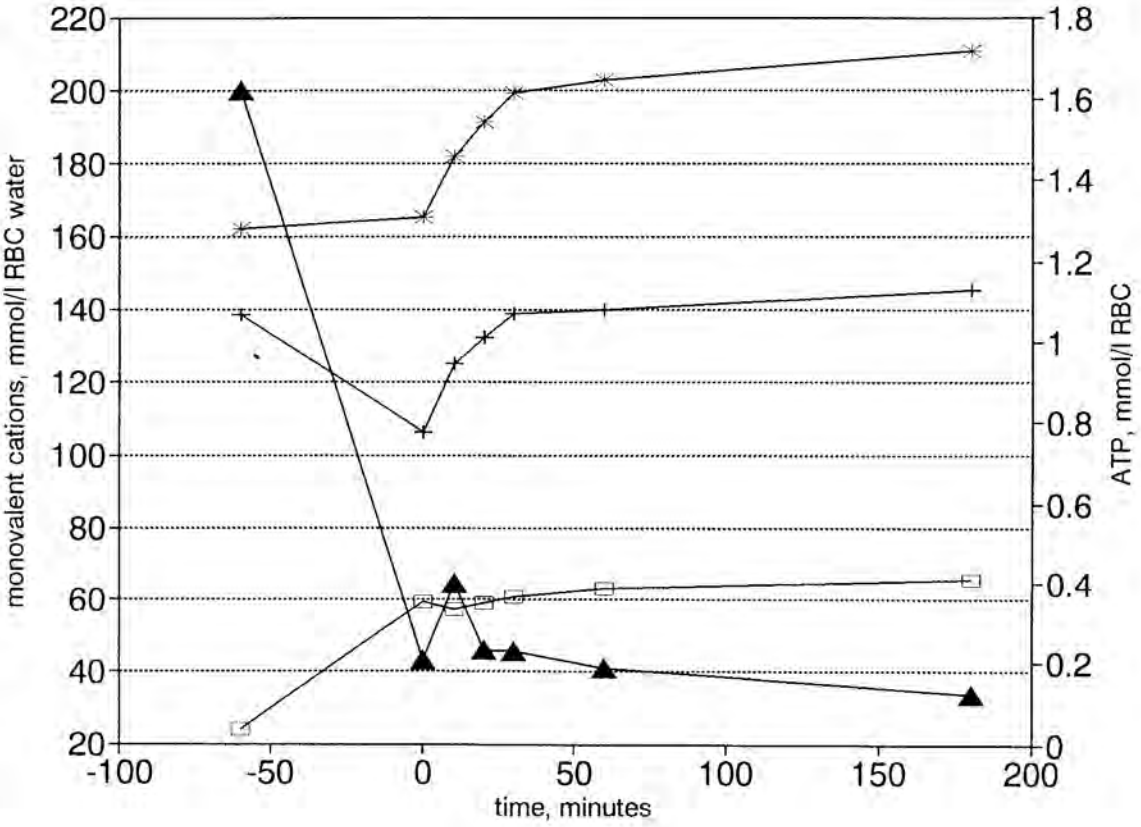
-50
0
50
100
150
200
time, minutes


220
200
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140
120
100
80
60
40
20
monovalent cations, mmol/l RBC water
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1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ATP, mmol/l RBC
-100
-50
0
50
100
150
200
time, minutes

JOURNAL OF CELLULAR PHYSIOLOGY 137:125-132 (1988)

Extent and Properties of Nonbulk "Bound" Water in Crystalline Lens Cells

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Crystalline lenses provided good material to study and measure the properties of cellular water. Different methods were used to establish the extent and properties of nonbulk water in mammalian lenses. These methods include: NMR titration analysis, a test of the osmotic properties, a test of dye exclusion in lenses with intact cell membranes and in lenses with disrupted cell membranes, and the water-holding capacity of lenses subjected to 40,000 × g for 1 hour with intact cell membranes and in lenses with disrupted cell membranes. The data from these methods, as well as other data from the literature, lead to the conclusion that most, if not all, of the water in lens cells (up to 2.2 g water/g dry mass) has motional and osmotic properties that distinguish it from bulk water. These findings call into question the common and convenient assumption that all but a small proportion of cellular water is like that in dilute solution.

Most cell physiologists state or assume that all but a small proportion of cellular water is like that in dilute solutions. If this assumption is not valid, then this conventional thinking must change (Macknight, 1987). Conversely some have taken the view that all or virtually all water in cells is perturbed by macromolecules such that essentially none is in the physical state expected were it pure "bulk" solvent water (Fisher and Suer, 1938; Ling, 1984). Ling (reviewed in 1984) has offered a polarized multilayer theory of cell water to explain how all or nearly all cell water differs from pure bulk water. The crystalline lens seems a favorable material to test the extent and properties of water in that it provides a rather large structural mass of well-characterized cells, with little extracellular space (estimates range from 1–12%; Kuck, 1970; Davson, 1980). The lens also has the advantage that it can be easily manipulated and studied. Perhaps the most important question at this time is: how much of the lens water differs from that of bulk water? It seems important that this question should be addressed by several different techniques as the individual techniques may give different quantitative values.

With this in mind we have applied several different methods to determine the extent and properties of nonbulklike water in the lens. The results of our studies as well as literature reports on the same subject, when taken together, indicate that most, if not all, of the water in the lens is nonbulklike in its motional and osmotic properties.

MATERIALS AND METHODS

Specimens

Pig and cow eyes from young adult animals were obtained from the slaughter house and the lenses were dissected from the eyeballs within 3 hours of killing. To avoid dehydration the lenses were immediately used for study. Likewise the lenses from young adult rats, weighing 250–300 grams, were dissected and immediately used in osmotic studies.

NMR measurements

Pulsed proton nuclear magnetic resonance (NMR) measurements on centrifugally packed cells combined with a fast proton diffusion (FPD) analytical approach have proved useful in the testing of various models of cellular water (Fullerton et al., 1983, 1985; Merta et al., 1986). The FPD approach proposes that multiple fast-exchanging hydration compartments constitute the physical status of water in cells. The careful stepwise dehydration, weighing, and NMR analysis of proton relaxation times allows hydration compartments to be isolated and characterized. The assumptions involved in the application of the FPD approach are that during the dehydration procedure the most mobile compartment of water is totally removed prior to the next most mobile water compartment and that the dehydration process is not responsible for the distinct water compartments observed. The validity of these assumptions has been discussed elsewhere (Fullerton et al., 1986, 1988; Merta et al., 1986).

Pulsed proton NMR relaxation analyses were conducted on a Praxis II instrument (Praxis Corp., San Antonio) with a 0.25 tesla magnetic field strength and a resonant frequency of 10.7 MHz. The spin-lattice relaxation time was measured using the saturation recovery

Received March 4, 1988; accepted May 9, 1988.

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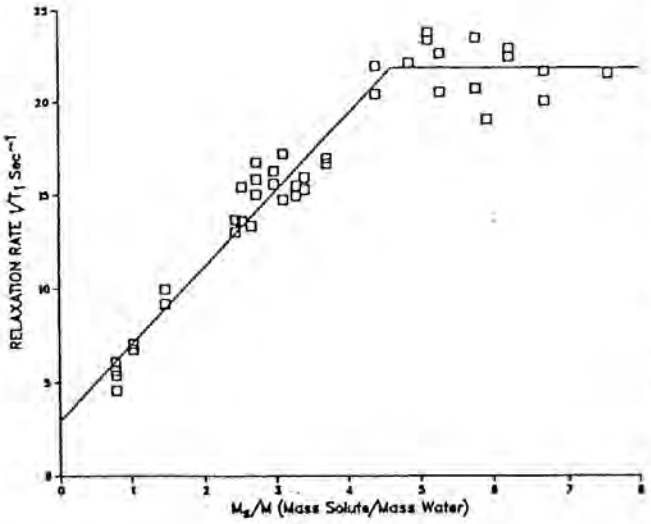

Fig. 1. Fast proton diffusion (FPD) model plots of the nucleus from a cow lens (initial water content 1.29 g water per g dry mass). Each point represents an independent T_1 (spin-lattice) relaxation rate measurement, $1/T_1$ (sec^{-1}), at its respective hydration level, (Ms/M = g dry mass per g water). Both $1/T_1$ and Ms/M valves can be readily converted to T_1 relaxation time (sec) and g water per g dry mass, respectively, by simply taking the reciprocal (1/x) of any coordinate value from the plots. Line and curve best fit regression analyses yielded two linear segments. The first segment extrapolates to an Ms/M axis at 2.94 and yields a T_1 relaxation time of 340 msec. Independent measures of bulk water (not shown) gave a relaxation time of 2,800 msec. The intercept point between the first and second linear segments occurred at an Ms/M value of 4.62, which converts to 0.22 g water per gram of dry mass.

pulse sequence (90-π-90). An interfaced microcomputer with software programs provided rapid data acquisition and analysis. The T_1 decay curve is the product of the resultant analysis of 30 free-induction-decay (FID) peak heights with a sequence of increasing interpulse delay times (Fullerton et al., 1986). All measurements were made at 23°C.

The samples were dehydrated in a vacuum chamber at room temperature, and evaluated at least daily for T_1 relaxation times and for mass in accordance with the NMR titration protocol employed elsewhere (Fullerton et al., 1986). The T_1 relaxation behavior throughout the experimental drying process yielded single exponential decay. These uniexponential decay curves permitted the determination of the T_1 relaxation times from the least-squares fit to the data points. On about day 25, dehydration was further promoted by temperature elevation in a vacuum oven; on a daily basis temperature was increased in 10-degree increments until a maximum of 100°C was obtained and a constant mass indicated the sample was dry.

Osmotic tests

To assess the osmotic properties of lens water, lenses from adult pigs and rats were exposed to solutions with different osmotic activity. The solutions selected for study were: dilutions of Hanks balanced salt solution (140 mM-NaCl, 5 mM-KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, 10 mM Tris HCl, pH 7.2, 290 m osm), dilutions of blood serum (pH 7.3, 300 m osm), and dextran solutions (10 mM Tris HCl, pH 7.2).

Four lenses were weighed and placed into 30 ml of each test solution. The 50-ml incubation containers were closed and agitated on a rocker table at 120 cycles per minute. The rocker was stopped at various times to reweigh the samples. The water content of the lenses was determined by weighing the wet lenses that had first been rolled around on a dry glass surface to remove the excess water. Dry weight was obtained by drying the cells at 100°C for the 3 to 4 days needed to establish constant weight. Osmotic activity of the Hanks and serum solutions were measured using a freezing point depression osmometer, whereas the osmotic pressure of the dextran solutions were done with a vapor pressure osmometer.

Dye exclusion tests

Fresh intact pig lens and pieces of sclera were placed in various solutions with or without nonionic detergents; see Table 1 for a list of the six different solutions that contained 0.1% concentrations of either nigrosin, trypan blue, or methylene blue. The tissues were agitated every 2–6 minutes and the tissues examined after 1 hour. At 1 hour the lens capsules were dissected from each lens and the tissues returned to its original dye solution for 2 additional hours. In a later experiment lenses that had been bisectioned into anterior and posterior halves were subjected to the same six dye solutions for 2–3 hours and then removed for observation.

Centrifugation studies

Pig lenses were individually weighed, sealed in 50-ml centrifuge tubes, centrifuged at 40,000 times gravity for 1 hour, removed, and reweighed.

RESULTS

Evaluation of nonbulklike water in the crystalline lens as determined by proton NMR titration

The results of sequential dehydration of the adult bovine lens nucleus on the spin-lattice relaxation rate ($1/T_1$) are summarized in Figure 1, which shows a graph of data on $1/T_1$ versus mass of solute per mass of water (Ms/M). The data can be described by two linear sections with a point of singularity between the linear sections. Such data are consistent with fast proton diffusion model interpretation (Fullerton et al., 1986).

The object of these studies was to determine the amount of water in the lenses that has osmotic properties similar to and different from that of bulk water.

The first linear segment on the left side of the graph, when extrapolated to the zero Ms/M axis, intercepts at a $1/T_1$ value of 2.94, which converts to a constant T_1 value in this water compartment of 340 msec. The T_1 relaxation time of bulk water measured in our machine gave a value of 2,800 msec. This value is eight times longer than the water T_1 relaxation time in this lens compartment. The total percentage of water in the freshly dissected lens nucleus is 56%. Thus the lens nucleus water content of the freshly dissected lens nucleus at the time of initial NMR measurements amounts to 1.29 g water per g dry mass. None of this water appears to have the relaxation rate of bulk water. The point of singularity, determined by the intersection of

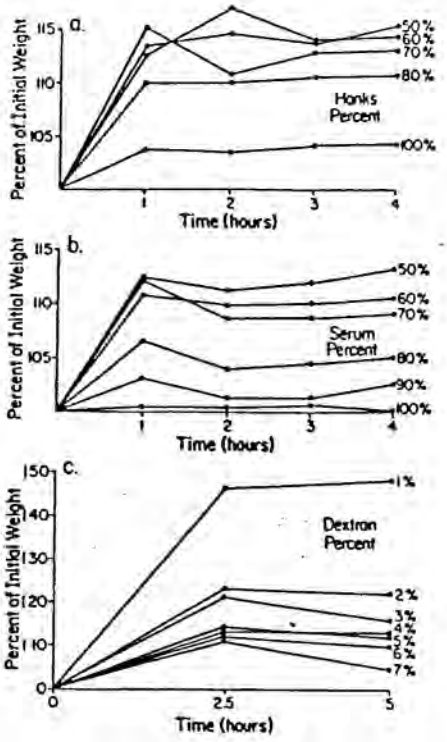

Fig. 2. Swelling of pig and rat lenses to equilibrium (expressed as the percent of initial weight) at 24°C. Each value is the mean from four intact lenses. (a) shows data from pig lenses in Hanks balanced salt solution diluted to different percentage of full strength (100%) with water. (b) shows the data from pig lenses in serum at full strength (100%) and in different degrees of dilution with water. (c) are data from the rat lenses incubated in various concentrations (w/v) of dextran (molecular weight of 150,000 daltons). The data indicate that equilibrium had occurred in all lenses by 2.5 hours of incubation.

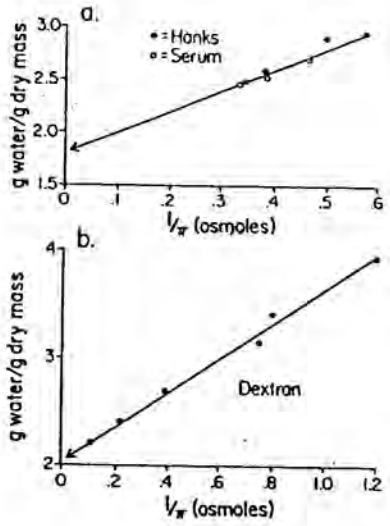

Fig. 3. Equilibrium water content (g water/g dry mass) of pig lenses (a) and of rat lenses (b) as a function of the inverse of osmotic pressure ($1/\pi$) in osmoles. The pig lenses were exposed to various dilutions of Hank's balanced salt solution or of blood serum. Each point is the mean of four lenses. The pig lens line equation extrapolates to a zero intercept of the $1/\pi$ axis at 1.81 g water/g dry mass. The rat lenses were equilibrated in various concentrations of dextran buffered with Tris HCl (150,000 molecular weight). The pig lens line equation extrapolates to a zero intercept of the $1/\pi$ axis to yield a value of 2.21 g water, g dry lens mass. The osmotic pressure of the solutions was measured by freezing point depression (a) or by vapor pressure (b).

the least-squares regression "best fit" lines to the data in each section is at an Ms/M value of 4.62, which amounts to 0.22 g water per 1.0 g of dry mass. The $1/T_1$ value of this intercept point is 22 sec^{-1}, which converts to a T_1 relaxation time of 46 msec for the water in this compartment.

These NMR data indicate the presence of two fractions or compartments of nonbulk water in the lens nucleus. One subfraction has 1.07 g water/g dry mass, whereas the other subfraction has 0.22 g water/g dry mass. The NMR titration data provide no evidence of bulk water in the lens nucleus.

Osmotic behavior of water in crystalline lenses

To make a determination of the osmotic behavior of lens water, it was first necessary to establish that the lenses come to equilibrium in each of the osmotic environments used in the study. Figure 2a–c indicates that the pig and rat lenses showed a progressive increase in weight, which is related directly to the osmotic activity of the incubation solution. The data in Figure 2a–c indicate that the lenses come essentially to osmotic equilibrium within the first 2 to 3 hours of incubation in each of the solutions. These findings indicate that osmotic equilibration of the lenses occurred within 3 hours of exposure to the different solutions being tested and that this is a sufficient duration for attaining equilibration.

To determine the extent of participation of lens water in the osmotic response reported in Figure 2a–c, it is customary to plot the water content of the tissue or the cells under study (in g water per g dry mass) versus the reciprocal of the osmotic pressure of the different bathing solutions ($1/\pi$ in osm) (Ling and Negendank, 1970). If all of the water in the lenses has the osmotic activity of bulk water, it should follow the familiar van't Hoff equation:

$$\pi V = nRT$$

where π is the osmotic pressure, V is the volume of water associated with nmoles of solute in the lens, R is the gas constant, and T is the absolute temperature. Thus if all of the water in the lenses is bulklike in its osmotic properties, it should participate in the van't Hoff equation. In such a case the plot of g water per g dry mass of lens versus $1/\pi$ should extrapolate linearly to zero g water per g dry mass at infinite osmotic pressure. The extent of deviation from zero g water/g dry mass would indicate the extent of water in the lenses that is not participating in the osmotic equation of van't Hoff; such a finding would therefore indicate that some of the lens water has reduced osmotic activity at least over the range of osmotic pressures studied.

Plots of the equilibrium water content of the pig and rat lenses versus the reciprocal of the osmotic pressure of the different solutions tested are shown in Figure 3a–

TABLE 1. Results of dye exclusion tests of the lens body, lens capsule, and sclera with and without treatment in nonionic detergents (+ stained, − = non-stained)[1]

Treatment	Lens			Capsule			Sclera		
	N[2]	TB[3]	MB[4]	N	TB	MB	N	TB	M
Hanks balanced salt	−	−	−	+	+	+	+	+	+
Hanks + 0.2% Brij-58	−	−	−	+	+	+	+	+	+
Hanks + 0.2% Triton X-100	−	−	−	+	+	+	+	+	+
0.25M Sucrose Tris HCl	−	−	−	+	+	+	+	+	+
Sucrose + 0.2% Brij-58	−	−	−	+	+	+	+	+	+
Sucrose + 0.2% Triton X-100	−	−	−	+	+	+	+	+	+

[1]The concentration of all three dyes was 0.1% and the tissues were left in the dyes for 3 hours with intermittent agitation. After the capsule began to stain, it w removed and the lens body was returned to the dye. Bisection of lenses into an anterior and posterior half prior to exposure to the dye did not cause a positi staining reaction in the lens, but heat denaturation (100°C for 5 min) of the lenses allowed a staining reaction at the surface.
[2]Nigrosin.
[3]Trypan blue.
[4]Methylene blue.

b. The results of exposure of pig lenses to dilutions of Hank's balanced salt solution and to dilutions of serum (Fig. 3a–b) show that the water content increases linearly with the decrease in osmotic pressure over the $1/\pi$ range of 0.3 to 0.58 osm. Extrapolation from this portion of the curve to $1/\pi = 0$ yields a lens water content value of 1.8 g water/g dry mass. Likewise extrapolation of the linear portion of the curve; from the data on rat lenses (Fig. 3b) incubated in dextran solutions, to $1/\pi = 0$ yields an amount of lens water equal to 2.05 g water/g dry mass, which did not participate in the osmotic equation of van't Hoff. As all water in the lenses was eventually removed by heating to 100°C in a vacuum oven, it is not proper to say that any fraction of lens water is "osmotically inactive," but the data do allow us to say that a specific amount of lens water has reduced osmotic properties compared to bulk water over the range of osmotic pressures tested (as illustrated in Fig. 3a–b).

Thus the fresh pig lens water content is 2.33 g water/g dry mass, and 1.8 g of this water has reduced osmotic activity compared to bulk water. The fresh rat lenses water content is 2.58 g of water/g of dry mass, and 2.05 g of this water appears to have reduced osmotic activity.

Dye exclusion studies in the crystalline lens

Because a large proportion of lens water had reduced motional properties, as shown by the NMR titration analysis, and also had reduced osmotic activity, it was decided to examine the ability of the lens fibers to exclude dyes both before and after disruption of the plasma membrane of lens cells that are exposed to the surface of the lens. The morphology of the lens is such that a significant proportion of the lens cells abut on the lens surface along the inner surface of the lens capsule. Thus exposure of the lens to agents that are known to disrupt the integrity of the plasma membrane (i.e., the nonionic detergents Brij 58 and Triton X-100; Schliwa et al., 1981; Kellermayer et al., 1984, 1986) should "open" at least the ends of these cells for penetration of dyes such as Nigrosin, Trypan blue, and Methylene blue.

Fresh intact pig lenses were incubated in the six different solutions listed in Table 1. A white portion of pig sclera, free of retina, was also incubated in the same dye solutions and served as a comparative reference tissue for the staining reaction. The water content of fresh sclera was 2.25 g water/g dry mass similar to that of the fresh pig lens (2.33).

Within 1 hour of exposure of intact lenses to the various dye solutions, the sclera and lenses all appeared to stain; however, removal of the lens capsule by manual dissection showed that the capsule alone had taken up dye, whereas the decapsulated lens had not. Return of the decapsulated lenses to their respective dye solutions for 2 more hours failed to show significant dye penetration into the lens. These observations are illustrated in the photographs of Figure 4A–C and are summarized in Table 1. The bisection of lenses into anterior and posterior halves prior to exposure to the dye solutions likewise failed to cause a positive staining reaction in the lenses.

Thus both nonplasma membrane penetrating dyes (nigrosin and trypan blue) as well as the membrane penetrating supravital dye (methylene blue) failed to show significant penetration into the lens body. Even the use of plasma membrane disruptive procedures failed to facilitate dye penetration into the lenses, as summarized in Table 1.

Centrifugation studies on crystalline lenses

To determine the water-holding capacity of lenses, they were individually weighed, sealed in centrifuge tubes, centrifuged at 40,000 times gravity for 1 hour, removed from the tubes, and reweighed. Two groups of pig lenses were subjected to this treatment. One group of lenses was incubated in Hanks balanced salt solution for 1 hour; then removed, weighed, centrifuged, and reweighed. The second group of lenses was incubated for 1 hour in Hank's balanced salt solution containing 0.2% Triton X-100 (a treatment designed to disrupt cell membranes). Half of these lenses were then bisected into an anterior and posterior half. The second group of lenses, or sections of lenses, were also weighed, centrifuged, and reweighed. The difference in weight before and after centrifugation was used to assess the water-holding ca

pacity of those lenses with and those lenses without intact cell membranes.

The lenses with intact cell membranes lost 5.93 ± 0.58 percent of their weight and the lenses with chemically and physically disrupted cell membranes lost 4.40 ± 0.29 percent of their weight. These mean values are not significantly different. It is not known if the loss in lens wet weight is due to: loss of water from the extracellular space, loss of water due to evaporation, or loss of water from the lens cells due to the centrifugation. Nevertheless this amount of water loss was a very small amount of the water originally present in the lenses.

A further observation on the centrifuged lenses revealed that they had the same shape after the centrifugation as before. In other words the lenses were not smashed to the bottom of the centrifuge tube by the centrifugal force. However, it took but slight pressure between the thumb and forefinger to destroy the intact lens.

These centrifugation studies indicate that most, if not all, of the lens cell water is in a physical state different from that of free or bulk liquid water.

DISCUSSION

Can differences in the methods of study account for the large reported differences in the extent of bound water in the lenses (as listed in Table 2)?

Notice in Table 2 that those methods that measured the extent of nonfreezing water (liquid residual water would be a better name) at temperatures of −9° to −30°C, give nonfreezing water content values of between 12.9 and 51% of the total lens water. On the other hand, those methods that do not rely on measurement of a nonfreezing liquid residual estimate total bound water at between 77.7 and 100% of total lens water. Data in the report of Rácz et al. (1979a,b) indicate that a smaller fraction of the nonfreezing, or liquid residual, still exists in lenses at temperatures as low as −70°C. This shows that subfractions of nonfreezing lens water exist in the lens and that the amount of nonfreezing water depends on the temperature at which the analysis was done. In this regard notice that the extent of nonfreezing water at −70° (Rácz et al., 1979a,b) is in close agreement with the less mobile bound water subfraction as measured by the NMR titration studies of this report (Table 2). Thus at least some of the variance in estimates between techniques may be due to different types of bound water subfractions, as discussed next.

To help answer the question posed at the beginning of the discussion, we have recently examined the question of nonfreezing water fractions in homogeneous polypeptide solutions (Kuntz, 1971, 1974; Derbyshire, 1982; Fullerton, 1988) in comparison to results obtained using the same NMR titration method we have used on the bovine lens in this report. We concluded, by comparison of results from the different methods, that the amount of nonfreezing water at temperatures of −30° to −35°C was related to water molecules that form ion bridges or multiple dipolar bridges in the polypeptides and also to water molecules in the primary hydration shell of the ionic groups on the polypeptides. At temperatures of −90° or below, only water that forms bridges remains as a nonfreezing fraction. At temperatures of −35°C to 0°C the nonfreezing or residual fraction should include all bound water molecules.

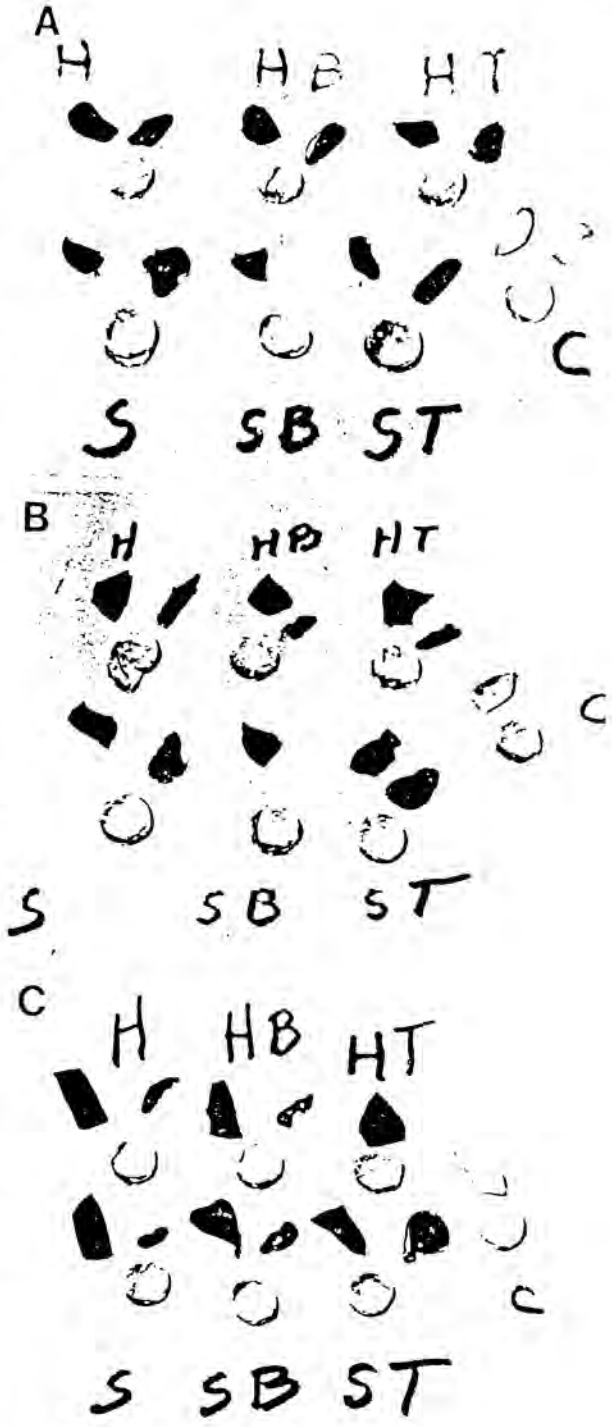

Fig. 4. Photographs illustrate the result of dye exclusion tests on the lens body, lens capsule, and sclera of pig eyes with and without treatment in nonionic detergents. Above each lens is a piece of sclera (upper left) and its dissected lens capsule (upper right). Abbreviations: H = incubation in Hanks balanced salt solution, S = incubation in 0.25 M sucrose, B = presence of 0.2% Brij-58, T = presence of 0.2% Triton X-100, and C = (controls) fresh unstained lens, capsule, and sclera. Specimens in A (top photograph), except the unstained controls were exposed to 0.1% nigrosin. Specimens in B (middle photograph) were exposed to 0.1% trypan blue, and in C (bottom photograph) were exposed to 0.1% methylene blue. After 1 hour in the dye solutions, the intact lenses were removed and their stained capsules removed. The lens body (less the capsules) was returned to the dye solution for 2 additional hours, then removed and placed on a glass plate along with its lens capsule and a piece of sclera. As shown here, none of the treatments resulted in dye uptake or staining of the lens body, whereas all of the lens capsule and sclera specimens took up the dye.

TABLE 2. Extent of total and nonbulk "bound" water in lenses of various mammals (gH_2O/g dry weight)

Species and region	Total amount of water	Extent of bound water	Bound water % of total water	Methods, comments, and reference
Rabbit (adult)				
Cortex	2.57	0.57	22.2	Differential scanning calorimetry (DSC) of nonfreezable water at −30°C; Bettelheim et al. (1986)
Nucleus	1.00	0.45	45.0	
Human (at birth)				DSC of nonfreezable water at −30°C ('−' = decrease with age); Lahm and Bettelheim (1985)
Cortex	3.05	1.39	45.6	
Intermediate	2.45	1.25	51.0	
Nucleus	2.52	1.16 − with age	46.0	
Human (adult)				DSC of nonfreezable water at −30°C; Bettelheim et al. (1986)
Cataractous cortex	2.58	0.61	23.6	
Cataractous nucleus	1.88	0.45	23.9	
Human (adult)				NMR amplitude of non-freezable water at above −9°C, at −70°C, the value in parentheses remains nonfrozen; Rácz et al. (1979)
Whole lens	2.41[1]	.58 (.18)	24.1	
Cataractous Whole lens	3.57[1]	.46 (.23)	12.9	
Rat (at birth)				DSC of nonfreezable water at −30°C ('+' = increase with age); Castoro and Bettelheim (1986)
Cortex	2.82	0.67	23.8	
Nucleus	1.58	0.42 + with age	26.6	
Bovine (adult)				NMR titration; Fullerton and Cameron (1988)
Nucleus	1.40	1.40 (.29)	100	
Nucleus	1.29	1.29 (.22)	100	NMR titration (this report) value in parentheses is a less mobile subfraction
Rat (adult)				Osmotic analysis (this report) in dextran solutions
Whole lens	2.58	2.05	79.5	
Pig (adult)				Osmotic analysis (this report) in diluted serum and in diluted Hanks solutions
Whole lens	2.33	1.81	77.7	
Whole lens	2.33	2.21	94.8	Water retained after centrifugation at 40,000 g for 1 hour (this report) (5.3% of total water lost)

[1]Based on previous published reports by Rácz & Kellermayer (1977).

We are therefore led to the conclusion that those methods that measure the nonfreezing residual water in lenses at temperatures of −30°C or below measure distinct subfractions of the total extent of bound water in the lenses and that the other methods used in this report (i.e., NMR titration, osmotic analysis, and water-holding capacity) better define the upper extent of bound water in the lens. Clearly the amount of bound water determined in the lens is a reflection of the fact that the different techniques have different criteria for "bound" water.

Table 2 reveals that estimates of the extent of bound lens water varies with age, region (nucleus vs. cortex), and the method of measurement. Nevertheless the biological trends with age, region, and cataract formation are the same regardless of the method of measurement.

Summary of evidence: Water in lens fibers is nonbulklike in physical properties

The NMR titration analyses of lens water presented in Figure 1 show no evidence of bulk water even though this method has detected bulk water in other cellular systems, i.e., sea urchin egg (Mertz et al., 1986), human erythrocytes (Cameron et al., 1988a,b), skeletal muscle (Mardini et al., 1987). It should be pointed out that a very small amount of bulk water, if present, might escape detection by the NMR titration method.

A previous NMR study of crystalline lens water had determined that the water diffusion coefficient in the lens was reduced by a third compared to the water diffusion coefficient of bulk water (Neville et al., 1977). These authors concluded that this degree of restriction of the translational motion involves the major proportion of lens water molecules. There exists an interpretation problem with NMR measures of water diffusion (D). The problem is that NMR measures of D are done over distances of several micrometers and the reduced D values in cells may therefore be due to physical obstructions that the water molecule encounters rather than the diffusive motion of water in water. To overcome this problem one should ideally measure D by a method that is fast enough so that water movement can be measured within a few angstroms space, thus ruling out most of the chance that the water molecule will encounter a physical obstruction. Although quasi elastic neutron scattering (QENS) provides such a method for measuring D, it has not yet been applied to lenses. This method has already been applied to *Artemia* cysts and the data indicate that all of the water differs from bulk water, i.e., 1.2–1.4 g water/g dry mass (Rorschach et al., 1982; Trantham et al., 1984). Clegg (1984) summarizes QENS and ultrahigh frequency dielectric measurements that support the idea that all or nearly all of water in fully hydrated *Artemia* cysts differs from pure bulk water (Clegg, 1984).

What do the dye exclusion studies tell us about the properties of lens water? What is the reason that the lenses, both with intact cell membranes and those with treatments intended to "open" up a large proportion of the cell membranes, fail to take up the dyes? Two interpretations are offered to explain these findings: (1) the solvent properties of essentially all of the lens water for the dye molecules was greatly reduced from that of bulk water, and (2) physical barriers to dye penetration exist even in the bisected lenses. The physical barriers could conceivably include electrostatic repulsion of charged dye molecules or physical obstructions in the lens fibers, which prevent significant diffusion of the dye molecules into the lens fibers. An alternate explanation to explain the failure to detect methylene blue coloration in the lens need not depend on its exclusion but could be due to its reduction to the colorless leucomethylene blue form. Further study is required to resolve the mechanism(s) involved with dye exclusion.

The data in this report that show a reduced osmotic activity of most of the water in the lenses appears to have no explanation other than it is perturbed from that of the osmotic activity of bulk water. That the amount of water with reduced osmotic properties in the lenses was somewhat less than 100% of the total lens water (i.e., 77.7–79.5%) does indicate that there is a small but measurable amount of water in the lenses that has osmotic properties like or similar to that of bulk water. An earlier study of the osmotic behavior of frog skeletal muscle also provided evidence that a large proportion of cellular water in this system is perturbed from that of the osmotic activity of bulk water (Ling and Negendank, 1970).

The lens water-holding capacity studies, done either with intact lenses or with lenses that have chemically or physically disrupted cell membranes, showed that less than 6% of the total lens water could be removed from the lenses by centrifugation. Ling (1984) had previously done centrifugation studies on the water-holding capacity of thin frog sartorius muscle fibers. He centrifuged thin intact fibers and those fibers that were transectioned into 2–4 mm-long segments. Although the membranes of the transected muscle fibers were disrupted the fiber segments that were centrifuged at 1,000 × g for 4 min lost only a small amount of water (equal to that in the extracellular space). In this regard the very small fraction of lens water lost after ultracentrifugation (5.3% of the total lens water present prior to centrifugation) may also be accounted for as loss of water in the extracellular space. It seems reasonable to conclude, from the centrifugation studies on lenses, that essentially all of the lens cell water must be in a physical state different from that of normal bulk water.

Another observation consistent with the idea that little if any of the lens fiber water is bulklike is that the quench freezing of rat crystalline lenses in liquid propane cooled in a liquid nitrogen bath did not allow growth of visible ice crystals, as revealed by scanning transmission electron microscopy of thin freeze-dried cryosections (Cameron et al., 1985, 1988a). Although the lens fiber cells did not show evidence of visible ice crystals, several other cell types studied by the same quench freezing procedure did reveal formation of ice crystals. This type of data strongly suggests that the lens fiber cells contain little if any water with the freezing characteristics of bulk water.

CONCLUSIONS

Prior studies on the extent of nonbulk water in lenses have been limited primarily to methods for determining the amount of nonfreezing or liquid residual water at some temperature below 0°C. These reports consistently showed a smaller fraction of nonbulklike water than the methods used in the present studies. The differences are explained by the fact that different subfractions of hydration water are revealed depending on the subzero

temperatures used (Derbyshire, 1982; Fullerton et al., 1988).

We propose, from the observations on lenses (present report) as well as those on blood plasma and serum (Cameron et al., 1988b) and on five different homopolypeptides (Fullerton et al., 1988), that amounts of water in the range of 1.8 to more than 2.4 g water per g of solute can demonstrate motional differences in the water hydrogen bonded in different ways to macromolecular surfaces. Use of the methods described in this report allow a determination of the extent and of the physical properties of water of hydration in the lens. Such experimental approaches, when applied to normal and pathological lenses, should help us understand the molecular mechanisms underlying lens transparency and disease states.

As noted at the outset, the demonstration that a large proportion of the water in mammalian lens cells (i.e., 77.7 to 100%) is not like water in dilute solutions disallows this convenient assumption. This demonstration calls for a revolution in our thinking concerning lens physiology. The finding that the extent of nonbulk water in the lens fibers amounts to as much as 2.2 g water per g of dry mass raises the possibility that other cell types may also have larger amounts of nonbulk water than previously thought by most cell physiologists.

ACKNOWLEDGMENTS

We thank Dr. Carlton F. Hazlewood for support and helpful discussions on this project. We also thank Dr. Tamas Koszegi for his photographic work. Suppported in part by joint grants from the National Hungarian Academy of Science #OTKA 83 and NSF #30.070/32/86 and #1N +850920.

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JOURNAL OF CELLULAR PHYSIOLOGY 146:394–398 (1991)

Proportional Equilibration of K, Na Ions, and Sucrose Molecules in Pig Lenses Incubated in the Presence of the Non-Ionic Detergent Triton X-100

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The release of sodium and potassium and the uptake of sucrose molecules was studied in pig lenses incubated in isosmotic sucrose solution in either the presence or absence of 1% Triton X-100 (a non-ionic detergent). This Triton X-100 treatment has been shown to cause severe disruptions of cell membrane integrity. If sodium and potassium were free in the lens fibers as in a dilute aqueous solution, they would be expected to diffuse three to four times faster than sucrose. However, measurements of sodium and potassium release and sucrose uptake in the Triton X-100 treated lenses show a 1:1 equilibration. When pig lenses were incubated in the same solution without detergent, the sucrose uptake was significantly less than the potassium and sodium release. It is postulated that a slow, detergent mediated collapse of protein-water-ion interactions within the lens is the rate-limiting step of the observed equilibration of monovalent cations and sucrose molecules.

The release of the two major monovalent cations of the ocular lens (K,Na) and the uptake of sucrose molecules were studied in pig lenses incubated in an isosmotic, sucrose containing solution in either the presence or the absence of 1% Triton X-100. Previously, the detergent Triton X-100 has been shown to permeabilize the membranes of bovine lymphocytes (Kellermayer et al., 1984), cultured mouse fibroblasts (Kellermayer et al., 1986), chicken erythrocytes (Cameron et al. 1988b), and was used to permeabilize the plasma membranes of the lens cells in the present work. Presumably, the metabolically active lens anterior surface epithelial cells are exposed and rapidly disintegrated by the detergent. The long lens fibers lying beneath the surface epithelial cells have little metabolic activity, and they are assumed to be osmoregulated by the cell membrane located at the lens surface (Bonting et al., 1963; Duncan, 1969; Duncan and Jacob, 1984). Disruption of the cell membrane at the lens surface by detergent treatment allows the characteristics of cytoplasm to be studied independently of membrane transport functions.

With this in mind we compared the release of the cellular sodium and potassium ions and the uptake of more slowly diffusing sucrose molecules. Lenses incubated in sucrose solution in the absence of detergent served as controls. What we would have expected to find if dilute solution conditions prevailed in the lens fibers was that potassium and sodium ions would diffuse four and three times faster, respectively, than the sucrose molecule. Therefore, the release of both potassium and sodium ions would be expected to exceed the uptake of sucrose molecules when lenses are incubated in 1% Triton X-100-containing sucrose solution.

Briefly, the findings indicate that there is a 1:1 relationship between the K^+Na efflux and sucrose influx when lenses are incubated in the presence of the detergent; however, in the absence of detergent the lenses lose monovalent cations significantly faster than the sucrose influx.

MATERIALS AND METHODS

Specimens

Eyes from 1-year-old pigs were obtained from the slaughterhouse. After the lenses were dissected from the eyeballs, they were used immediately for the experiments.

Incubation and preparation of lenses for measurements

Thirty milliliters of 10 mmol/L TRIS-HCl (pH 7.2) containing 250 mmol/L sucrose solution with (ST solution) or without (S solution) 1% Triton X-100 detergent was added to each lens in separate containers. The weight of the lenses selected for study was measured before and after incubation. The incubation was per-

Received May 29, 1990; accepted November 19, 1990.

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formed at room temperature on a rocker table (120 cycle/min) for up to 5 hours. At the end of incubation, the lenses were carefully taken out of the containers, and the excess water removed by rolling the lenses on a clean dry glass surface.

Some of the lenses were digested in 4 ml 50% tetramethylammoniumhydroxide (TMAH, Merck) for 24 hours at room temperature. Aliquots of these sample stocks were used for Na, K, and sucrose measurements.

Water, sodium and potassium concentration measurements

To determine the water content of each of the incubated lenses, their weight was measured gravimetrically with an analytical balance before and after heating them at 104°C for 48 hours. Water is given in g water/l g dry mass. Na and K measurements were carried out with an OMSZOV (Hungary) flame photometer. All data were given in mmol Na or K/liter cell water, and as the percentage of the sodium or potassium contents of the non-incubated normal pig lens.

Sucrose concentration measurements

The well-known ortho-toluidine method (Pryce, 1967) was modified for sucrose measurements. The serial dilutions of the 250 mmol/L (8.5%) sucrose containing incubation buffer served as standard. A linear absorbtion curve was obtained at 630 nm when the sample sucrose concentration was between 6.25 mmol (0.21%) and 25.0 mmol/L (0.85%) (Fig. 1). Addition of 1% Triton X-100 and/or 15% TMAH to the reaction mixture did not influence the results. Duplicate measurements were performed from dilutions of the stock samples.

Protein release measurements, and measurement of the concentration of Triton X-100 in the lens

The protein released from the lenses to the incubation solution was measured by Lowry's procedure (Lowry et al., 1951). When Triton X-100 is present in the sample used for protein measurements, it is necessary to add 1 ml 10% sodium-dodecyl-sulfate (SDS) before adding the Folin reagent to avoid precipitation. When SDS was added, data were read from a standard curve of serial dilutions with a protein solution of known concentration. All measurements were therefore carried out in the presence of SDS.

RESULTS

Sucrose uptake and Na, K release from pig lenses in the presence or absence of 1% Triton X-100 detergent

Pig lenses were incubated in an isosmotic sucrose solution (pH 7.2) with or without 1% Triton X-100 non-ionic detergent at room temperature for up to 5 hours. After 3 hours' incubation in sucrose solution containing Triton X-100 (ST), the amount of sucrose measured inside the lens was 6.06% (Table 1), which is 71.4% of the sucrose concentration of the ST solution. The monovalent cation concentration of the ST treated lens at 3 hours' incubation was 29% of the total monovalent cation concentration of the intact, non-incubated lens (Table 1). Thus, 71% of the total initial

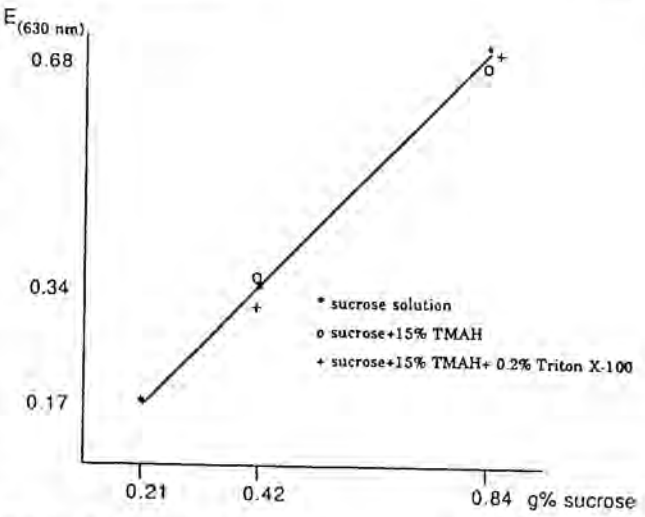

Fig. 1. Relationship between extinction at 630 nm and sucrose concentration. Sucrose was measured by the colorimetric ortho-toluidine method (Pryce, 1967). Tetramethyl-hydroxiammonium was used to digest lenses, and Triton X-100 present in the incubation solution of lenses did not interfere with the measurement.

monovalent cation content of the ST treated lens was released. After 5 hours' incubation the sucrose concentration measured inside the ST treated lens was 7.02% equal to the 82.7% of the sucrose concentration of the incubation solution. The residual sodium plus potassium concentration was 19.4% of the initial monovalent cation content of the ST treated lens, or, 80.6% of the monovalent cation content of the intact non-incubated lens was released. When these data are taken together (Fig. 2), one may conclude that there is a close to 1:1 relationship between the sucrose uptake and the monovalent cation release. Although our incubation solution contained 10 mM/L TRIS-HCl at pH 7.2, the release of K and Na ions did not change if TRIS-HCL was omitted (data not shown). Therefore an exchange mechanism between TRIS and K or Na ions did not play a significant role in the release of K and Na ions.

Control pig lenses were incubated in the same solution without detergent (S solution); both the sucrose uptake and monovalent cation release were smaller than in the case of lenses kept in the ST solution (Table 1; Fig. 2). However, the monovalent cation release (50.8%) was significantly more than the sucrose influx (36%).

The potassium/sodium ratio was 2.68 in the intact, control lens (Table 1). Due to a relatively faster sodium efflux, a significant increase in this ratio was observed when the lenses were incubated in ST solution for 3 hours (K/Na=3.54), but there was no further increase in this ratio when the lenses were incubated for 5 hours. The K/Na ratio of the S incubated lenses was 3.17, which is higher than in the control lenses, but not as high as in the ST treated lenses.

Protein release, Triton X-100 uptake

The amount of released proteins was measured in the incubation solution and compared with the initial dry

TABLE 1. Sodium, potassium, sucrose, and water contents of pig lenses incubated in isosmotic sucrose solution at pH 7.2

Sample	n	Sodium (mmol/L lens water)	Potassium (mmol/L lens water)	K/Na	Sucrose (g%)	Water (g/g dry)
Intact lens	4	41.4 ± 3.4[1]	111.0 ± 4.2[1]	2.68		2.33
ST 3 hours	5	9.74 ± 0.92	34.5 ± 3.0	3.54	6.06 ± 0.38[1]	2.64
ST 5 hours	4	6.50 ± 0.80	23.1 ± 1.8	3.55	7.02 ± 0.15	2.65
S 5 hours	4	18.5 ± 1.10	58.8 ± 3.1	3.17	3.07 ± 0.05	2.45

[1]Standard deviations.

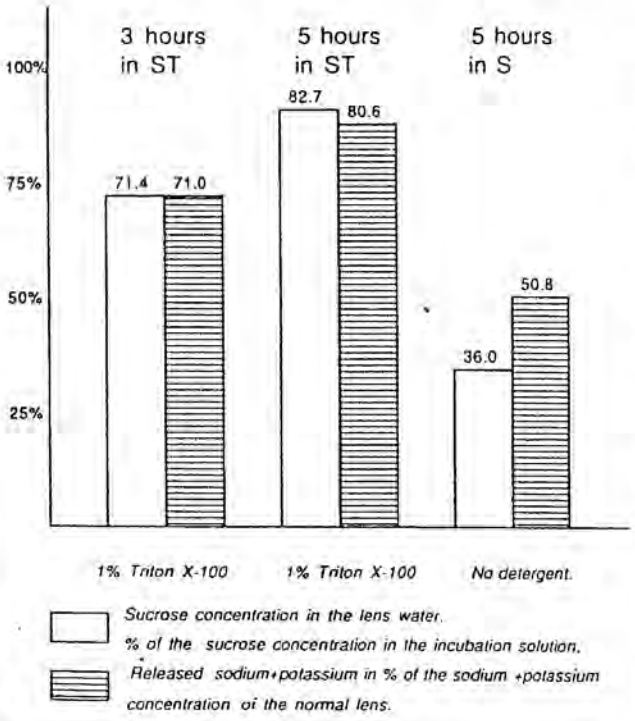

Fig. 2. The release of monovalent sodium and potassium ions and the uptake of sucrose molecules run together when pig lenses are incubated in the presence of 1% Triton X-100. The loss of monovalent cations is significantly faster than the sucrose uptake when lenses are incubated in the absence of Triton X-100.

mass of the lens. The dry mass of the non-incubated intact pig lens was 30% of its total weight (2.33 g water/g dry mass). The amount of the released proteins was 3.05% of the dry mass of the intact lens after 3 hours' incubation in ST, and went up to 5.25% after 5 hours' incubation. It is important to note that pig lenses appear to maintain their macroscopic structural integrity at least for 24 hours in ST solution, except that they lost their capsule within 5 minutes of incubation.

In spite of the release of materials (ions, proteins, lipoids, etc.) from the ST treated lens, the total lens wet and dry weight increased after incubation in ST (Table 2). The water contents of the ST treated lenses increased from an initial 2.33 g water/g dry mass to 2.65 g water/g dry mass after 5 hours' incubation. The net increase of the dry mass of the lenses is the consequence of the massive sucrose and Triton X-100 influx. The sucrose uptake may be calculated from the data of sucrose concentration and the water content of the lens. Additional increase of the lens dry weight is the result of detergent influx. On the basis of these data the Triton X-100 concentration in the lens is 0.94% at 3 hours' incubation.

DISCUSSION

The question of whether the sodium and potassium content of the cellular interior are regulated solely by cell membranes or by the cytoplasm may be studied by following the release of monovalent cations under certain experimental conditions. The ocular lens provided an excellent experimental model to study this question since the "active transport activity for sodium and potassium" was located in the lens surface epithelial cells by Bonting et al. (1963), Duncan (1969), and Jacob and Duncan (1981). The lens cortex and nucleus are assumed to be osmoregulated passively by these epithelial cells via communicating junctions (Kuszak et al., 1978; Duncan and Jacob, 1984). The anatomical structure of the lens gave us an opportunity to disrupt the membranes of the anterior surface epithelial cells and to study the lens as a simple physico-chemical system. Earlier studies showed that the Triton X-100 non-ionic detergent disrupts the integrity of plasma membranes of simian virus infected H-50 monolayer cells (Kellermayer et al., 1986), chicken erythrocytes (Cameron et al., 1988b), or bovine thymus lymphocytes (Kellermayer et al., 1984) within 2 minutes, probably within seconds as the concentration of detergent used in this study (1%) was far above the critical micellar concentration (the CMC of Triton X-100 is 0.015%).

In our experiments intact pig lenses were incubated in "isosmotic" sucrose solution (pH 7.2) either with or without 1% Triton X-100 detergent. In the presence of detergent, the basement lamina covering the lens surface epithelial cells was lost within about 5 minutes. On the basis of the law of diffusion, the number of molecules passing the lens surface is linearly correlated with their diffusion coefficients and the concentration gradient. Since the diffusion coefficient for potassium ions is four times higher and that of sodium three times higher than that of the sucrose molecules in free aqueous solutions (Fig. 3) the rate of release of both ions would be expected to be four or three times faster than the rate of sucrose uptake. However, this assumption holds true only if the lens water behaves as a simple solvent for sodium, potassium, and sucrose and if there is no significant interaction of the ions with the cytoplasmic macromolecules.

Fifty percent of the initial sodium plus potassium content of the lens was lost after incubation in the

TABLE 2. The increase of the dry weight of the lenses, and the uptake of sucrose and of Triton X-100 during incubation in ST solution

	Lens weight (g)	n	Dry mass (g)	Sucrose (g%)	Triton X-100 (g%)
Before incubation	0.4437 ± 0.0389[1]	5	0.1331 ± 0.0117[1]	—	—
At 3 hours incubation	0.5775 ± 0.0449	5	0.1586 ± 0.0123	6.06 ± 0.38[1]	0.94
Before incubation	0.4680 ± 0.0324	4	0.1404 ± 0.0097	—	—
At 5 hours incubation	0.6203 ± 0.0356	4	0.1692 ± 0.0098	7.02 ± 0.15	0.96

[1]Standard deviations.

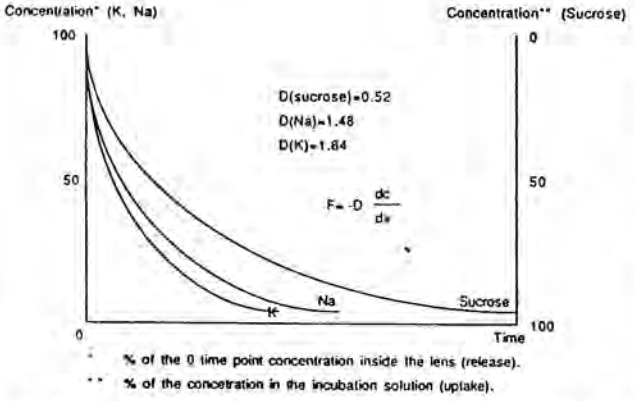

Fig. 3. Fick's law of diffusion predicts that the flux of small molecules and ions in dilute aqueous solution decreases/increases linearly with the diffusion coefficient and the concentration gradient. Diffusion coefficients for K and Na are given in 0.1 M KCl or NaCl solution. Diffusion coefficient for sucrose is given in 0.38% solution. (CRC Handbook of Chemistry and Physics, 1968, 49th ed., p. F-47). Diffusion coefficients are expressed as $10^{-5}/cm^2sec^{-1}$.

absence of detergent for 5 hours (Fig. 2). The sucrose uptake was 36% at this time point. One may expect an even faster monovalent cation release relative to the uptake of the slower diffusing sucrose molecules when lenses are permeabilized by 1% Triton X-100 detergent. Our results indicate that the sucrose uptake was similar to the release of the monovalent cations both after 3 or after 5 hours' incubation (Fig. 2). Since in the presence of detergent the selectivity of lipoid membranes may not be improved between the faster diffusing potassium/sodium ions and slower diffusing sucrose, the observed 1:1 equilibration of these molecules indicates that the cytoplasm is responsible for this phenomenon.

The result of the examination of the K/Na ratio of the ST treated lenses between 3 and 5 hours' incubation is also consistent with this hypothesis, since the faster diffusing potassium ions and the slower diffusing sodium ions were released to the same extent. In other words, no change of the potassium/sodium ratio was observed between 3 and 5 hours in the ST solution (Table 1).

The initial increase of the K/Na ratio both in S and in ST incubated lenses is probably the result of two mechanisms: 1) The extracellular space inside the ocular lens is rich in sodium and poor in potassium, as in other tissues. A faster release of this sodium may be a factor contributing to the increase of the K/Na ratio. 2) The existence of faster and slower equilibrating intracellular sodium fractions is known from sodium release and uptake experiments on frog sartorius muscle (Levi and Ussing, 1949; Ling, 1980), on amphibian oocytes, (Horowitz and Feinichel, 1970), on lymphocytes, and other cell types (Negendank, 1982). The faster equilibrating sodium fraction might be another source of the initially faster intracellular sodium release.

Unfortunately, data on the size of the lens extracellular space range from 1% to 12% of the total lens volume, depending on the measuring method and the species (Kuck, 1970; Davson, 1980). Although our previous measurements on the lens water support the lower estimate (Cameron et al., 1988a), in the absence of reliable data on the size of the extracellular space of pig lens, we cannot estimate how the two mechanisms share the responsibility for this preferential sodium release.

From our present data it appears that the release characteristics of sodium and potassium ions may not be explained on the basis of their diffusional mobilities in free aqueous solution even if the lenses are exposed to a lipolytic concentration of a detergent molecule. The 1:1 relationship between monovalent cation release and sucrose uptake in the case of the detergent incubated lenses is in agreement with our previous experiments on the state of lens water (Cameron et al., 1988a). These data showed that little if any of the cell water has characteristics similar to the free, "bulk" water, while in the present study we show that the monovalent cations are not as free to diffuse as expected under dilute aqueous solution conditions. The results are also consistent with our earlier observations on the release of potassium ions out of Brij-58 and out of Triton X-100 detergent incubated bovine thymocytes, simian virus infected H-50 cells, and chicken erythrocytes. According to these observations the release of K ions correlates with a restricted release of proteins after the permeabilization of cell membranes (Kellermayer et al., 1984, 1986; Cameron et al., 1988b), and probably corresponds with a detergent mediated disintegration of the cellular protein network (Bogner et al., 1989). Unlike monolayer cultures and suspended cells, in the case of the ocular lens the release of proteins is limited not only by their slow diffusion but also by the size of the lens. This might account for the

high monovalent cation concentration of lenses even after several hours' incubation in potassium- and sodium-free 1% Triton X-100 containing solution.

In conclusion, the proportional equilibration of sucrose molecules and monovalent cations could not be explained on the basis of the membrane solute theory. The two possible answers based on this theory are in contradiction with the facts as follows: 1) The arrangement of lens fibers as well as physico-chemical measurements detailed above prove that a sequential opening of lens cells may not be responsible for the phenomena. 2) Although K and Na ions interact with the carboxyl side chains of globular proteins, this interaction cannot significantly delay their release. Monovalent cations dissociate from the free carboxyl groups of globular proteins easily. Due to this fact, a protein containing dialysis bag and its neutral polysaccharide containing control release K and Na ions similarly.

Our suggested explanation for the 1:1 equilibration of monovalent cations and sucrose is in line with Ling's association-induction hypothesis (Ling and Cope, 1969; Ling, 1984).

According to this theory the solubility of those small molecules which are unable to interact with the cellular proteins (here sucrose) is determined by the accessible solvent water. In addition to this, the amount of K and Na within the cell is affected by an interaction with cellular proteins. Proteins behave like a fixed charge system, similar to an ion exchange resin. Therefore the interaction between the monovalent cations and proteins is enhanced. The potassium and sodium ions may not be free to leave the lens, while the sucrose molecules may not be entirely free to enter into the lens after the permeabilization of lens epithelial cells. Prior to the quantitative equilibration of small molecules between the lens and the incubation solution, the cellular water-protein-ion interactions break down because of a detergent mediated destruction.

The detergent mediated collapse of the fixed charge system affects the cell water and the association of monovalent cations simultaneously. Therefore the lens compartment in which the water becomes solvent for sucrose is identical with the lens compartment where proteins release both K and Na. When equilibration of small molecules is studied in the absence of detergent the observed release kinetics are probably due to membrane effects and to the destruction of the water-protein-ion interactions inside the living cells. Unfortunately our measurements do not provide information on how the co-compartmentalization of ions, water, and proteins is broken down. Probably both the water-protein and the ion-protein interactions change at the same time. Furthermore, the motional freedom of the monovalent cations should be different depending upon the state of water.

ACKNOWLEDGMENTS

This work was supported by the joint grant of the Hungarian Academy of Sciences OTKA 83 and NSF 30.070/32/86.

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Ion and water distribution in pig lenses incubated at 0°C to disable ion transport pumps

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Received January 10, 1991

CAMERON, I. L., HARDMAN, W. E., FULLERTON, G. D., KELLERMAYER, M., LUDANY, A., and MISETA, A. 1991. Ion and water distribution in pig lenses incubated at 0°C to disable ion transport pumps. Biochem. Cell Biol. 69: 742–746.

This study was designed to test how extended exposure of lenses to sera with different ionic strengths influences the distribution of ions and water in the lens. Pig lenses were incubated in cold sera (0°C), which were adjusted to variable concentrations of NaCl, and their K^+, Na^+, Cl^-, and water contents were measured. Incubation at 0°C inhibits active transport processes and thereby allows equilibration of the mobile ions and water. The hypothesis was that lens water content (volume) would follow the ion-induced protein changes predicted by a model derived from previous osmotic studies on proteins. As expected, exposure of the lens to cold caused a gain of sodium and a partial loss of potassium. However, the potassium concentration in the lens remained several fold higher than that in the bathing solution (about 41 vs. 1.8–4.6 mM/kg H_2O), indicating that a portion of the potassium within the cold-exposed lens was not free to diffuse. That the water content of the lens showed a negative rather than a positive relationship with the concentration of NaCl within the lens was explained by the idea that an increase in NaCl within the lens (up to at least 250 mM/kg H_2O) causes a decrease in the osmotically unresponsive water volume associated with lens proteins.
Key words: pig lens, cell water, Na^+, K^+, Cl^-, osmotic pressure, ion distribution, cell volume regulation, inhibition of active transport.

CAMERON, I. L., HARDMAN, W. E., FULLERTON, G. D., KELLERMAYER, M., LUDANY, A., et MISETA, A. 1991. Ion and water distribution in pig lenses incubated at 0°C to disable ion transport pumps. Biochem. Cell Biol. 69: 742–746.

Dans ce travail, nous tentons de vérifier comment une exposition prolongée du cristallin au sérum avec une force ionique différente influence la distribution des ions et de l'eau dans le cristallin. Des cristallins de porc sont incubés dans le sérum froid (0°C) ajusté à des concentrations variables de NaCl et nous mesurons leurs teneurs en K^+, en Na^+, en Cl^- et en eau. L'incubation est effectuée à 0°C pour inhiber les processus de transport actif et permettre ainsi l'équilibration des ions mobiles et de l'eau. L'hypothèse était que la teneur (volume) en eau des cristallins suivrait les changements protéiques induits par la force ionique et prédits par un modèle issu d'études osmotiques antérieures sur les protéines. Tel que prévu, l'exposition du cristallin au froid augmente le sodium et diminue partiellement le potassium. Toutefois, le potassium du cristallin demeure beaucoup plus élevé que le potassium de la solution d'incubation (environ 41 vs 1,8–4,6 mM/kg H_2O), preuve qu'une portion du potassium dans le cristallin exposé au froid n'est pas libre de diffuser. Le fait que la teneur en eau du cristallin montre une relation négative plutôt que positive avec la concentration du NaCl dans le cristallin s'expliquerait par l'hypothèse qu'une augmentation (allant au moins jusqu'à 250 mM/kg H_2O) du NaCl dans le cristallin entraînerait une diminution du volume d'eau osmotiquement insensible associé aux protéines du cristallin.
Mots clés : cristallin de porc, Na^+, K^+, Cl^+, pression osmotique, distribution ionique, régulation du volume cellulaire, inhibition du transport actif.

[Traduit par la rédaction]

Introduction

Conventional thought is that volume regulation in the mammalian lens as a whole is provided by the same mechanism proposed to function for volume regulation of mammalian cells in general (Davson 1980). Specifically, cell volume control has been ascribed to a "pump-leak" model system. This model calls for the active transport of potassium into the lens cells which is linked to the simultaneous extrusion of sodium from the cells by an ATP-requiring Na^+/K^+-transporting ATPase "pump" located in the cell membrane. The net effect of this process is to generate a high potassium content (110–130 mM/kg water) and low sodium content (15–30 mM/kg water) in lens cells, even though the natural environment surrounding the lens cells has the reverse ratio of these two monovalent cations (Davson 1980; Bettelheim and Siew 1982). The pump-leak model further proposes that ion movement occurs by active transport against the chemical and electrical gradients that exist between the inside and the outside of the cell, and against the passive diffusion of mobile ions down their chemical and electrical gradients. Harris and Gehrsitz in 1951 reported that cooling the lens *in vitro* inhibited metabolism and caused the lens potassium content to fall and the sodium concentration to rise, processes that were reversed by rewarming. Hightower and Kinsey (1977) studied the effects of inhibiting the Na^+/K^+-transporting ATPase pump by lowering the temperature and by using ouabain

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Printed in Canada / Imprimé au Canada

pecific inhibitor of the Na^+/K^+-transporting ATPase np) on rabbit lens. They observed that such inhibition lted in a decrease in potassium and an increase in sodium hin the lens, but they also observed that there was no ct on the diffusion fluxes of these cations.

ogether the above observations are consistent with the a that lens volume is controlled by an active transport cess. Thus sodium is thought to be pumped out of the so that the total cellular concentration of inorganic ions ss than the surrounding extra-lens environment, thereby mpensating for the excess of intracellular organic solutes ., proteins and nonelectrolytes) within the lens cells. In s model a molecular pump (or pumps) located within the act plasma membrane is (or are) required to lower the al concentration of solutes within the cell to prevent the motic movement of water into the cell. A prediction of s model is that inhibition of the pump(s) by cooling (0°C) uld allow lens swelling (Bettelheim and Siew 1982).

However, recent research on the state of water in the lens s led to the consideration of other mechanisms in lens lume regulation. For example, it was recently reported at 80% of lens water (greater than 2 g of water per gram dry mass) in the lens is unresponsive to osmotic changes oduced by varying the concentration of a nonpermeant lute (dextran) in the environment surrounding the lens Cameron *et al.* 1988*a*). Such a large volume of osmotically nresponsive water in the lens has been attributed to water olecules that are motionally perturbed by interaction with the surface of proteins (Fullerton and Cameron 1986; Cameron *et al.* 1988*b*; Cameron and Fullerton 1990). There is also evidence that the extent of the osmotically unresponsive water volume associated with proteins is variable depending on the NaCl concentration and on the pH (Cameron and Fullerton 1990). The present study was designed to test how changes in the concentration of NaCl within the lens would influence the equilibrium distribution of water and inorganic ions. To eliminate the involvement of active ion transport pumps, the osmotic equilibration of the lenses was carried out in the cold (0°C).

Materials and methods

Specimens

Eyes from 1-year-old pigs were obtained from the slaughterhouse. The lenses were dissected from the eyeball within 3 h of death. To avoid dehydration the lenses were immediately used for the study.

Incubation and preparation of lenses for measurements

The dissected pig lenses were weighed before and after incubation in serum that was cooled to 0°C, and that was either diluted with distilled water or had increasing amounts of NaCl added. The incubation, as described below, was continued for 20 h. Prior to weighing, each lens was rolled on a clean dry glass surface to remove excess fluid. Some of the fresh lenses were digested in 4 mL of 40% tetramethyl-ammonium hydroxide (TMAH; Merck) for 24 h at room temperature. Aliquots of these sample stocks were used for Na^+, K^+, and Cl^- measurements. All Na^+ and K^+ measurements were carried out with an OMSZOV flame photometer, while Cl^- was measured by a colorimetric procedure. To determine the water content of the lenses, their weight was measured gravimetrically with an analytical balance before and after baking at 104°C for 48–72 h. Lens water content is reported in grams of water per kilogram of dry mass. Ionic data are reported in mM/kg water. The amount of protein released from the incubated lenses was measured by Lowry's procedure (Lowry *et al.* 1951). Samples were taken from the incubation solution to measure Na^+, K^+, Cl^-, protein, and osmolarity. Osmotic activity of the incubation solutions was measured by a freezing point depression osmometer. Thirty millilitres of incubation solution at 0°C was placed in specimen bottles, four preweighed lenses were added, and the bottles were sealed. The lenses were incubated at 0°C on a rocker table (120 cycle/min) for 20 h.

Calculation of lens ion concentration changes upon equilibration in the incubation solutions

Because of the higher concentration of K^+ in the fresh lenses than in the incubation solution (serum), any increase in K^+ that occurs in the incubation solution after exposure to the fresh lenses must have diffused from the lenses. Therefore, measurement of the net increase in K^+ in the incubation solution during equilibration in the cold can be used to calculate the amount of K^+ lost from the lenses, given the water volume of the incubation solution and the water content of the lenses. As the initial K^+ concentration of the lens was known by measurement, one can further calculate the final K^+ concentration in the lens by subtraction of the amount of K^+ lost from the lens.

The net influx of Na^+ or Cl^- into the lenses from the incubation solution was also calculated given: the initial ion concentration in the incubation solution prior to addition of the lenses (mM/kg water), the final ion concentration in the incubation solution (mM/kg water), the final wet weight of lens (kg), the final dry weight of lens (kg), and the mass of incubation solution (0.03 kg).

Statistical evaluations

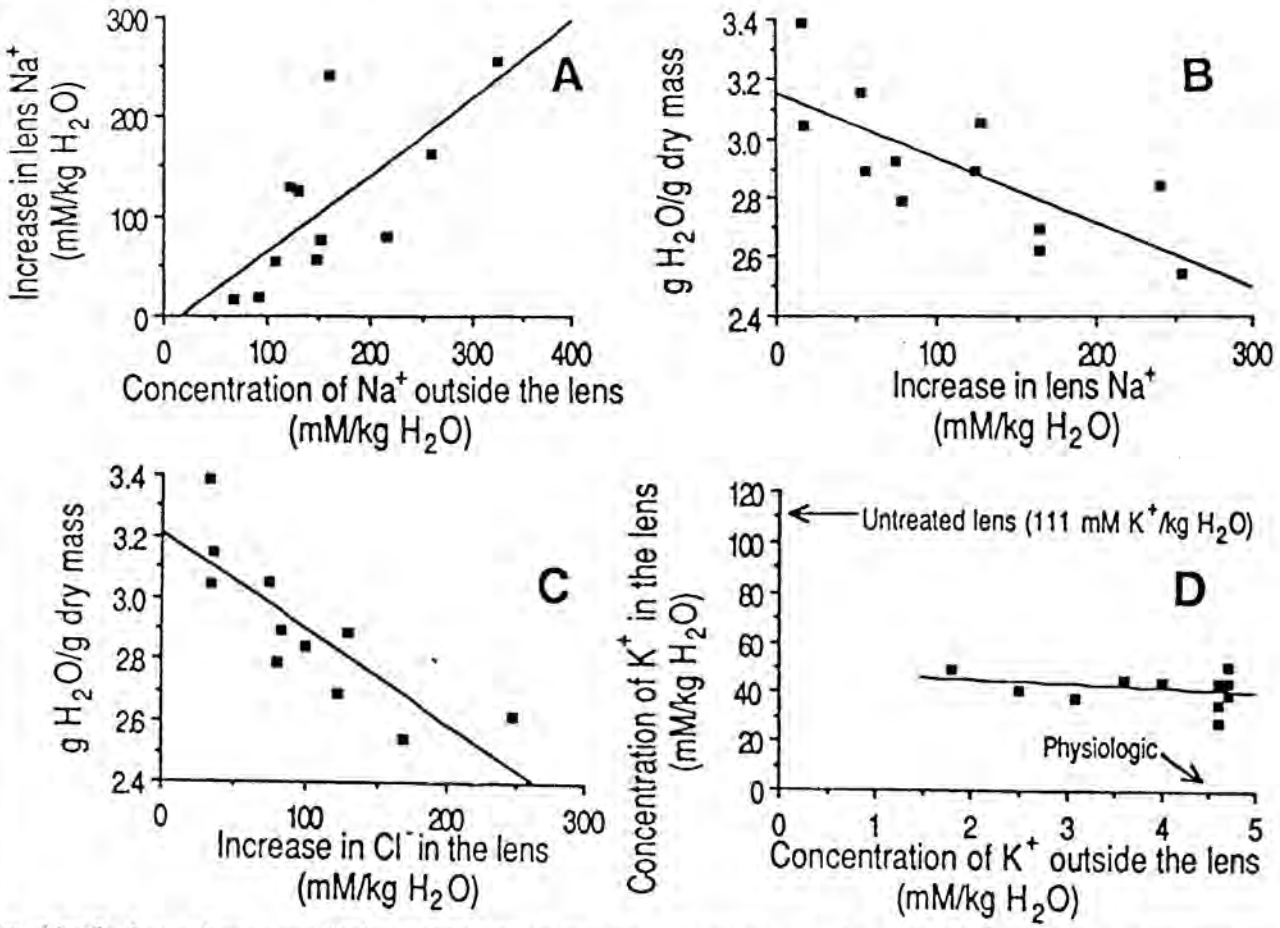
Data were subjected to analysis of variance and to least squares linear regression analysis. The statistical significance of each regression analysis was established by Student's *t*-test.

Results

Twelve fresh lenses were weighed then incubated in serum (pH 7.3, 300 mosm, Na^+ 146, K^+ 4.66, and Cl^- 105 mM/kg water) for 20 h at 0°C. There was no significant difference in the mean lens weight between 15 and 20 h of incubation, which gave an indication that the lenses had reached weight equilibrium by 15 h. Although the overall mean lens weight increased between 3 and 4% when exposed to the cold for 20 h (0.433 ± 0.008 vs. 0.451 ± 0.009 g), this difference was not statistically significant ($p > 0.05$). At the time of removal of the lenses from the cold, they were all observed to show a nuclear opacity (nuclear cold cataract) that quickly disappeared as the lenses warmed to room temperature.

Figures 1A–1D illustrate results of the attempt to change lens water content by modification of the intra-lens NaCl concentration when the lenses were equilibrated in the cold (0°C) for 20 h. Lens weight equilibration was demonstrated because no significant change occurred in mean lens weight in any of the cold-exposed lens samples between 15 and 20 h. Figure 1A shows that the Na^+ concentration outside the lens affects the Na^+ concentration within the lens when lens metabolism is inhibited by cooling. The lens Na^+ concentration is shown to increase with an increase in Na^+ concentration outside the lens (up to at least 250 mM/kg H_2O). Similar results were found for Cl^- (not illustrated). Figures 1B and 1C reveal a statistically significant decrease in lens water content as a function of increase in concentration of NaCl in the lens.

The same cold-exposed lenses decreased K^+ from 111 mM to about 41 mM/kg H_2O, but this loss did not decrease to that in the extra-lens environment (4.66 mM/kg



FIGS. 1A–1D. Ion and water equilibrium distributions in cold-exposed pig lenses incubated at 0°C to disable active transport pump(s). The salt concentration was varied by adding salt to serum or by diluting normal serum in an attempt to change the levels of Na^+, Cl^-, K^+, and water within the lens. Four lenses were used to determine each point on the graphs. Figure 1A illustrates the relationship between Na^+ measured outside the lens and the increase in Na^+ level in the lens (the slope shows a significant increase, $p < 0.01$ and correlation coefficient = r value of 0.72). Figure 1B shows the relationship between increase in lens Na^+ and lens water content (the slope shows a significant negative slope, p value < 0.01 and r of 0.78). Figure 1C shows the relationship between the increase in lens Cl^- and lens water content (the negative slope is significant, $p < 0.01$ and $r = 0.81$). Figure 1D shows the relationship between the measured K^+ concentration outside the lens and the concentration of K^+ in the lens (the slope is not significantly different than a slope of zero). Physiologic in Fig. 1D refers to the K^+ concentration of undiluted serum (i.e., 4.66 mM/kg H_2O); the K^+ concentration in fresh untreated lenses is also indicated in Fig. 1D as 111 mM/kg H_2O.

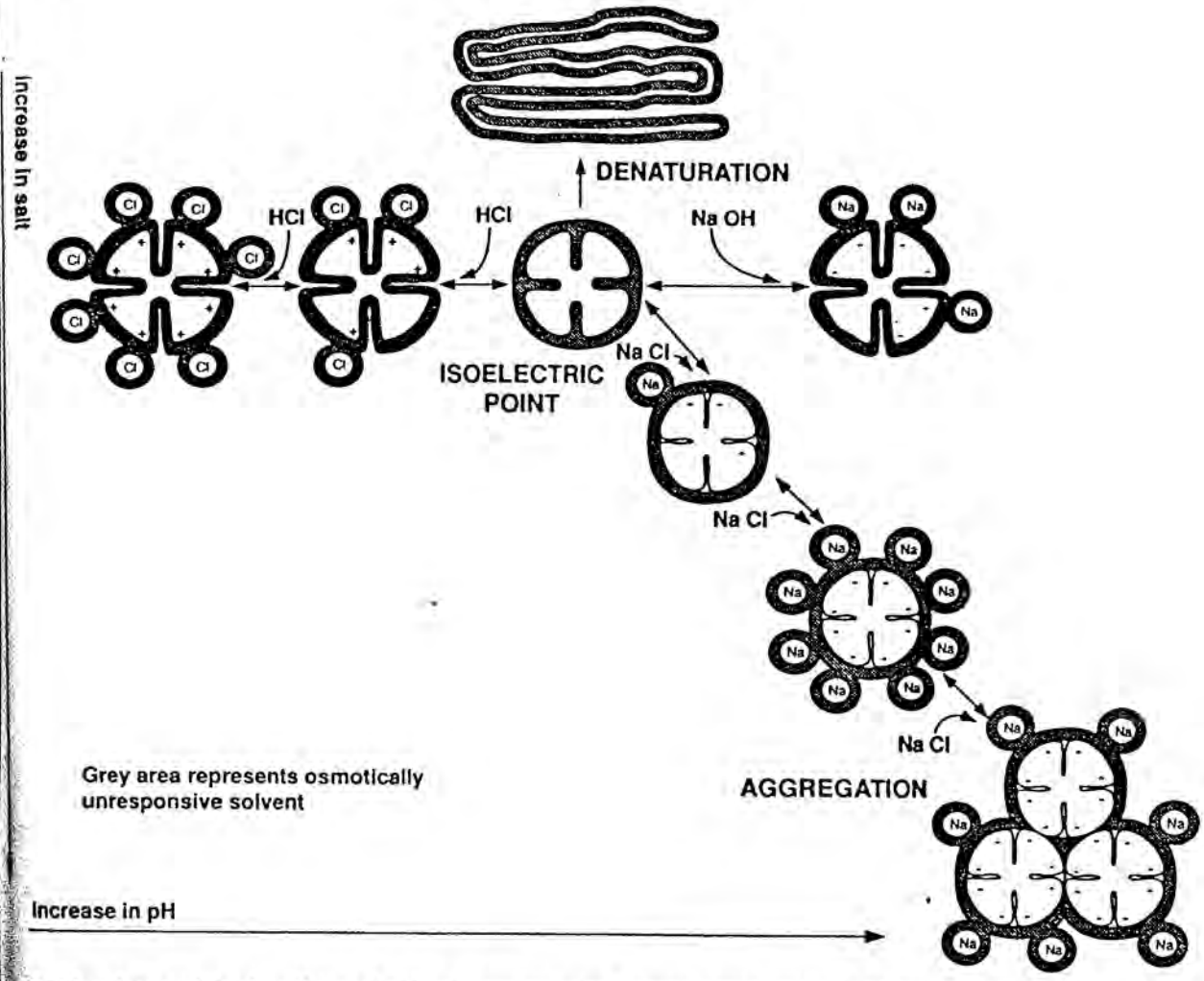
H_2O), and the lens K^+ content in the applied range was not dependent on the concentration of K^+ outside the lens (Fig. 1D). Analysis of the protein content of the incubation fluids before and after incubation showed no increase in protein concentration; this indicates retention of lens proteins regardless of the ionic strength of the lens environment used in these studies.

Discussion

The first section of the discussion gives a brief account of the advantages of the ocular lens as a cell system to study ion and water distribution; the second section summarizes the key experimental observations on the water and ion distribution in lenses equilibrated to constant weight; and the third section explains the observations on the basis of a working model of how NaCl and pH change the water of hydration of proteins within the lens.

Lenses were selected for study because they consists of a large mass of homogeneous cells (mainly extremely elongated fiber cells), with no nuclei, mitochondria, or other organelles that might absorb or scatter light and compromise lens function; lenses have but a small amount of extracellular space (average estimated at less than 5% (Davson 1980; Cameron *et al.* 1988*a*)) with no complicating blood vessels, lymphatics, or nerves; and because essentially all of the lens fiber cells (with the possible exception of a few in the central area) have a portion of their plasma membrane that interfaces with the lens surface. Thus the lens provides a unique cellular system favorable for solute flux studies.

As expected from past experiments (Harris and Gehrsitz 1951; Hightower and Kinsey 1977), cold-exposed lenses showed increased Na^+ and Cl^- concentrations. This increase in lens NaCl was positively related to the NaCl concentration in the extra-lens environment. Cold exposure also caused a decrease in K^+ concentration within the lens. However, the K^+ concentration in the cold-exposed lenses still remained about 9-fold higher than that in the extra-lens environment. It is argued that the intra-lens K^+ of 41 mM/kg H_2O is the equilibrium value under the experimental conditions used, because this 41 mM value was observed in all of the lenses over a range of external K^+ concentrations of 1.8 to 4.6 mM/kg H_2O, and because



2. Model to explain the effects of pH, salt concentration, protein denaturation, and protein aggregation on the osmotically ponsive solvent fraction on proteins (from Cameron and Fullerton. 1990. Biochem. Cell Biol. 68: 894-898, reproduced with per-n). This model can be used to help explain the lens water content observations in this study. The grey area on the figure represents motically unresponsive solvent fraction on the protein.

h at 0°C is enough time for free K^+ (diffusion coef-t, about 1 or 2 × 10^5/cm^2 per second) to reach equi-m with the extra-lens environment. Thus most, if not this 41 mM K^+ in the lens was not free to diffuse would have done so within 20 h. Also the overall se in the major monovalent cations within the lens (up um of 291 mM/kg H_2O for K^+ plus Na^+) did not in a statistically significant swelling of the lens as might pected upon cold inhibition of the active transport ion of the Na^+/K^+-transporting ATPase pump d in the plasma membrane. Instead, the lens volume content) actually decreased in a direct linear relation-o an increase in the lens NaCl concentration over the of 30 to about 250 mM.

w can these observations help explain ion distribution olume regulation in the lens? Two factors that may erational are (*i*) regulation of the number of ically active particles within the lens by adsorption to ns and (*ii*) changes in the osmotically unresponsive t fraction. In this regard, that exposure of the lens inhibitor of metabolism (cooling) and therefore to ion of active transport processes did not result in ete equilibration of lens K^+ with the extra-lens environment strongly suggests that adsorption of K^+ to proteins plays a significant role in ionic distribution (see Negendank and Edelmann, 1988, and Ling, 1984, for reviews on this subject). That at least a portion of lens Na^+ is also adsorbed under room temperature conditions is clear from NMR studies which show that a substantial fraction of lens Na^+ is "NMR invisible" and that lens Na^+ displays relaxation characteristics that can only be accounted for by adsorption (Pettegrew *et al.* 1985). The failure of the lens to act as a perfect osmometer with a nonpenetrating solute clearly indicates that a large proportion of lens water is osmotically unresponsive (Cameron *et al.* 1988*a*) and otherwise differs from unperturbed bulk water (Racz *et al.* 1979*a*, 1979*b*; Neville *et al.* 1974; Bettelheim and Siew 1982; Castoro and Bettelheim 1986; Haner *et al.* 1989; Lahm *et al.* 1988).

The following is offered as one possible explanation of how ionic strength and pH within the lens affects the water content of the lens. This explanation is based on a model that explains the osmotic pressure behavior of proteins under variable pH or ionic strength conditions (Cameron and Fullerton 1990). The model, as depicted in Fig. 2, shows that changes in pH, either above or below the isoelectric point

of the protein, increase the osmotically unresponsive solvent fraction by increasing the surface area of the protein for interaction with water molecules. As the great majority of lens proteins have an isoelectric point below pH 7.0 (Davson 1980), one might expect the amount of osmotically unresponsive water in the lens to increase as the intra-lens pH increases above the isoelectric point of the lens proteins. Increasing ionic strength (NaCl concentration) at first reduces the osmotically unresponsive solvent fraction (as occurs over the relatively low NaCl concentration range used in this study) by reducing the protein surface area for interaction with water molecules, but it is predicted that a sufficiently high salt concentration would eventually increase the nonspecifically bound hydrated ion to the charged protein.

Regulation of lens water content (volume) is therefore thought to include changes in the adsorption of osmotically active ionic particles and in the osmotically unresponsive solvent fraction. Measures of the state of ions and water in the lens by NMR techniques are needed to further test the proposed model.

It is concluded from this and past studies (Cameron *et al.* 1988*a*; Miseta *et al.* 1991) that the classical membrane – osmotic pump – leak concept of ionic distributions and volume maintenance in lenses (Davson 1980) is not adequate by itself to explain the water and ion distribution results. It is now apparent from several measurement techniques that a large proportion of lens water exists in a bound or osmotically unresponsive state (up to 2+ g H_2O/g dry mass, Cameron *et al.* 1988*a*; Lahm *et al.* 1988; Bettelheim and Siew 1982; Neville *et al.* 1974). Likewise, there is evidence that much of the Na^+ (Pettegrew *et al.* 1985) and K^+ within the lens (Miseta *et al.* 1991) and K^+ within the cold-exposed lens (this report) are adsorbed. The data in this report show the inadequacy of the common assumption that inorganic monovalent ions (K^+, Na^+, and Cl^-) are totally free to diffuse within cells. It also indicates that ion concentration (expressed per unit cellular water) does not directly indicate ionic activity as it would under dilute solution conditions. Therefore it is not correct to assume dilute or ideal solution conditions for the intracellular environment in the lens.

The results from this report are interpreted to indicate that the NaCl concentration within the lens influences the water content in cold-exposed lenses. The proposed mechanism is that NaCl concentration alters the conformation of proteins within the lens and thereby increases or decreases the available surface area of the protein for interaction with water molecules. By this mechanism, the osmotically unresponsive solvent fraction in the lens can be increased or decreased. Bettelheim and Siew (1982) previously suggested that an increase in low molecular weight sugars or ions in the lens might result in an effective competition for water between the lens proteins and the smaller solute molecules. They propose that this competition for water would decrease the hydrodynamic radius of lens proteins, and we propose that it also reduces the osmotically unresponsive water fraction in the lens. These suggestions are consistent with the findings in this study and the model shown in Fig. 2.

Acknowledgments

The authors thank Dr. Carlton F. Hazlewood for support and helpful discussion. Supported in part by joint grants from the National Hungarian Academy of Science (no. OTKA83) and United States National Science Foundation (no. 3[illegible]8d and no. 1N+850920). They also thank Lita Chumbum for her expert typing of the manuscript

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Összefoglalás, tervek:

Összfüggést találtunk különböző fajok vörösvértestjeinek "steady state" monovalens kation tartalmai és az ugyancsak "steady state" ATP szintek között (táblázatok és grafikonok a 39, 44 és 45. oldalakon).
Hasonlóan alakul a monovalens kationok és ATP szintek közötti összefüggés akkor is, ha egy faj eltérő egyedei között vizsgálódunk (táblázatok és grafikonok a 46 és 47. oldalakon). A fenti jelenséget azóta további vizsgálatoknak vetettük alá, melyek korábbi már közölt megfigyelésünket alátámasztják. Ezeket ebben az értekezésben még nem mutattuk be. Ezek az eredmények nem hozhatók maradéktalanul összhangba sem a klasszikus "membrán-pumpa" sem pedig az "asszociációs-indukciós" hipotézisekkel, ahogyan azt a közlemények diszkussziójában szerepeltetjük is.
Jelenleg az ATP mellett ADP és piridin nukleotid szintekről is adatokat gyűjtünk. Ugyanígy kiterjesztettük a vizsgálatainkat az aktív ás passzív ionáramlási paraméterekre is.
Egyedi vörösvértesteket is vizsgáltunk röntgensugár mikropróba analízissel (50-55. oldalak) abból a szempontból, hogy miként alakul azok ion tartalma az öregedésük során. Megállapítottuk, hogy az öregedő vörösvértestek vízvesztéséhez társultan csökken a K és Cl tartalom, nő a Ca és kéntartalom. Érdekes módon a Na, Mg és P tartalmakban nem találtunk értékelhető eltéréseket.

Mellékleletként találtuk meg, de eredeti felfedezésnek bizonyult a szamarakban található K és Na polimorfizmus. Tudomásunk szerint ez az első nem kérődző fajban leírt polimorfizmus. Öröklésmenete viszont hasonló a juhokban tapasztalthoz (56-59. oldalak). Sajnos továbbvizsgálatra regisztrált állomány hiányában nincs lehetőség.

Munkánk kissé elkülönülő részét adják detergensekkel kapcsolt vizsgálataink. Itt is a lipolítikus koncentráció feletti hatásvizsgálatok elkülöníthetők, amennyiben itt a plazma membrán folyamatossága megszakad, jól mérhető lipid és fehérje rilíz van (60-67. oldalak). Legfőbb megállapításunk, hogy a kálium kiáramlás késleltetett a plazma membrán lipid komponenseinek leoldása után.

Detergensek sub-CMC koncentrációkban is fokozzák a plazma membránok ion áteresztő képességét. Kevés ismeret volt azonban arról , hogy ez miként függ a detergensek hidrofób és hidrofil kémiai komponenseitől.
Megfigyeléseink szerint a Brij széria detergenseinek esetében ez elsősorban az oxyethylén egységek számától függ, de viszonylag kevés befolyása van a zsírsav résznek. Viszont telítetlen zsírsavak (olajsav) származékok jobban facilitálják a vörösvértest és környezete közötti ion kicserélődést (68-83. oldalak).
Megállapítottuk, hogy a detergensek eltávolítására in vitro használt albumin in vivo is alkalmas erre a célra (84-109.) oldalak.

Részletes vizsgálatokat végeztünk a szemlencse víz kötöttségi állapotának meghtaározására, mely vizsgálatok átvezettek detergensekkel kapcsolatos kutatásainkhoz is. Megállapítottuk, hogy a szemlencse víztartalom 77-95% van kötött állapotban (110-117. oldalak), az eltérések a megközelítések jellegéből adódnak. Hidegen (0°C) inkubált szemlencsékben a Na felvétel nem indukált duzzadást ami arra utalhat, hogy az ozmotikusan aktív és inaktív frakcók arányában következhetett be változás (123-127. oldalak). A szemlencséket erőteljes detergens kezeléssel sem lehetett szupravitális festékek számára átjárhatóvá tenni (114. oldal). Szaharóz viszont bejut a szemlencsékbe és ott -amennyiben detergens hatás van - azonos nagyságú kompartmentet foglal el a kálium ionok által kiürültel (118-122. oldalak). Ez felveti a lehetőségét annak, hogy csak azt a kompartmentet hagyják el a K ionok, melyet a detergens hatás megelőzően destruált.

Végezetül leírhatom, hogy szerteágazó kutatásiunk során melyeknek csak egy kis részét mutattam be itt (lásd publikációs lista) számos érdekes felfedezést tettünk, melyek legtöbbje azonban még több új továbbvizsgálatra érdemes kérdést vetett fel.

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Effect of non-hemolytic concentrations of Brij-series non-ionic detergents on the metabolism-independent ion-permeabilty properties of human erythrocytes.
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Preferential utilisation of exogenous phenylalanine in *Escherichia coli* cells
A. Miseta, D. N. Wheatley, K. Sipos
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The acid extractable amino acid pool in Escherichia coli: its role in amino acid accumulation and protein synthesis
A. Miseta, P. Csutora, K. Sipos and D. N. Wheatley
submitted to **Biochimica et Biophysica Acta**

Köszönetnyilvánítás:

Amikor 1979-ben első éves medikusként a Központi Klinikai Kémiai Laboratórium tudományos diákköröse lehettem, alig volt sejthető az, hogy **Dr. Kellermayer Miklós** és **Dr. Ludány Andrea** tanítványaként később dolgozni is itt fogok. Legelőször nekik kell megköszönjem, hogy tanítványként, munkatársként és barátként is folytonosan segítettek és segítenek. Itthon és külföldi tanulmányutaim során kerültem kapcsolatba **Dr. Ivan L. Cameronnal, Dr. Carlton F. Hazlewood-dal, Dr. Lawrence I. Slobinnal, Dr. Trombitás Károllyal, Dr. Denys N. Wheatley-vel és Dr. Gilbert N. Lingg-el**, szakterületük kiváló és eredeti müvelőivel, akik sokban befolyásolták gondolkozásomat. Ezért Őket is köszönet illeti.

Közvetlen munkatársaim közül **Dr. Kőszegi Tamás** az, akivel számos tudományos problémát próbáltunk együtt megfejteni, de minden más munkatársamat is köszönet illeti irántam tanúsított türelméért és jóindulatáért. Sok köszönettel tartozom a vezetésem alatt álló és az intézetünkben dolgozó diákkörösöknek, elsősorban **Dr. Bogner Péternek, Dr. Jilling Tamásnak, Dr. Kollár Balázsnak, Dr. Berényi Ervinnek, Dr. Kopcsányi Ritának, Dr. Galambos Csabának, Csutora Péternek, Borbély Csabának**, hogy a teljesség igénye nélkül felsoroljam néhányukat.

Különösen adósa vagyok **Stocker Lajosné, Pappné Bácskai Sarolta** és **Fábián Jánosné** laboratóriumi szakasszisztenseknek, akik az értekezés anyagát képező tudományos munka számos pontján segítettek. **Kövecs Ferenc** és **Szabó Zsolt** kiváló technikusaink sok gyakorlati probléma elhárításában segítettek, amiért feltétlen köszönet illeti **Őket**.

Rövidítésjegyzék:

RBC - Vörösvértest szám

Ht - Hematokrit

MCV - Átlagos vörösvértest térfogat

MCH - Átlagos vörösvértest hemoglobin tartalom

CMC - Kritkus micelláris koncentráció

HLB - Hidrofil-lipofil balansz szám

NMR - Magmágneses rezonancia

ATP - adenozin-trifoszfát

HPLC- Nagy hatékonyságú folyadékkromatográfia