Egyértékű ionok megoszlása permeabilizált sejtekben

Ph.D. disszertáció

Dr. Bogner Péter

Pécs, 1996

Tartalom

Rövidítések és kémiai nevek 3

Bevezetés 4

Kérdésfelvetés 13

Megközelítés és módszerek 16

Eredmények és megbeszélés (publikációk) 19

I. Motional characteristics of K^+ and Na^+ in intact and sucrose-permeabilized rat lymphocytes. 19

II. Release of potassium, lipids and proteins from non-ionic detergent treated chicken red blood cells. 27

III. Release of hemoglobin and potassium from human red blood cells treated under the critical micellar concentration. 35

IV. Effect of non-lytic concentrations of Brij-series detergents on the metabolism-independent ion permeability properties of human erythrocytes. 40

V. Albumin-mediated reversal of potassium depletion in human erythrocytes treated with the non-ionic detergent, Brij 58. 46

Összefoglalás 77

Idézett közlemények 79

A jelölt publikációi 87

Köszönetnyilvánítás 90

Rövidítések és kémiai nevek

CMC - critical micellar concentration

HLB - hydrophile-lipophile balance

Hb - hemoglobin

K^+ - kálium

Na^+ - nátrium

Ca^{2+} - kálcium

NMR - nuclear magnetic resonance

Brij 58 - polioxietilén-20-cetil-éter

Triton X-100 - polioxietilén-9-10-oktil-éter

DPH - 1,6-difenil-1,3,5-hexatrién

TMA-DPH - 1-(4-trimetilammonium-fenil)-6-fenil-1,3,5-hexatrién

CHO - chinese hamster ovary sejtek

L 929 - egér fibroblaszt sejtek

BSC 1 - African green monkey kidney sejtek

Bevezetés

Tény, hogy az élő anyag legnagyobb arányban három alkotórészt tartalmaz: vizet, fehérjéket és anorganikus ionokat. Ezen összetevők aránya, az élet adott formáját tekintve, viszonylag állandó, fiziológiás és pathológiás folyamatok során azonban változhat. Ugyancsak jelentős variáció található e három összetevőt illetően az élő különböző formáiban. Minden élő szervezet arra törekszik, hogy a belső összetételének, valamint néhány kritikusan fontos paraméterének (pl. ozmolalitás, pH stb.) állandóságát megőrizze, mely számos funkciót involvál. Ha konkrétan emlős sejtekről beszélünk, a sejt és környezete közti kapcsolat is pontosan definiált; ez egyrészt azt jelenti, hogy a sejt az életműködése szempontjából fontos szerves és szervetlen anyagok közül megőrzi azt, ami benn van, másrészt a környezetében lévő molekulákat szelektíven engedi be. Ha úgy tetszik ez létkérdés, szigorúan szabályozott, és talán nem véletlen a rákos sejtek azon tulajdonsága, hogy a permeabilitásuk szelektivitása csökken.

Az oldott anyagok egyenlőtlen megoszlása és azok regulációja tehát életjelnek tekinthető.

A reguláció kérdése sok szempontból ma is megválaszolatlan, bár sok munka fekszik benne, gondoljunk csak a számos pumpára, transzporterre, csatornára stb. (11). A megszerzett ismeretek többsége arra az elképzelésre épül, hogy a sejt egy szabályozottan zárt rendszer, mely felszínén "dönti el", hogy mi kerülhet be és mi nem (11,32). Más szóval a sejt és környezete közti anyag-transzport mennyiségi és minőségi paraméterei a sejtmembrántól és a vele kapcsolatban lévő mechanizmusoktól függenek.[1]
Az élő sejt belső organizációjának megismerésében a nem-ionos detergensek alkalmazása fontos szerepet játszott/játszik (1,16,25,40,44,49). A nem-ionos

[1] Érdekes megjegyezni azt a tényt is, hogy pl. a K^+ és Na^+ ionok szabályozó mechanizmusaiban olyan fiziko-kémiai törvényeket is figyelembe vesznek (pl. Donnan, van't Hoff), melyek híg vizes rendszerekben működnek, holott a sejt egyáltalán nem tekinthető annak.

detergensek megjelenése után mind a biokémiai és biológiai metodikákban elterjedtté vált használatuk, - gondolok elsősorban a különböző membránfehérjék izolálására -, vagy a citoszkeleton felfedezésére, melynek definíciója is a detergensekkel összekapcsolt (16,17,21,25). A membránfehérjék izolálása során kiderült, hogy az egyes detergensek fiziko-kémiai karakterüktől függően különféle fehérjék izolálásra optimalizálhatók, és ez a megállapítás nem csupán nagy általánosságokra igaz, hanem azt is jelenti, hogy a detergensek diszkrét szerkezeti különbségei meglepően nagy fiziko-kémiai hatásokat idézhetnek elő (12,13,17,20,45,51,59). Hasonlóképpen a citoszkeleton kutatásban is ismertté vált, hogy a különböző detergensekkel a szolubilizálható fehérjék aránya eltérő (23,34,50,56). Ezen túlmenően "enyhébb" hatású nem-ionos detergensek alkalmazásával sikerült kimutatni a sokat vitatott és többször megkérdőjelezett microtrabecularis hálózatot (lattice) (18,44,48). Mindenesetre nyugodtan mondhatjuk, hogy a detergensek jelentős szerepet kaptak a sejten belüli kompartmentalizáció kutatásában, gondoljunk csak a citoszkeletonhoz különböző erősséggel asszociált fehérjékre, melyeket az izoláló médium paramétereinek változtatásával lehet frakcionálni (16,47).

Az a kép, melyet a klasszikus sejttan az élő sejtről illetve annak struktúrálódásáról alkotott, a detergens-rezisztens citoszkeleton felfedezése után változni látszik (2,26,33,44,53). A hagyományos, korábban uralkodó elképzelés szerint a sejtmembránnal körülvett sejtben az egyes alkotórészek és organellumok egy vizes oldatban úsznak, némileg a véletlennek kiszolgáltatva. Ma már persze tudjuk, hogy ez közel sem így van, hiszen a sejtorganellumok szinte kipányvázva, minden bizonnyal szigorú törvényszerűségek szerint helyezkednek el és mozognak az intracelluláris térben (11,26,38,40,44). A fentebb említettek alapján arra is komoly bizonyítékok vannak, hogy nem csupán organellum szinten létezik kompartmentalizáció, hanem a fehérjék egy része is szorosan, vagy kevéssé szorosan kapcsolódik a szkeletonhoz (26,34,49,52,55). A két dimenzió - t.i.

organellum és fehérje lépték - közötti kapcsolódás kiváló példája a fehérjeszintetizáló rendszer, mely a riboszómákon keresztül szintén a citoszkeletonhoz van horgonyozva (2). Ismert, hogy CHO sejteket szaponinnal permeabilizálva, azok tripán kékre, sőt exogén tRNS-re is permeabilissá válnak, ugyanakkor endogén makromolekulák nem szabadulnak fel szignifikáns mértékben. Ebben a preparátumban a fehérjeszintézis sebessége az intakt sejtekkel megegyező, és 40x gyorsabb a sejtmentes rendszereknél. A szintetizált fehérjespektrum az intakt sejtekben szintetizálttal gyakorlatilag megegyezik (40).

Vajon a fehérjéknél kisebb molekulák szintén részei lehetnek a citoszkeletonhoz kapcsolódó organizációnak?

Figyelemre méltóak Srere és munkacsoportja eredményei, akik a glikolitikus intermedierek ún. "channelling"-jét feltételezik, természetesen a glikolitikus enzimek pontos organizációja ill. kompartmentalizációja mellett (53). Az, hogy a glikolitikus enzimek szintén a citoszkeletonhoz asszociáltak ma már jól ismert tény; így pl. vörösvértestekben, ahol csak membránszkeleton van jelen, a glikolitikus enzimek a membránszkeletonnal együtt izolálhatók (26,55).

Schliwa és munkatársai írták le, hogy Brij 58 nem ionos detergens hatására BSC 1 sejtek lipid membránjai a kezelés megkezdésétől számított 5 perc alatt lizálódtak, de a mikrotrabekuláris rendszer csak ezután néhány perccel dezintegrálódott (48,49). Ezt a jelenséget a szerkezetileg rokon Triton X-100 detergenssel nem lehetett produkálni. Ezen ultrastrukturális megfigyelésekből kiindulva Intézetünkben a Brij 58 és Triton X-100 detergenseket több szempontból összehasonlítottuk. CMC (kritikus micellaképző koncentráció) fölötti koncentrációt alkalmazva mindkét detergens membranolitikus hatású volt rövid időn belül borjú thymus limfocitákon és H-50 fibroblaszt sejteken is (22-24). A citoplazmolitikus

hatás és sejtmagfehérjék mobilizációja gyorsabb volt Triton X-100 detergens jelenlétében és a K^+ ionok teljes equilibrációja két percen belül bekövetkezett. Meglepő módon Brij 58 detergenssel kezelt sejtek esetén a K^+ equilibráció sokkal lassabban zajlott és szigmoid kinetikát mutatott (22-24). Ezeket az eredményeket az ionok diffuziós tulajdonságaival és a fehérjékkel történő intracelluláris ko-kompartmentalizációjával magyaráztuk. Mathur és mtsai szemlencsén hasonlították össze a Triton X-100 és a Brij 58 fehérjemobilizáló hatását és azt találták, hogy az előbbi a glikolitikus enzimeket jóval hatásosabban mobilizálta, mint a Brij 58 (34).

Más Brij szériához tartozó detergenseknek, melyeknek a HLB (hydrophile-lipophile balance) értéke a Triton X-100 detergenséhez hasonlóak, például a Brij 56, valójában nagyon hasonlóan viselkednek a Triton X-100-hoz. Ugyanígy a Triton szériához tartozó, de az utóbbitól eltérő HLB értékű detergensek igen eltérő hatásúak. Ezen megfigyeléseink arra utalnak, hogy a membranolitikus és a citoplazma destabilizáló hatás, a detergensek fizikokémiai tulajdonságai közül elsősorban a HLB értékekkel függ össze (24,25,37,51,59).

Cameron és munkacsoportunk kísérletei alapján jelent meg azok a röntgensugár mikropróba analízis eredményekről szóló közlemény, mely azt bizonyította, hogy az egyes vörösvértesteken belül a K^+ iontartalom eltérő lehet (8). A vörösvértestek detergens hatásra bekövetkező átlagos K^+ tartalom csökkenése az alacsony K^+ tartalmú vörösvértestek számának növekedésével arányos, de még 15 perces detergens inkubálás után is maradnak olyan vörösvértestek, melyek K^+ tartalma hasonló a kiindulási értékhez. A magvas csirke vörösvértesteken belül a mag K^+ tartalma együtt változott a citoplazma K^+ tartalmával. Igen fontos megemlíteni, hogy a fehérje (hemoglobin) kiáramlás együtt következett be a K^+ kiáramlásával. Munkacsoportunk szemlencsék Triton X-100 detergenssel való inkubációja után is a K^+ és Na^+ ionok elnyújtott equilibrációját tapasztalta, holott a

lencsesejtek membránja néhány percen belül morfológiailag destruálódott (9,35). Az egyértékű ionok diffúziós koefficiensét figyelembe véve a jelenség nem lenne magyarázható, ha az ionokat a szemlencsében szabadon, oldott állapotban feltételeznénk.

Ezekhez a megfigyelésekhez kapcsolódnak azok a kísérleteink, melyekben izotóniás szacharózban izolált limfocitamagok ionmegoszlását vizsgáltuk (5). A sejtmembrán dezintegrációját elektronmikroszkópiával bizonyítottuk, de kevés citoplazma szennyeződés gyakran megfigyelhető volt a homogenizálást alkalmazó izolálás után. Ezeket a sejtmag preparátumokat K^+-mentes közegben inkubálva azt találtuk, hogy az intranukleáris K^+ ionok egy része nem mobilizálódik, ugyanakkor a Na^+ ionok szabadon permeálták a limfocita magokat.

Ezeket az eredményeket együtt szemlélve úgy tűnik, hogy a plazmamembrán nem lehet önmagában felelős az intracelluláris fehérjék és kálium ionok megtartásáért.

Clegg és munkatársai (46) további bizonyítékot szolgáltattak arra vonatkozólag, hogy a Brij 58 detergenssel kezelt sejtek, fehérje és K^+ vesztése kooperatív jellegű folyamat. Sűrűség grádiens centrifugálást használtak annak vizsgálatára, hogy a detergens kezelés folyamán miként alakulnak át a normál denzitású L929 sejtek egy kevésbé denz formába. Lényegében ezek az eredmények megerősítették Cameron és mtsai észleleteit. (8,10).

A Brij 58 detergens hatásra bekövetkező hemoglobin (Hb) mobilizációt D_2O-dal feltöltött sejteken NMR segítségével is monitorozni lehet (10). A fentebb említett Hb és K^+ kiáramlás előtt a Hb molekulák mozgási állapota jelentősen megváltozik. Nyitott kérdés marad azonban, hogy a Hb mozgásának felgyorsulása miként függ össze a K^+ ionok elvesztésével. Ponder megfigyeléseiből tudjuk, hogy a K^+ ionok kiáramlása a hemolízist

időben megelőzi (4). Saját kísérleteinkben CMC alatti Triton X-100 detergens kezeléssel, adott vörösvértest populáció lízisét időben nagymértékben el lehetett nyújtani és megerősítve az imént említett megfigylést, a K^+ ionok prelitikus elvesztését igazolni tudtuk[2] (4,37).

Jól ismert, hogy a detergensek lipolitikus hatása a CMC értékekkel függ össze (39). Transzmissziós elektronmikroszkópiával bizonyítottuk, hogy a csirke vörösvértestek plazmamembránjai effektív módon megnyílnak mintegy ½ perccel a Brij 58 detergens (CMC feletti koncentráció) hozzáadás után (8,24). Ezeket a megfigyeléseket tovább támogatták vékonyréteg kromatográfiás meghatározásaink (24). A plazmamembránt alkotó főbb lipidféleségek kivétel nélkül megjelennek az inkubációs médiumban. Normális körülmények között, nem permeábilis "szupravitális" festékek, mint pl. a tripán kék könnyedén bejutnak a Brij 58-cal kezelt sejtekbe akkor is, ha az expoziciós idő csak néhány másodperc (23,46).

A sejt és környezete között ugyanakkor, az ionkicserélődés akkor is felgyorsul, hogyha a sejtek környezetében a CMC értéket el nem érő detergens koncentráció van (3,4,6,19,37,43,57). Ezeknél az alacsony koncentrációknál a detergensek nem nyitják meg a plazma membránt, hanem hatásuk inkább ionophore-szerű (7,30,42). Igen érdekes volt az a megfigyelésünk, hogy azok a vörösvértestek, melyek CMC alatti detergens koncentrációk jelenlétében, 0 és 4 °C között inkubálva jóllehet sok K^+-t vesztettek, plazmában vagy szérumban történő reinkubálás után, visszanyerték eredeti K^+ koncentrációjukat (6). Amennyiben a második inkubáció fehérjementes minimál médiumokkal történt (Krebs-Ringer vagy Hank's oldatok) a vörösvértestek tökéletesen megsemmisültek, hemolizáltak. Indirekt bizonyítékot szolgáltattunk arra, hogy az albumin magas affinitással kötődik a polioxietilén-detergens molekulákhoz és kivédi

[2] Megjegyeznénk, hogy az ATP molekulák a hemoglobinnal teljesen azonos kiáramlási kinetikát mutattak [unpublished]

annak hemolitikus hatását. A közel eredeti vörösvértest K^+ szint helyreállításában egyrészt az aktív transzport, másrészt pedig zsugorodási fenomén játszott szerepet. E kettő szerepét glikolízis inhibítorokkal és ouabain inhibícióval vizsgáltuk (6).

A különböző izolálási metodikákban használt detergens koncentráció általában a tized százalékos tartományban van (16,25). A detergenshatás itt a jól ismert micellaképzés révén valósul meg, tehát vizes rendszerekben a mobilizált apoláros karakterü molekularészek a micella belseje felé orientálódnak, míg a poláros rész a micella felületét alkotó hidrofil láncok közé ékelődik (16,28,31,60). Ez adott detergenstől függően facilitálja a sejtmembrán ‚szétesését. Alacsonyabb koncentrációnál, nevezetesen a micellaképző koncentráció alatti tartományban (sub-CMC) a detergensek monomer formában vannak jelen és ezek a monomerek lépnek kölcsöhatásba a membránnal (4,15,30). Pontosabban fogalmazva ez azt jelenti, hogy detergens monomerek épülnek be a sejtmembránba és változtatják meg a membrán bizonyos funkcióit és tulajdonságait, a membrán permeabilissá válik és pl. a monovalens ionok kicserélődése megkezdődik (4,30,37). A detergensek és a lipid kettősrétegek kölcsönhatása egy adott detergens-lipid arány esetén a lipidfázisban lévő detergens mennyiségétől függ (30). Ez azért fontos, mert bizonyos detergensek partíciós koefficiense, míg a detergens koncentráció a lipid koncentrációnál jóval alacsonyabb, lineáris, viszont emelkedni fog, ha a detergens koncentráció a lipid koncentrációt megközelíti. Ez a jelenség jóval a CMC alatt (1/10) is létrejöhet. Lasch és mtsai carboxyfluorescein-nel töltött liposzómákat kezeltek sub-CMC Triton X-100 detergenssel, mely 1/4 CMC fölött jelentős festék kiáramlást okozott (30). A detergens beépülést bizonyították a liposzómák méretének meghatározásával, mely detergens hozzáadása esetén nőtt. Ezekben az esetekben lipid mobilizáció biztosan nem következett be. Gonzales-Manas csoportja kimutatta, hogy a Triton X-100 már a litikus koncentráció alatt is kötődik a Halobacterium purple

membránokhoz, ugyanolyan gyorsan, mint más membránok esetén, és amíg a foszfolipid 75%-a a membránban van, a fehérjemobilizáció nem indul meg (15). Ha a fehérje kiáramlás egyszer már megkezdődik, egy viszonylag szűk detergens koncentráció tartományban végbemegy.

Ismert ugyanakkor az is, hogy sub-CMC koncentrációjú Triton X-100 kezelt vvt-k ozmotikus rezisztenciája megnő hipozmotikus közegben (57). Saját kísérleteinkben bizonyítottuk, hogyha sub-CMC koncentrációnál az inkubáló médium cserélgetésével újabb és újabb monomereket viszünk a rendszerbe, azok szintén beépülnek a membránba, kumulálódnak és előbb-utóbb vvt-k esetén hemolízist kapunk (4). Felmerül tehát a kérdés: a nem-ionos detergensek által okozott citolízis oka elsődlegesen miben áll? Mi lehet az a hatás, amely a megfelelő számú detergens monomer beépülése után a sejt belső organizációját úgy zavarja meg, hogy a citoszkeletonhoz asszociált molekulák és ionok kiáramlását előidézi?

Egy további kísérletünkben a membrántulajdonságok detergens kezelésre bekövetkező változásainak detektálását tűztük ki célul, nevezetesen a steady-state fluoreszcens anizotrópia mérésével, mely leegyszerűsítve a membránfluiditásról ad információt. Kérdésünk az volt, hogy milyen összefüggés van a membrántulajdonságok megváltozása és az egyértékű ionok permeabilitásának növekedése között? Emellett a Brij detergensszériát használva lehetőségünk volt a kémiai karakter és a detergens hatás szisztematikus tanulmányozására, mivel ebben a csoportban hozzáférhetők különböző hosszúságú hidrofil és hidrofób láncú molekulák. Eredményeink azt mutatták, hogy humán vörösvértestekben az ionpermeabilitás fokozódásáért a hidrofil lánc hosszúsága a felelős, a hidrofób tag hosszúsága nem, de szterikus változása döntően befolyásolja a detergenshatást (37). Izolált vörösvértest "ghost"-okon mértük a detergensek által előidézett fluoreszcens anizotrópia változásait, mely azt mutatta, hogy a detergensek ionpermeabilitást fokozó hatása és az ezzel párhuzamosan mért anizotrópia változás szorosan korrelál. Az anizotrópia változást kétféle

fluoreszcens festék, DPH és TMA-DPH is mutatta, melyek membrán-lokalizációjukban (hidrofobicitás) különböznek. Ezek az eredmények arra utalnak, hogy a detergensek membránba való beépülése a membrán fizikai állapotát (fluiditás) módosítják, a beépült festékmolekulák mozgási szabadsága nő, mely az ionpermeabilitás növekedéséhez vezet. Kimutattuk azt is, hogy albumin hozzáadásával a fluoreszcens anizotrópia is reverzibilisen visszaáll a kontroll értékekre (6,58).

Összefoglalva, a nem-ionos detergensekkel elvégzett kísérletek alkalmasnak tűnnek a sejt belső struktúrálódásának megismerésére. Fiziko-kémiai jellegüktől (valamint adott sejtféleségtől) függően a nem-ionos detergensek egy komplex, részleteiben ismert mechanizmussal indítják meg a sejt dezorganizációját. Saját és mások kísérletei meglehetős variációt mutatnak a detergenshatást illetően, melyekből más és más konkluzió vonható le. Ebben persze a kísérleti körülményekben meglévő jelentős különbségek is szerepet játszanak. Nyilvánvaló ugyanakkor, hogy az élő csupán egy meghatározó elv szerint organizálódik. A nem-ionos detergensek használata egyfajta metodikának tekinthető, és az ezzel a metodikával kapott eredmények, valamint más módszerekkel elvégzett kísérletek szintézise közelebb vihet a valósághoz (32,33,36,54). A disszertációban ismertetendő és korábbi kísérletek arra utalnak, hogy az élő sejtben az egyértékű ionok nem szabadon, oldott formában vannak jelen. Hasonlóan a kisebb-nagyobb makromolekulákhoz, ezek az ionok is valószínűleg a citoszkeleton és a hozzá kapcsolódó fehérjékhez asszociáltak. Bizonyos, hogy a sejtfelszín kitüntett szerepet kap ezen struktúrák stabilitásának megőrzésében.

Kérdésfelvetések

A disszertáció a nem-ionos detergensekkel és más permeabilizálási módszerrel végzett kísérletek alapján négy megjelent és egy közlésre elküldött dolgozatot foglal magában.

I. *Vajon equilibrálódnak-e a K^+ és Na^+ ionok thymus limfocitákban, miután a sejtmembránt és a citoplazma nagy részét mechanikusan eltávolítottuk? Ha nem, milyen mobilitást mutatnak ionos és ionmentes médiumokban?*
Motional characteristics of K^+ and Na^+ in intact and sucrose-permeabilized rat lymphocytes. (1992) Physiological Chemistry and Physics & Medical NMR 24: 281-288.

II. *A kritikus micellaképző koncentráció felett milyen fiziko-kémiai paraméterek határozzák meg a nem-ionos detergensek citolitikus hatását? Miként viszonyul egymáshoz a különböző sejtkomponensek kiáramlása detergenskezelés után? Mobilizálódanak-e az intracelluláris K^+ és Na^+ ionok a plazmamembrán fenesztrációját követően?*
Release of potassium, lipids and proteins from non-ionic detergent treated chicken red blood cells. (1994) Journal of Cellular Physiology 159: 197-204.

III. Ahogy a bevezetőben említettem (és az előző (II.) közleményben is bizonyítást nyert), a Triton X-100 és Brij 58 detergensek release kinetikai vizsgálatánál a membranolitikus hatásban lényeges különbség nem mutatkozott, azonban a K^+ és a citoplazmatikus fehérjék kiáramlásában igen. Bár a Brij 58 detergens jelenlétében a fehérje és ion-release jóval elhúzódóbb volt, mint Triton X-100 esetén, a kiáramló fehérjék időbeli frakcionálására nem volt lehetőség. *Ezért egy olyan kísérletes modell*

kidolgozását terveztük, ahol a detergens okozta fehérje kiáramlás időben elnyújtható és ezáltal a kiáramló fehérjék frakcionálhatók. Triton X-100 nem-ionos detergens monomerek humán vörösvértestekben képesek a K^+ ionok és a hemoglobin elnyújtott kiáramlását előidézni. A K^+ ionok a fehérjével azonos kinetikával, de azt megelőzve távoznak a sejtekből.

Release of hemoglobin and potassium from human red blood cells treated under the critical micellar concentration. (1989) Scanning Microscopy 3:1241-1245.

IV. A detergenshatás tanulmányozására további vizsgálatokat végeztünk. *Érdeklődésünk kiterjedt a detergensek kémiai karaktere és a detergenshatás összefüggésének vizsgálatára. Különösen érdekesnek tűnt az a kérdés, hogy adott detergens által előidézett, a prelitikus ionkiáramlásnak megfelelő fázisban miként változik a plazma membrán fizikai állapota?*

Effect of non-lytic concentrations of Brij-series detergents on the metabolism-independent ion permeability properties of human erythrocytes. (1995) Biophysical Journal 69: 2563-2568.

V. A két előző publikációban összefoglalt kísérletekből tudjuk, hogy a kritikus micellaképző koncentráció alatt akár a Triton X-100 vagy a Brij 58 detergensek képesek előidézni a vörösvértestek teljes szétesését. *Vajon a sub-CMC detergens koncentrációval előidézett sejtszétesés irreverzibilisnek tekinthető? Kísérleteink azt mutatták, hogy a detergens-indukált ionpermeabilitás növekedés, - mely a citolízis első jelének tűnik - reverzibilis, illetve a vörösvértestek képesek a víztartalomra számított K^+-szint visszaállítására.*

Albumin-mediated Reversal of Potassium Depletion in Human Erythrocytes Treated with the Non-Ionic Detergent, Brij 58. (submitted to Cell Biology International)

A vázolt főbb kísérletek célja az volt, hogy a detergensek tulajdonságairól és a detergens - sejt, illetve detergens - sejtmembrán kölcsönhatásról újabb ismeretekre tegyünk szert. Korábbi közleményeinkben főleg a jelenség leírására szorítkoztunk, újabban kutatásainkat a háttérben álló biokémiai és biofizikai mechanizmusokra is kiterjesztettük. Úgy gondoljuk ez azért is fontos, mivel a sejtek permeabilizációját széles körben alkalmazzák a biokémiai, molekuláris genetikai és sejtélettani kutatásokban is. A kísérletek rávilágíthatnak olyan újabb tényekre is, melyek a citoplazma állapotáról adnak információt, valamint a sejt és környezete között fennálló egyenlőtlen ion megoszlás jobb megértését segítik.

Megközelítés és módszerek

Legtöbb kísérletünkhöz heparinizált emberi vért használtunk (125 NE heparin/ml vér), amit fiatal egészséges egyénektől nyertünk. Felhasználás előtt a vörösvértesteket izotóniás NaCl oldatban mostuk kétszer. A mosás során szupernatanssal együtt a fehérvérsejtekben gazdag felszíni réteget (buffy coat) is eltávolítottuk. Amennyiben tisztább vörösvértest frakcióra volt szükség, Percoll grádiens centrifugálást végeztünk. A kísérletekre a vörösvértesteket a megfelelő oldatban állandó hematokrit mellett szuszpendáltuk. A hőmérséklet, amelyen a vörösvértesteket, illetve az oldatokat tartottuk 37 vagy 4 °C, amennyiben másként nem jelöljük.

A CMC érték lehetséges eltolódásai miatt egy kísérleten belül az ion összetétel, pH, ionerősség és hőmérséklet ugyanaz volt, amennyiben azt másként nem jelöljük (39). A CMC értéket adott detergensre laser-nephelometriás módszerrel határoztuk meg ill. ellenőriztük.

A thymus limfocitákat fiatal patkányokból nyertük, a kísérletben használt sejtpreparátumot izotóniás szacharózban történő homogenizálással kaptuk. A homogenizálás mértékének és effektivitásának "meghatározására" elektronmikroszkópos vizsgálatokat végeztünk a metodika kidolgozása során. Vizsgálataink azt mutatták, hogy a sejtpreparátumok iontartalmát számos (az I. közleményben sem taglalt) tényezőtől függ, melyeket természetesen figyelembe vettünk. Ugyanakkor az "ionmozgások" jellege és tendenciája az egyes kísérletekben nem változott.

A minták elektrolit szintjeit vagy lángfotometriával vagy pedig atomabszorpciós spektrofotométerrel végezzük (24). A klorid meghatározásokat a laboratóriumunkban használt fotometriás metodikával végezzük. Lehetőségünk van ezeket a komponenseket individuális

vörösvértestekben is megmérni, melyhez segítséget Dr. I. L. Cameron nyújt a Texas-i Egyetemről (San Antonio).

A fluoreszcens anizotrópia meghatározásokat a POTE Biofizika Intézetben Szarka Ágnessel kollaborációban végezzük. A fluoreszcens anizotrópia méréseket Dodge szerint izolált szellemsejteken végezzük, mivel a hemoglobin jelenléte ezeket a méréseket nem tenné lehetővé (10). A fluoreszcens mérésekhez az igen gyakran használt DPH és TMA-DPH fluoreszcens festékeket használjuk.

Az ATP tartalmakat biolumineszcens metodikával a Boehringer cég által fejlesztett luciferin/luciferáz rendszer segítségével mértük. Glukóz, laktát és hemoglobin szinteket a glukóz oxidáz/peroxidáz metodikával, laktát dehidrogenáz, valamint a Drabkin reagens segítségével mértünk.

Kísérleteinket minden egyes mérési pontnál több mintán végeztük. Meghatároztuk méréseink átlagát, szórását és az egyes mérési pontok közötti eltérések szignifikanciáit. Minden kísérletet többször megismételtünk és ellenőriztük ezek reprodukálhatóságát.

A fehérje analízist egy- és kétdimenziós poliakrilamid gélelektroforézissel végezzük. Az egydimenziós elektroforézishez a Laemmli, a kétdimenzióshoz az O´Farrell technikát használjuk. (21,26) A lipid analízishez egydimenziós vékonyréteg kromatográfiát alkalmazunk.

A felhasznált vegyszerek a Sigma, Serva és Reanal cégek által forgalmazott termékek.

Strukturális adatokat továbbra is transzmissziós elektron-mikroszkópiával nyerünk.

További metodikai részleteket illetően a mellékelt publikációk Materials and Methods részeire utalnék.

Physiol. Chem. Phys. & Med. NMR (1992) *24*:281–288

Motional Characteristics of K^+ and Na^+ in Intact and Sucrose-Permeabilized Rat Lymphocytes

P. Bogner,⁺ T. Berke and M. Kellermayer

Department of Clinical Chemistry, University Medical School, Pécs, 7643 Pécs, Hungary
⁺Present and corresponding address: CT-Biological Center, Faculty of Animal Production, Pannon University of Agriculture, Kaposvár, P.O. Box 16, 7401 Kaposvár, Hungary

Abstract: Most, if not all, cells maintain an unequal distribution of Na^+ and K^+ against their environment. These two monovalent ions are in constant exchange between the cell and the extracellular space since both ions have proved to be permeable through the cell membrane.

The distribution of Na^+ and K^+ in intact and "sucrose-permeabilized" rat lymphocytes were studied ("sucrose-permeabilization" means homogenization in isotonic sucrose solution). Both the intact and the permeabilized lymphocytes were incubated in Hanks' solution and then transferred into K^+, Na^+-free isotonic sucrose solution. Alternatively, the cells were incubated only in the sucrose solution or in Hanks' solution. The Na^+ and K^+ content of the cells were determined at the conclusion of each period of incubation in the same or different medium. We found that K^+ did not equilibrate under any conditions in intact lymphocytes but Na^+ responded to changes of the incubation media. In the permeabilized cells Na^+ freely equilibrated with the extracellular medium while K^+ did not, although its concentration decreased compared to that of intact cells.

IT HAS BEEN KNOWN for a long time that Na^+ and K^+ are essential components of every living organism and that their characteristic distribution is very much connected to life phenomena. With a few exceptions most eukaryotic cells maintain high concentration of K^+ and low levels of Na^+ intracellularly. The mechanism maintaining this unequal distribution has been a major question for physiologist in this century and in recent times the Na^+, K^+-ATPase is thought as the primary candidate. Nevertheless, the problem of unequal distribution for many solutes seems rather complicated in the view of the membrane-pump hypothesis.

We used permeabilized rat thymus lymphocytes to test the equilibration of Na^+ and K^+ in a physiological salt solution (Hanks') and in a K^+, Na^+-free isotonic sucrose solution. According to our findings neither of the two monovalent ions behave as if they were complete-

ly freely dissolved in the cell. In spite of the damaged membrane structures much of the K^+ and a minor portion of Na^+ is retained inside the cells. Our results can not be explained on the basis of the membrane-pump hypothesis but they are in good accordance with association-induction hypothesis of Ling.

Materials and Methods

All chemicals of analytical grade were purchased from Reanal, Budapest. For the experiments albino rats were used at 100–150 g weight. The animals were anesthetized with urethan and exsanguinated after isolating and severing the carotid arteries. The thymus was removed from the chest and the cells were carefully released from the stroma by teasing the gland with dissecting needles in isotonic sucrose solution (0.25 M sucrose, 0.2 mM Ca-acetate, 20 mM Tris-HCl pH 7.4) (*intact cells*). The thymus cells literally poured out from the disrupted stroma. After the stroma was "emptied" the cell suspension was filtered through 4 layers of gauze. Suspended cells were collected as a pellet by centrifugation at 2000 rpm for 5 minutes (Sorvall RC-5, rotor SS-34).

For permeabilization the previously suspended cells were homogenized in the isotonic sucrose solution (see also 1,2) (*permeabilized cells*). Homogenization was performed with a Braun-Melsungen blendor for 3 minutes at 2000 rpm. The homogenate was filtered through 4 layers of gauze and the filtrate was spun down at 2000 rpm for 10 minutes (Sorvall RC-5, rotor SS-34).

The pellets collected (100–150 mg wet weight) were resuspended in 8 ml of either the sucrose solution which was used for the isolation procedure or Hanks' solution (3).

The permeabilization and incubation were performed at room temperature (22–23°C). The intact and permeabilized cells were incubated either in Hanks' solution or in isotonic sucrose solution for three hours. In other cases cells were incubated in Hanks' solution for two hours and then centrifuged at 2000 rpm for 5 minutes (Sorvall RC-5, rotor SS-34) and the pellet resuspended in sucrose solution. These cells were then incubated for another hour in the sucrose solution. At all steps, i.e. at the end of the two or three (or 2+1) hour incubation period, the cells were centrifuged at 13,000 rpm (15000 g) for 20 minutes and the pellets were analyzed for Na^+, K^+ and water contents.

Water content was determined by weighing the samples before and after drying at 105°C for 48 hours. After dissolving the dried samples in 1N HCl, Na^+ and K^+ contents were measured by flame photometry (OMSZÖV-Digit). Measurement for intact tissue was made from freshly excised thymus; no correction for the extracellular space was made at the calculation.

We examined the effectiveness of the homogenization, i.e. the damage of the cell surface and/or cytoplasm and nucleus by electron microscopy. For electron microscopy the specimens were fixed in 2.5% glutaraldehyde for one hour and postfixed with 2% OsO4 (pH 7.2). After fixation the dehydration was carried out in graded solutions of ethanol and embedded in Epon resin. Ultrathin sections were stained with uranyl acetate and examined on a Jeol-100C electron microscope.

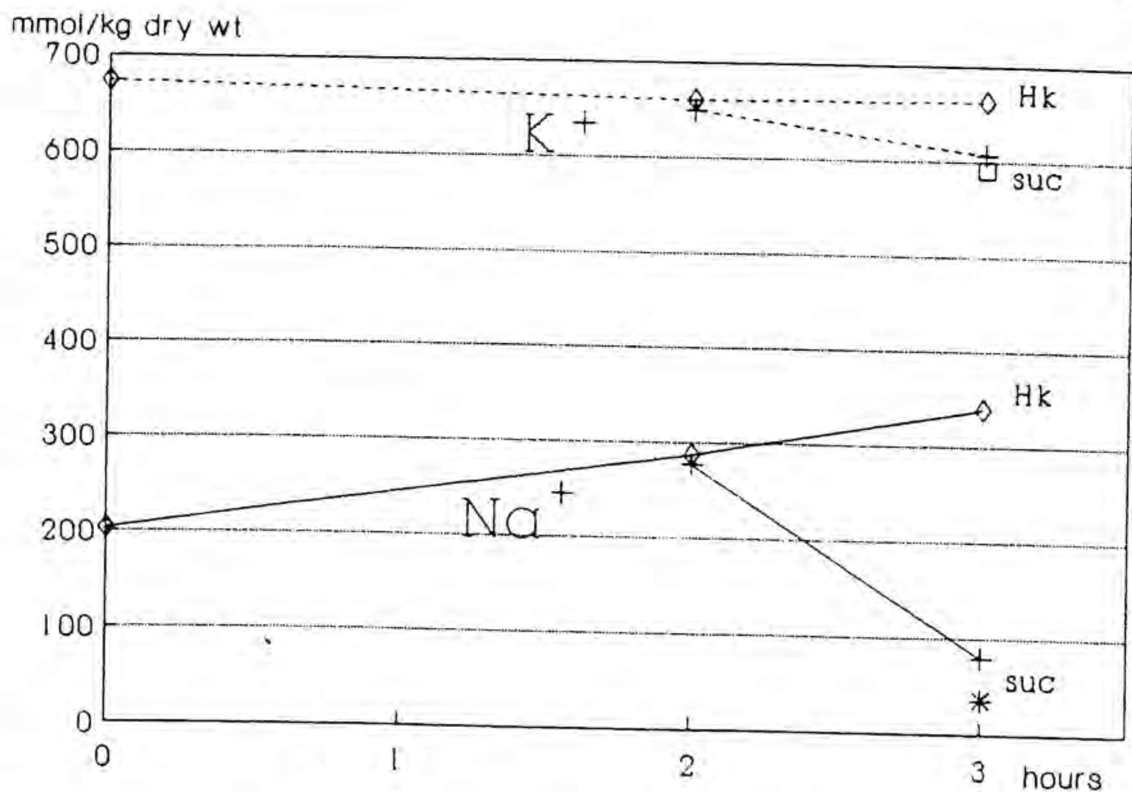


FIGURE 1. The release of K^+ (dashed line) and Na^+ (solid line) from intact (unhomogenized) rat thymus lymphocytes. 0 hour refers to the intact tissue. The K^+ or Na^+ concentration from cells incubated in Hanks' solution (Hk) for three hours is represented by diamonds. The K^+ or Na^+ concentration from cells incubated for two hours first in Hanks' solution and then transferred into sucrose (suc) solution for one hour is represented by crosses. The data obtained for cells incubated continuously in sucrose solution for three hours are represented with square (K^+) and star (Na^+), respectively. The K^+ and Na^+ contents of cells are expressed on a dry weight basis.

Results

Intact cells

Rat thymus lymphocytes isolated in isotonic sucrose solution were incubated in Hanks' solution for three hours. Through this period the K^+ content of the cells remained constant (Figure 1). (The values of K^+ and Na^+ at 0 hour refer to the intact tissue.) Lymphocytes kept in Hanks' solution for two hours and then transferred to K^+, Na^+-free sucrose solution for one hour showed a slight decrease in K^+ content. It is important to note that the same degree of change was found in cells incubated continuously in K^+, Na^+-free isotonic sucrose solution for three hours (Figure 1.).

The Na^+ content of the intact lymphocytes increased linearly during the three hour incubation period in Hanks' solution up to about 1.7 times of the initial value. The cells which were transferred to sucrose solution after the second hour in Hanks' lost more than 200

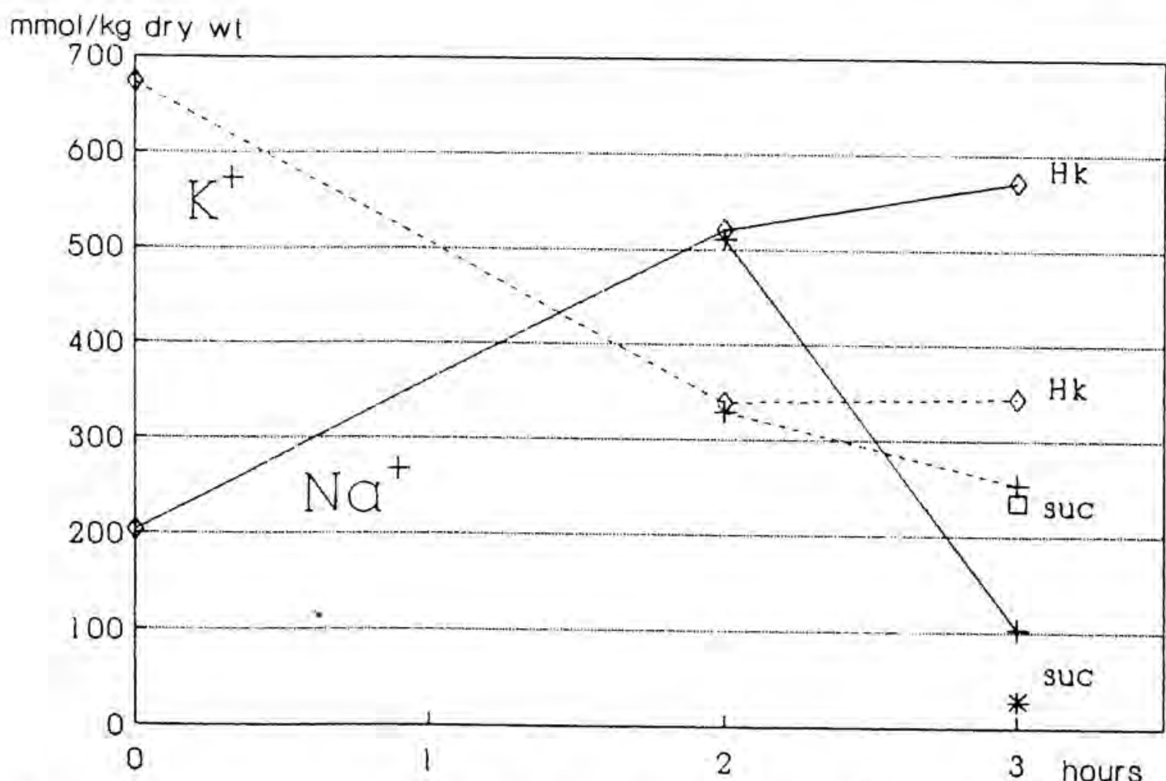


FIGURE 2. The release of potassium and sodium from permeabilized rat lymphocytes. (Symbols are the same as in Figure 1).

mmol/g dry wt. Na^+. The final Na^+ concentration in cells which were incubated in sucrose solution for these hours went even lower (35.18 mmol/g dry wt.). Nevertheless, this value is comparable to what was found after transferring the cells from Hanks' solution to sucrose solution for one hour (79.73 mmol/kg dry wt.) (Figure 1).

Permeabilized cells

Permeabilized rat thymus lymphocytes (homogenized in sucrose) lost half of their K^+ content during the preparation and the first two hours of incubation in the Hanks' solution (Figure 2). Additional incubation in Hanks' solution for another hour did not result in further loss of K^+ from these homogenized cells. Transferring them at two hours of incubation in Hanks' solution to isotonic sucrose solution for one hour caused an additional decrease in the K^+ content of about 85 mmol/kg dry wt. Interestingly, those permeabilized cells which were incubated continuously in sucrose solution for three hours contained just as much K^+ as the permeabilized cells incubated for two hours in Hanks' then transferred to sucrose solution for one hour only (Figure 2).

The Na^+ content of the permeabilized cells increased similarly to the intact cells but this increase was much pronounced. Three hours of incubation of the homogenized cells in Hanks' solution seemed to be almost sufficient time to reach an equilibrium with the incubation medium (Table I). Transferring the homogenized cells to the K^+, Na^+-free sucrose solution led to a massive efflux of Na^+ from the cells. However, even lower Na^+ concentration was measured in cells incubated continuously in K^+, Na^+-free sucrose solution for three hours after permeabilization. It is worth noting that the levels of Na^+ are quite similar in the

Table I. Results of Na^+ and K^+ measurements calculated on mmol/kg H_2O basis and of water contents of the cells.

	n	$[K^+]$	$[Na^+]$	$\%H_2O$
		mmol/l		
Intact tissues	10	182.21 ± 6.26	54.87 ± 13.63	78.71 ± 1.37
Intact cells				
Hanks' 2h	4	170.03 ± 11.87	74.06 ± 4.89	79.52 ± 0.38
Hanks' 3h	4	157.87 ± 16.42	81.05 ± 9.56	80.75 ± 0.97
Hanks' 2h + sucrose 1h	4	171.21 ± 8.69	22.62 ± 5.01	77.90 ± 0.56
Sucrose 3h	4	194.02 ± 16.15	11.33 ± 5.27	75.64 ± 0.86
Homogenized cells				
Hanks' 2h	4	87.81 ± 16.23	134.57 ± 5.92	79.45 ± 0.57
Hanks' 3h	4	84.22 ± 14.55	139.62 ± 7.65	80.37 ± 0.37
Hanks' 2h + sucrose 1h	4	56.73 ± 5.24	22.98 ± 1.88	81.76 ± 0.48
Sucrose 3h	4	63.15 ± 7.16	7.52 ± 1.03	78.79 ± 1.06

permeabilized and in the intact (unhomogenized) cells at the end of the three hour incubation in isotonic sucrose solution (Figures 1 and 2).

Electron microscopy

To ensure the effectiveness of permeabilization, the electronmicroscopic morphology of the homogenized lymphocytes was studied. Figure 3 shows that the lymphocytes did not loose the cytoplasm but the cell surface is damaged in different degrees. At certain parts remnants of the cell membrane are visible but no continuous membrane structures can be seen. Thorough examination of a few hundred cells under the electron micrographs revealed no intact cells.

Discussion

Isolation of calf thymus nuclei was first worked out by Allfrey and his coworkers (1, 2). This method preserved most of the nuclear proteins and enzymes and the nuclei were able to execute several metabolic and synthetic processes (4, 5, 6, 7). However, other authors were not convinced about the purity of the isolated nuclei and demonstrated significant amount of remaining cytoplasm (8, 9). From a different point of view lymphocytes processed by the Allfrey method can definitely be considered as membrane-disrupted cells: several electron-microscopic studies as well as our micrographs demonstrated this observa-

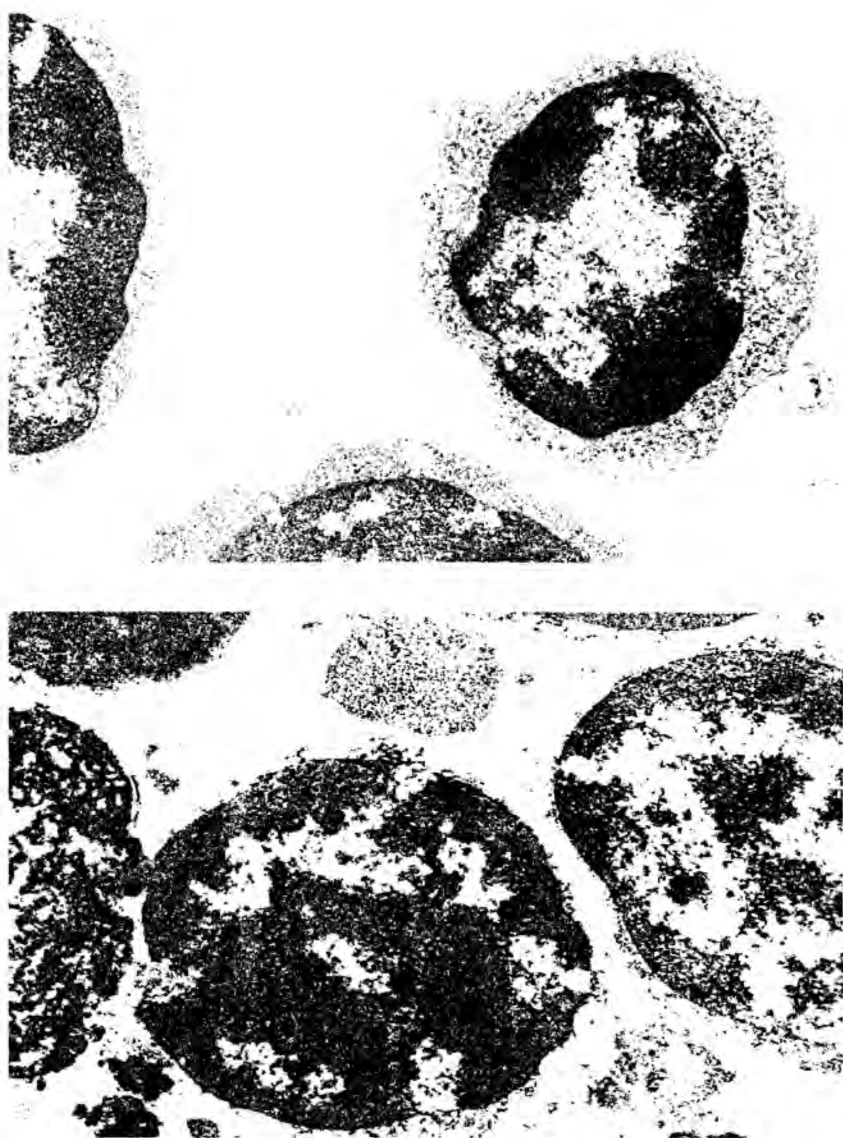


FIGURE 3. Electron micrograph of intact (suspended) (*top*) and homogenized (*bottom*) rat thymus lymphocytes (magnification: 22,000x). In the homogenized sample the nuclei are more or less contaminated with cytoplasm but no continuous plasma membrane structures can be seen.

tion (8, 9). The success in getting relatively clean thymus nuclei will depend on several factors; additionally lymphocytes of another species probably will show a different fragility as well. In the case of rat thymus and under the conditions we used the permeabilization was perfect, i.e. the membrane damage in consequence of homogenization could be observed. With this statement we come to a basic and unresolved problem of cell physiology, namely what parameters or features define a cell permeable (10, 11)? (On the other hand our permeabilized lymphocytes could not be considered as clean nuclei because cytoplasmic debris always was found around the nuclei.)

In our study we followed the movement of the monovalent K^+ and Na^+ ions in different ionic environments in intact and "sucrose-permeabilized" lymphocytes. Comparing the influx of Na^+ in intact and sucrose permeabilized lymphocytes we observed a continuous increase in both cases but at three hours the homogenized cells equilibrated with the Hanks' solution. Changing the ionic medium to the K^+, Na^+-free sucrose solution caused the efflux of Na^+ in cells with or without homogenization. A minor portion of Na^+ still remained inside the cells even when the cells were incubated in the sucrose solution for three hours. As the amount of the remaining Na^+ is very similar in the intact and permeabilized cells (35.82 and 27.93 mmol/kg dry wt., respectively) we are prone to believe that these ions are bound to certain cellular structures (12, 13, 14).

In contrast to Na^+, K^+ showed a much more restricted movement in both intact and permeabilized cells. In intact cells about 90% of K^+ did not leave the cells following incubation in the isotonic sucrose solution for three hours or for one hour after transferring them from the Hanks' solution. Cells lost half of their K^+ content through the homogenization but the remaining half was constant in the Hanks' solution. If these cells were incubated in sucrose solution an additional 15% (of the total) of K^+ was lost. This change was also independent of the time of incubation in the sucrose solution.

The maintenance of the intracellular ionic milieu is generally believed to be the function of the cell membrane and different ion pumps situated in the cell membrane; the one responsible for K^+-Na^+ balance is hypothesized to be the Na^+, K^+-ATPase (15, 16, 17, 18). Along with the membrane-pump hypothesis K^+ and Na^+ are considered to be freely dissolved in the cytosol. According to this, one would expect a quick equilibration of the Na^+ and K^+ after the cell membrane is disrupted and after the sodium pump is inhibited (e.g., by incubating the cells in a K^+, Na^+-free solution). However, our results are not in accordance with this prediction of the membrane-pump theory for the following reasons: 1. The sucrose-permeabilized cells could maintain their K^+ content in Hanks' solution and in K^+, Na^+-free sucrose solution for hours. 2. At the same time Na^+ moved freely in these cells (leaked and/or equilibrated). 3. The "leakage" of Na^+ or K^+ from intact vs. permeabilized (homogenized) cells are comparable.

It was demonstrated by several authors that the intercellular K^+ is retained intracellularly in lymphocytes and in other tissues even though the cell membrane is disrupted and/or the Na^+,K^+-ATPase is not functional (14, 19, 20, 21, 22, 23, 24, 25, 26). The best explanation of their and our observations is that the intercellular K^+ is not freely diffusable. This is exactly predicted by Ling's association-induction hypothesis which makes intercellular proteins responsible for K^+ accumulation (27).

This work was supported by the Hungarian Academy of Sciences, grant #OTKA 83.

References

1. Allfrey, V.G. (1954) *Proc. Natl. Acad. Sci. USA 40*:881.
2. Allfrey, V.G., Littau, V.C. and Mirsky, A.E. (1964) *J. Cell. Biol. 21*:213.
3. Paul, J. (1965) In: *Cell and Tissue Culture*. E. & S. Livingstone Ltd., 3rd ed., p. 83.
4. McEwen, B.S., Allfrey, V.G. and Mirsky, A.E. (1963) *J. Biol. Chem. 238*:758.
5. Allfrey, V.G., Hopkins, J.H., Frenster, J.H. and Mirsky, A.E. (1960) *Annal. N.Y. Acad. Sci. 88*:722.
6. Roof, B.S. and Aub, J.C. (1960) *Canc. Res. 20*:1426.

7. Stern, H. and Mirsky, A.E. (1953) *J. Gen. Physiol. 37*:177.
8. Komada, R.M. and Tedeshi, H. (1963) *J. Cell Biol. 18*:541.
9. Allfrey, V.G. and Mirsky, A.E. (1955) *Science, 121*:879.
10. Hersey, S.J. and Perez, A. (1990) *Annu. Rev. Physiol. 52*:345.
11. Gomperts, B.D. and Fernandez, J.M. (1985) *TIBS 10*:414.
12. von Zglinicki, T., Ziervogel, H. and Bimmler, M. (1989) *Scanning Microsc. 3*:1231.
13. Negendank, W. (1984) *Physiol. Chem. Phys. & Med. NMR 16*:3.
14. Itoh, S. and Schwartz, I.L. (1957) *Am. J. Physiol. 188*:490.
15. Darnell, J., Lodish, H. and Baltimore, D. (1986) In: *Molecular Cell Biology*. Scientific American Books Inc., 1st ed. pp. 625–632.
16. Skou, J.C. (1988) *Methods in Enzymol. 156*:1.
17. Skou, J.C. (1990) *FEBS Lett. 268*:314.
18. Cohen, B.J. and Lechene, C. (1989) *Biol. Cell. 66*:191.
19. Ling, G.N. (1990) *Scanning Microsc. 4*:737.
20. Negendank, W. (1989) *Scanning Microsc. 3*:865.
21. Cameron, I.L., Hardman, W.E., Hunter, K.E., Haskin, C., Smith, N.K.R. and Fullerton, G.D. (1990) *Scanning Microsc. 4*:89.
22. Kellermayer, M., Rouse, D., Gyorkey, F. and Hazlewood, C.F. (1984) *Physiol. Chem. Phys. & Med. NMR 16*:503.
23. Kellermayer, M., Ludany, A., Jobst, K., Szücs, G., Trombitas, K. and Hazlewood, C.F. (1986) *Proc. Natl. Acad. Sci. USA 83*:1011.
24. Miseta, A., Kellermayer, M., Ludany, A., Cameron, I.L. and Hazlewood, C.F. (1991) *J. Cell. Physiol. 146*:394.
25. Hazlewood, C.F. and Kellermayer, M. (1990) *Scanning Microsc. 4*:457.
26. Edelmann, L. (1989) *Scanning Microsc. 4*:1219.
27. Ling, G.N. (1984) In: *The Search of the Physical Basis of Life*, Plenum Press, New York.

Received February 13, 1992;
accepted March 13, 1992.

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

MIKLÓS KELLERMAYER,* ANDREA LUDÁNY, ATTILA MISETA, TAMÁS KÖSZEGI, GÁBOR BERTA, PÉTER BOGNER, CARLTON F. HAZLEWOOD, IVAN L. CAMERON, AND DENYS N. WHEATLEY

Department of Clinical Chemistry, University Medical School, Pécs, Ifjúság u. 13. H-7643 Hungary (M.K., A.L., A.M., T.K., G.B., P.B.); Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, Texas 77030 (C.F.H.); Department of Cellular and Structural Biology, The University of Texas, Health Science Center at San Antonio, San Antonio, Texas 78284 (I.L.C.); Department of Pathology, University of Aberdeen, Foresterhill, Aberdeen, AB9 2ZD, Scotland (D.N.W.)

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).
© 1994 Wiley-Liss, Inc.

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

© 1994 WILEY-LISS, INC.

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

*To whom reprint requests/correspondence should be addressed.

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 8 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.

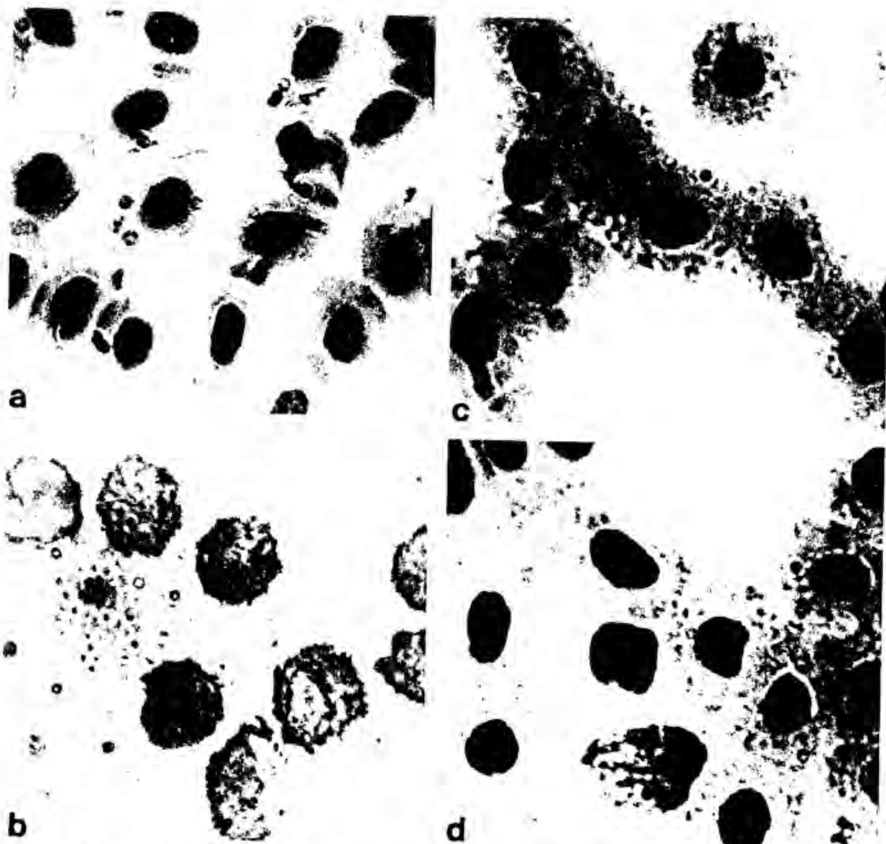


Fig. 1. Light micrographs of chicken erythrocytes incubated in the absence and in the presence of nonionic detergents. **a:** Control, no detergent. **b:** Erythrocytes exposed to 0.2% Triton X-100 for 2 min. **c:** Erythrocytes exposed to 0.2% Brij 58 for 2 min. **d:** Erythrocytes exposed to 0.2% Brij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

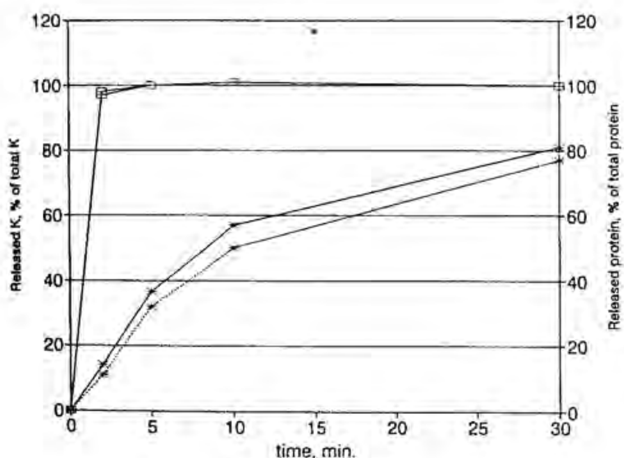


Fig. 3. Detergent-induced release of potassium (continuous line) and proteins (dashed line) from chicken erythrocytes exposed to 0.2% Triton X-100 (open squares) or 0.2% Brij 58 detergents (stars).

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).

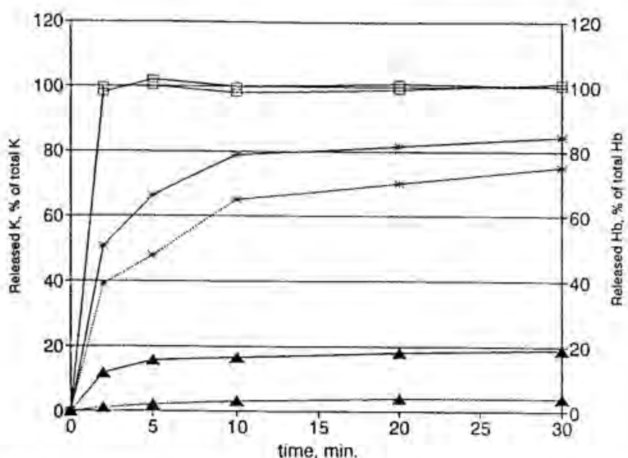


Fig. 4. Detergent-induced release of potassium (continuous line) and proteins (dashed line) from chicken erythrocytes exposed to 1.9 mmol/l Brij 35 (triangles), Brij 58 (stars), and Brij 56 (open squares).

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

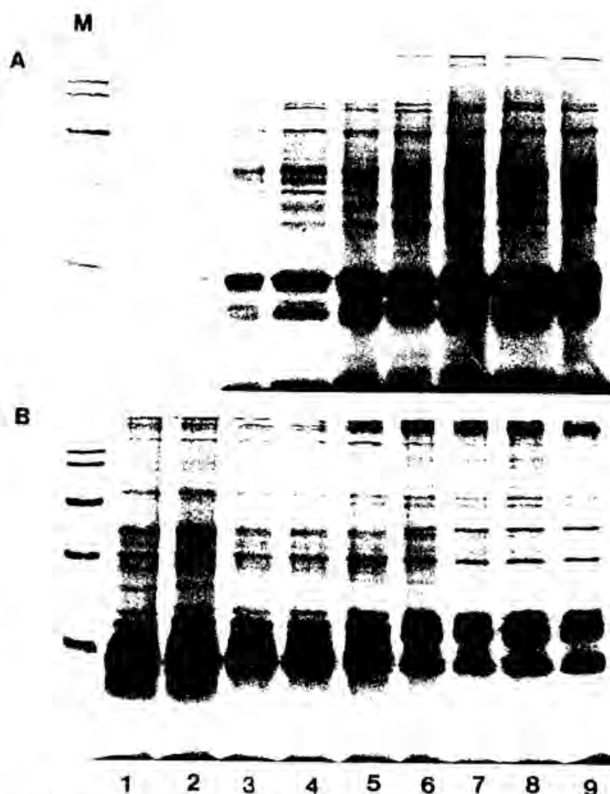


Fig. 5. SDS-PAGE electrophoretograms of proteins separated from control and detergent treated chicken erythrocytes. Proteins were stained with Coomassie Blue. **A:** Supernatant fractions. **B:** Control and detergent-resistant pellets. **Lane 1:** Control, no detergent. **Lanes 2–5:** Brij 58 treatments 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-

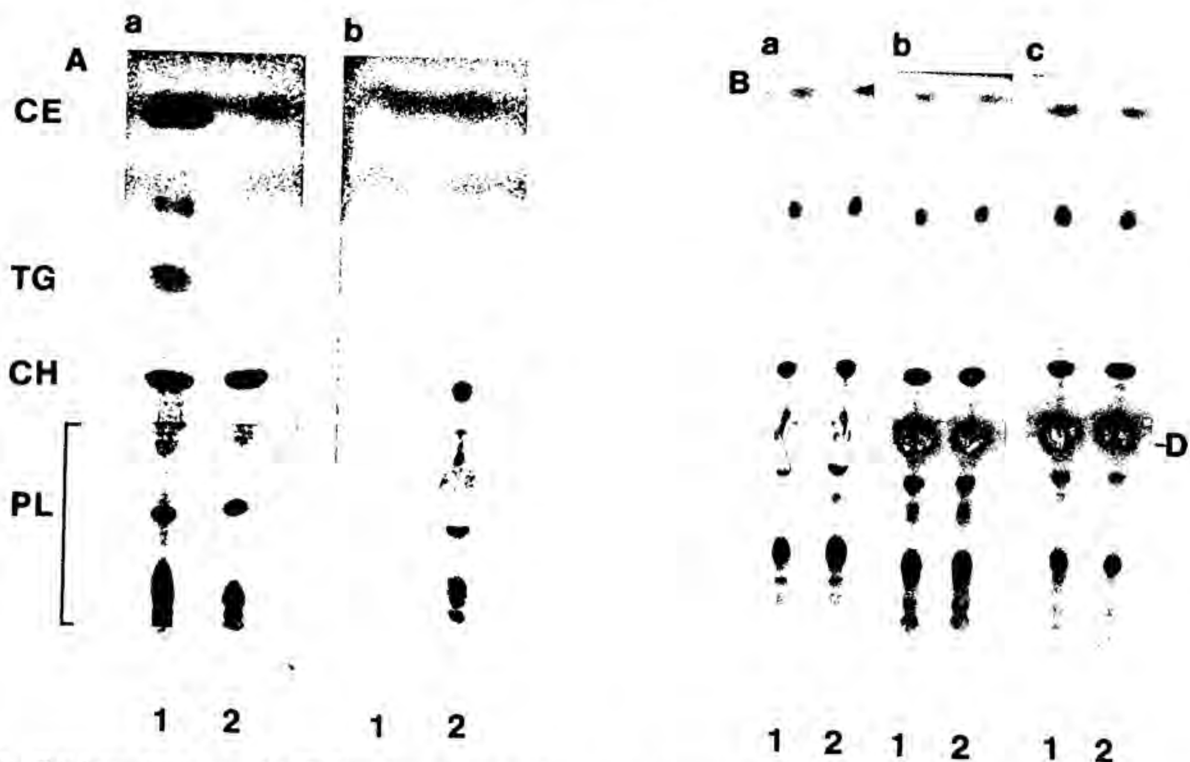


Fig. 6. Thin-layer chromatographic (TLC) pattern of total lipid in control chicken erythrocytes and lipids mobilized by Brij 58 detergent. **A: a/1:** Whole chicken blood. **a/2:** Washed erythrocytes (control erythrocytes). **b/1:** Supernatant fraction of control cells (no detergent). **b/2:** Supernatant 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 (Kőszegi et al., 1987) and human erythrocytes (Kőszegi 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.

LITERATURE CITED

Cameron, I.L., Hunter, K.E., Smith, N.K.R., Hazlewood, C.F., Ludány, A., and Kellermayer, M. (1988) Role of plasma membrane and of cytomatrix in maintenance of intracellular to extracellular ion gradients in chicken erythrocytes. J. Cell. Physiol., *137*:299–304.

Cameron, I.L., Cox, L.A., Liu, X.R., and Fullerton, G.D. (1991) Maintenance and mobility of hemoglobin and water within the human erythrocyte after detergent disruption of the plasma membrane. J. Cell. Physiol., *149*:365–374.

Cameron, I.L., Hardmann, W.E., Smith, N.K.R., Fullerton, G.D., and Miseta, A. (1993) Changes in the concentration of ions during senescence of the human erythrocyte. Cell Biol. Int., *17*:93–98.

Carroll, R.C., Butler, R.G., Morris, P.A., and Gerrand, J.M. (1982) Separable assembly of platelets pseudopodal and contractile cytoskeletons. Cell, *30*:385–393.

Chedid, A., Haux, P., and Natelson, S. (1972) Use of thin-layer chromatography on silica gel for serum lipid fractionation and measurement in the routine clinical laboratory. Clin. Chem., *18*:384–390.

Clegg, J.S. (1991) Metabolic organization and the ultrastructure of animal cells. Biochem. Soc. Trans., *19*:986–991.

Edelmann, L. (1988) The cell water problem posed by electron microscopic studies of ion binding in muscle. Scanning Microsc., *2*:851–865.

Folch, J., Lees, M., and Sloan-Stanley, G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., *226*:497–509.

Freedman, F.C. (1976) Partial restoration of sodium and potassium gradients by human erythrocyte membranes. Biochim. Biophys. Acta, *455*:989.

Hazlewood, C.F. (1979) A view of the significance and understanding of the physical properties of cell water. In: Cell Associated Water. W. Drost-Hansen and J.S. Clegg, eds. Academic Press, New York, pp. 165–259.

Hazlewood, C.F., and Kellermayer, M. (1988) Ion and water retention by permeabilized cells. Scanning Microsc., *2*:267–273.

Kellermayer, M. (1991) ATP-dependent co-compartmentation of proteins and K^+ within the living cell. In: The Physical Aspect of the Living Cell. J. Tigyi, M. Kellermayer, and C.F. Hazlewood, eds. Academic Press, Budapest. pp. 165–192.

Kellermayer, M., and Hazlewood, C.F. (1979) Dynamic inorganic ion-protein interactions in structural organization of DNA of living cell nuclei. Cancer Biochem. Biophys., *3*:181–188.

Kellermayer, M., Rouse, D., Györkey, F., and Hazlewood, C.F. (1984) Potassium retention in membraneless thymus nuclei. Physiol. Chem. Phys. Med. NMR, *16*:503–511.

Kellermayer, M., Ludány, A., Jobst, K., Szücs, Gy., Trombitás, K., and Hazlewood, C.F. (1986) Cocompartmentation of protein and K^+ within the living cell. Proc. Natl. Acad. Sci. U.S.A., *83*:1011–1015.

Kőszegi, T., Kellermayer, M., Berényi, E., Jobst, K., and Hazlewood, C.F. (1987) The bulk of ATP is associated to proteins in the living cell: A release kinetics study. Phys. Chem. Phys. Med. NMR, *19*:143–146.

Kőszegi, T., Kellermayer, M., Kövecs, F., and Jobst, K. (1988) Bioluminescent monitoring of ATP release from human red blood cells treated with nonionic detergent. J. Clin. Chem. Clin. Biochem., *28*:599–604.

Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature, *227*:680–685.

Ling, G.N. (1978) Maintenance of low sodium and high potassium levels in resting muscle cells. J. Physiol., *280*:105–123.

Ling, G.N. (1984) In Search of the Physical Basis of Life. Plenum Press, New York, pp. 227–268.

Ling, G.N. (1992) A Revolution in the Physiology of the Living Cell. Krieger Publishing Co., Florida, pp. 39–65.

Ling, G.N., and Cope, F.W. (1969) Potassium ions: Is the bulk of intracellular K absorbed? Science, *163*:1335–1336.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with Folin phenol reagent. J. Biol. Chem., *193*:265–275.

Miseta, A., Kellermayer, M., Ludány, A., Cameron, I.L., and Hazlewood, C.F. (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. J. Cell. Physiol., *146*:394–398.

Post, R.L. (1989) Seeds of sodium, potassium ATPase. Annu. Rev. Physiol., *51*:1–15.

Ridsdale, J.A., and Clegg, J.S. (1991) Evidence for the cooperativity of protein dissolution in Brij 58 permeabilized L929 cells. J. Cell. Physiol., *147*:242–247.

Schliwa, M., van Blerkom, J., and Porter, K.R. (1981) Stabilization of the cytoplasmic ground substance in detergent opened cells and a structural and biochemical analysis of its comparison. Proc. Natl. Acad. Sci. U.S.A., *78*:4329–4333.

Slinde, E., and Flatmark, T. (1976) Effect of the hydrophylelipophile balance of non-ionic detergents (Triton X-series) on the solubilization of biological membranes and their integral *b*-type cytochromes. Biochim. Biophys. Acta, *455*:796–805.

Tompkins, R.K., and King, W. (1974) Investigations of the enterobiliary metabolism of lecithin. Surgery, *75*:243–252.

Traub, P., Perides, G., Schimmel, H., and Scherbarth, A. (1986) Interaction in vitro of nonepithelial intermediate filament proteins with total cellular lipids, individual phospholipids and a phospholipid mixture. J. Biol. Chem., *262*:13742–13748.

Umbreit, J.N., and Strominger, J.L. (1973) Relation of detergent HLB number to solubilization and stabilization of D-alanine carboxypeptidase from Bacillus subtilis membranes. Proc. Natl. Acad. Sci. U.S.A., *70*:2997–3001.

Scanning Microscopy, Vol. 3, No. 4, 1989 (Pages 1241-1245)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/89$3.00+.00

RELEASE OF HEMOGLOBIN AND POTASSIUM FROM HUMAN RED BLOOD CELLS TREATED WITH TRITON X-100 UNDER THE CRITICAL MICELLAR CONCENTRATION

P. Bogner,* M. Kellermayer, and C.F. Hazlewood+

Department of Clinical Chemistry, University Medical School, Pecs, Hungary

+Department of Physiology and Molecular Biophysics,
Baylor College of Medicine, Houston, TX, USA

(Received for publication June 17, 1989, and in revised form December 06, 1989)

Abstract

The action of detergents is thought to be connected primarily with micelle formation. However, detergent monomers can also effect biological systems. It was found in this study that human red blood cells can be disintegrated with Triton X-100 non-ionic detergent at a concentration of 0.007%, lower than the critical micellar concentration (CMC). The time dependent release of hemoglobin and potassium was detected at 37°C and both were sigmoid in character. Although potassium was released faster than hemoglobin, a cooperative relationship between potassium and hemoglobin within the intact red blood cell is suggested by this observation.

Key words: Hemoglobin, Potassium, Red Blood Cells, Triton X-100.

*Address for correspondence:
Peter Bogner,
National Institutes of Health,
Building 37, Room 5D-12
Bethesda, MD 20892

Phone Number: (301) 496-4116

Introduction

The use of non-ionic detergents in cell permeabilization experiments has become common in the last few years [10]. Their ability to remove membrane lipids and proteins (i.e. to permeabilize) is generally attributed to micelle formation which occurs only above the critical micellar concentration (CMC) [6, 11]. However, below this concentration, detergent monomers can attach to biological membranes which might influence the local molecular organization [11, 13, 28].

Triton X-100 is one of the most widely used non-ionic detergents. Its CMC is 0.24mM (0.015%) [6] and is commonly used far above this concentration (0.1-0.5%) which effectively mobilizes not only the surface-localized structures but also the majority of internal proteins and lipids [14, 19, 25]. The remaining structures are referred to as the detergent resistant cytoskeleton [22]. In the case of human red blood cells (RBC) the detergent resistant framework comprises only a few percent of the total protein content [4]. The majority of RBC proteins can easily be removed, being almost equal to the hemoglobin content.

In this paper we report a sequential decomposition of human red blood cells which can be achieved at a concentration of Triton X-100 below the critical micellar concentration (i.e. 0.007% and 37°C). In this case the time required for the release of the total amount of hemoglobin is about 2 hours as opposed to seconds for concentrations above CMC. The release of hemoglobin and potassium may both be described by sigmoid shape curves, suggesting cooperative processes with potassium release being the faster of the two. These release kinetics are consistent with the idea that hemoglobin and potassium are associated to structures within the intact red blood cells [7]. Thus, an intricate interaction between hemoglobin, potassium and the detergent-resistant frame (ghost) within the red cells is suspected. The possible co-compartmentalization of these major components will be the major subject of this paper.

Materials and Methods

Purification of RBC: Blood samples were collected from healthy adult patients of both sexes. Clotting was inhibited with heparin. All chemicals were obtained from Reanal (Hungary, Budapest) at analytical grade. The freshly drawn samples were processed within an hour. Plasma was separated by

centrifugation for 10 minutes at 1000g (Sorvall RC-5, rotor HB-4). The plasma was drawn off and the cells were washed once with buffered physiological salt solution (PSS) consisting of 145 mM NaCl and 10 mM Tris-HCl pH 7.4. Cells were pelleted at 1000g for 10 minutes.

Experimental procedure: 100 microliter RBC, containing about 10^9 cells, were incubated in 5 ml of medium (PSS) at 37°C. The incubation medium contained Triton X-100 at a final concentration of 0.007% in PSS. In the case of controls no detergent was added. The incubation consisted of 15 minute cycles of 10 minutes of incubation plus 5 minutes of centrifugation at 2000g in a Hettich EBA 3S table centrifuge. After each pelleting the supernatant was removed carefully and the cells were resuspended in fresh medium. The incubation was continued until the supernatant was no longer colored, i.e., no more hemoglobin was released. The supernatants were collected separately. At the end of incubation the remaining pellet was treated with 1% Triton X-100 to liberate the remaining detergent-mobile proteins.

Determination of hemoglobin and potassium: The hemoglobin was determined photometrically at 542 nm. Potassium was measured by flame photometry with a digital flame photometer (OMSZOV, Hungary).

Results

Human red blood cells incubated in a physiological salt solution maintain their integrity, as far as their hemoglobin and potassium contents are concerned, for at least two hours. Even after repeated centrifugation the release of hemoglobin and potassium from the untreated (control) red blood cells did not exceed 6-8% up to the longest incubation time (Fig. 1.). On the other hand, red blood cells treated with 0.007% Triton X-100 in the same solution lost both potassium and hemoglobin, sequentially. The release kinetics of potassium and hemoglobin are described by curves of similar characteristics. In the case of hemoglobin the 50% release occurs at 75 minutes while half of the potassium of red cells was lost before 60 minutes of incubation. The two curves converge at 120 minutes where the release of both components is essentially 100% (Figure 1). The points in Figure 1 are the means of 8 determinations.

If red blood cells were incubated in the detergent containing solution without changing the incubation medium for two hours, not more than 10% of hemoglobin and 40% of potassium ions was lost (data not shown). When the concentration of Triton X-100 was raised above the CMC to 0.1% the release of hemoglobin and potassium was achieved within seconds.

Discussion

We have investigated the release kinetics of hemoglobin and potassium from human red blood cells exposed to Triton X-100 non-ionic detergent under the critical micellar concentration at 37°C. The release kinetics of both hemoglobin and potassium from red blood cells treated with Triton X-100 at [illegible] concentration showed a sigmoid character; however, there was a slight time shift with a half-time of release of less than 60 and more than 75 minutes for potassium and hemoglobin, respectively. The time required for a complete release of hemoglobin and potassium under these conditions was about 120 minutes.

In preliminary experiments, when ATP release was followed with the chemiluminescence method of Koszegi [16], we found that the release of intracellular ATP coincided with hemoglobin release (unpublished observation). The so-called hemolytic activity of non-ionic detergents is well known and used in experimental and laboratory practice [1, 3, 16]. In the case of Triton X-100, the commonly used concentration is between 0.1-0.5%; this concentration range is rather effective in solubilizing different cells, cell organelles and macromolecules. This range is far above the CMC and there is a good agreement that the detergent action is connected to micelle formation [19].

Although it has been well documented that proteins and lipids can interact with detergent molecules [5, 9, 18, 19, 27], the action of detergents under the CMC is more uncertain [11]. In a more complex system, like the living cell, the interaction between single detergent molecules and structures on the cell surface and interior are multiple. Thus, it is not possible to determine the primary site of cell decomposition or to fully know the mechanism of detergent induced disintegration of cells. However, the periodical change of incubation medium that contained the detergent must result in an increasing amount of "cell-bound" detergent molecules that is responsible for the advancing loss of cell components. If red cells were incubated in detergent containing medium without changing it periodically, the degree of cell decomposition was much lower.

The time-dependence of the release of potassium showed a parallel with the hemoglobin release. Though the prolytic loss of potassium was observed previously by Ponder and others [23, 24], the mechanism of the phenomenon has not been fully understood yet. This observation however, cannot be understood on the basis of a destroyed membrane-barrier function, but the compartmentalization of potassium and hemoglobin would explain the parallel of hemoglobin and potassium release. If so, this supports the idea that within the living cell potassium may be selectively adsorbed to cellular proteins [15, 20:chapter 8].

The observation that the release kinetics may be described by sigmoidal shaped curves is suggestive of cooperative interactions between cellular constituents. The skeletal architecture of red blood cells is established and is highly organized, particularly in the marginal region of the cells where protein interactions are the subject of intensive investigation [2, 8, 12, 21]. Our study shows an overall response of red cells to the disruption of multiple protein (and lipid) interactions which involves membrane skeleton-cytoskeleton and hemoglobin-cytoskeleton connections as well [8, 17, 26].

In conclusion, our experiments provide indirect evidence of a conceivable potassium-hemoglobin compartmentalization. The nature of this interaction though remains to be identified. As ATP is released simultaneously with hemoglobin (preliminary data), one may suppose a direct connection between ATP and hemoglobin which may provide an enhanced stability of protein association.

Acknowledgements

The authors acknowledge the support by the

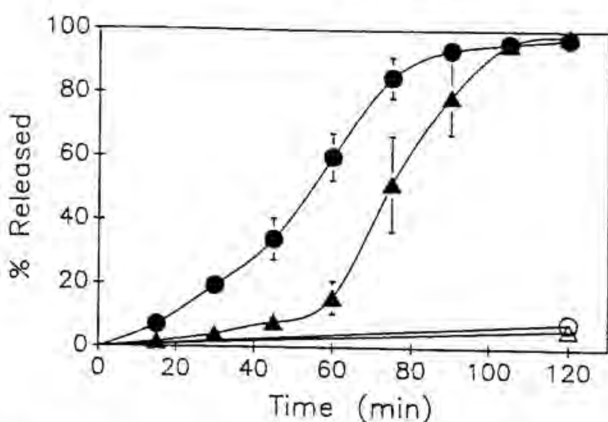


Figure 1. Release of hemoglobin (closed triangles) and potassium (closed circles) from red blood cells treated with Triton X-100 non-ionic detergent at 0.007% concentration at 37°C. The release of hemoglobin (open triangle) and potassium (open circle) from untreated cells is also shown. Each point represents the mean value of 8 separate experiments.

Hungarian Academy of Sciences - National Science Foundation (USA) Joint Grant No. 30.07032/86, OTKA 83, and the Robert A. Welch Foundation. CHF acknowledges a gift given in the memory of Stanley T. Weisner.

References

1. Azaz E, Segal R, Milo-Goldzweig I (1981). Hemolysis caused by polyoxyethylene-derived surfactants. Evidence for peroxide participation. Biochim. Biophys. Acta 646, 444-449.

2. Backman L (1988). Functional or futile phosphorus? Nature 334, 653-654.

3. Balatoni M, Fodor F (1980). Anionos es nem ionos szintetikus detergensek hatasa kulonbozo speciesek vorosvertestjeinek ozmotikus rezisztenciajara - in vitro (The effect of anionic and non-ionic synthetic detergents on the osmotic resistance of red blood cells of different species - in vitro (English Abstr.). Acta Pharm. Hung. 50, 233-237.

4. Bennett V (1985). The membrane skeleton of human erythrocytes and its implications for more complex cells. Annu. Rev. Biochem. 54, 273-304.

5. Bordier C (1981). Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. 256, 1604-1607.

6. Brito RM (1986). Determination of critical micellar concentration of surfactants using fluorescent probe N-phenyl-naphthylamine. Anal. Biochem. 152, 250-255.

7. Cameron IL, Hunter KE, Smith NKR, Hazlewood CF, Ludany A, Kellermayer, M (1988). Role of plasma membrane and of cytomatrix in maintenance of intracellular to extracellular ion gradients in chicken erythrocytes. J. Cell. Physiol. 137, 299-304.

8. Carraway KL, Carraway CAC (1989). Membrane-cytoskeleton interactions in animal cells. Biochim. Biophys. Acta 988, 147-171.

9. Clarke S (1977). The interaction of Triton X-100 with soluble proteins: Possible implication for the transport of proteins across the membranes. Biochem. Biophys. Res. Commun. 79, 46-52.

10. Felix H (1982). Permeabilized cells. Anal. Biochem. 120, 211-234.

11. Helenius A, Simons K (1975). Solubilization of membranes by detergents. Biochim. Biophys. Acta 415, 29-79.

12. Hsu L, Morrison M (1983). The interaction of human erythrocyte Band 3 with cytoskeletal components. Arch. Biochem. Biophys. 227, 31-38.

13. Isomaa B, Hagerstrand H (1988). Effects of nonionic amphiphiles at sublytic concentrations on the erythrocyte membrane. Cell Biochem. Funct. 6, 183-190.

14. Kellermayer M, Ludany A, Jobst K, Szucs G, Trombitas K, Hazlewood CF (1986). Co-compartmentalization of proteins and K^+ within the living cell. Proc. Natl. Acad. Sci. USA 83, 1011-1015.

15. Kellermayer M, Rouse D, Gyorkey F, Hazlewood CF (1984). Potassium retention in membraneless thymus nuclei. Physiol. Chem. Phys. Med. NMR 16, 503-511.

16. Koszegi T (1988). Rapid bioluminescent measurement of human erythrocyte ATP content. Clin. Chem. 34, 2578.

17. Lebbar I, Stetzkowski-Marden F, Mauffret O, Cassoly R (1987). Interaction of actin and tubulin with human deoxyhemoglobin. Their possible occurrence within erythrocytes. Eur. J. Biochem. 170, 273-277.

18. LeMaire M, Kwee S, Andersen JP, Moller JV (1983). Mode of interaction of polyethyleneglycol detergents with membrane proteins. Eur. J. Biochem. 129, 525-532.

19. Lichtenberg D, Robson RJ, Dennis EA (1983). Solubilization of phospholipids by detergents. Structural and kinetic aspects. Biochim. Biophys. Acta 737, 285-304.

20. Ling GN (1984). In search of the physical basis of life. Plenum Press, New York.

21. Nermut MV (1981). Visualization of membrane skeleton in human erythrocytes by freeze etching. Eur. J. Cell Biol. 25, 265-271.

22. Osborn M, Weber K (1977). The detergent-resistant cytoskeleton of tissue culture cell includes the nucleus and the microfilament bundles. Exp. Cell Res. 106, 339-349.

23. Ponder E (1947). The prolytic loss of K^+ from human red cells. J. Gen. Physiol. 30, 235-246.

24. Ponder E (1947). K^+-Na^+ exchange accompanying the prolytic loss of K^+ from human red cells. J. Gen. Physiol. 30, 379-387.

25. Schliwa M, Euteneuer U, Porter KR (1987). Release of enzymes of intermediary metabolism from permeabilized cells: Evidence in support of a structural organization of cytoplasmic matrix. Eur. J. Cell Biol. 44, 214-218.

26. Shaklai N, Yguerabide J, Ranney HM (1977). Interaction of hemoglobin with red blood cell membranes as shown by a fluorescent chromophore. Biochemistry, 16, 5585-5592.

27. Sukow WW, Bailey J (1981). Characterization of the binding of Triton X-100 to equine and rabbit serum albumin. Physiol. Chem. Phys. 13, 455-459.

28. Tragner D, Csordas A (1987). Biphasic interaction of Triton detergents with erythrocyte membrane. Biochem. J. 244, 605-609.

Discussion with Reviewers

W. Negendank: How do you know that the sigmoid release kinetics of potassium and hemoglobin are characteristics of most of the individual cells in the preparation, as implicitly assumed in your conclusion. Is it possible that only a few cells are affected at earlier times, and more cells are affected at later times? Do you have any data that might address this issue?

J.S. Clegg: The sigmoidal release of K^+ and hemoglobin (Hb) is interpreted exclusively as cooperative interactions of K^+-Hb and these with other cell structures. Have you considered any other interpretations? Could different sensitivities to Triton of cells within the cell population be a consideration?

Authors: The red cell population is not homogenous, there are older and younger cells and their resistance to hemolytic agents or osmotic insult is not the same. There is no question that this circumstance must be involved in our results as well. According to our light microscopic studies on cells, at different time intervals of treatment, there are hemoglobin-free ghosts and still "intact" cells. This is seen with 15 to 30 minutes but the majority of red cells shows a sequential loss of hemoglobin i.e., their "redness" decreases continuously with time. In a similar type of experiment (described above) electrophoretic analysis was made of the different fractions. These results showed the accumulation of certain proteins in certain time periods, which suggests a rather uniform behavior of cell disintegration. Please also refer to Ponder's work (ref. 24) who, in studying the volume changes of red cell, came to a similar conclusion.

W. Negendank: How do you know that the release of potassium and hemoglobin (Figure 1) is not caused by an effect of the detergent on plasma membrane, with small "holes" permitting potassium release earlier, and larger "holes" permitting hemoglobin release later?

I.L. Cameron: It appears to me that the observed sigmoidal shaped release kinetic curves of K^+ and of hemoglobin obtained from the Triton X-100 treated erythrocytes has an alternate explanation to the cytomatrix binding one that you offer in your report. How, for example, can you rule out the possibility that the Triton X-100 treatment slowly makes small "holes and pores" in the plasma membrane? These holes would allow initial escape of the smaller K^+ but as these holes eventually grow larger would allow escape of larger hemoglobin molecules? It seems to me that with this explanation of your data the plasma membrane, not sorption of K^+ or hemoglobin in the cytoplasm, becomes the main mechanism for maintenance of the K^+ and hemoglobin gradients.

Authors: A "hole" big enough for the leakage of hemoglobin is more than suitable for potassium to pass through. It could be possible that initially small "holes" permit only the electrolytes to leave but as the "hole"-size reached a certain magnitude, no distinction according to size can be supposed. In other words, if potassium ions were freely dissolved in the cell, considering the diffusion coefficient of K^+ in solution, one should observe complete equilibrium with the surrounding medium by the time hemoglobin started to leak from the cells.

J.S. Clegg: With regard to Figure 1, and your experimental procedures: you say you collect the supernatants separately but Figure 1 indicates that you add the values of the separate K^+ and Hb measurements. Please clarify.

Authors: At fifteen minute intervals the samples were centrifuged, the supernatants decanted, and the potassium and hemoglobin concentrations were determined. (Knowing the volume, the amount of potassium and hemoglobin released during that fifteen minute segment was determined). Also at each fifteen minute interval, the pelleted cells were resuspended in fresh medium containing the detergent. Thus, at the end of the complete experiment, we were able to determine the percentage of the total at each fifteen minute interval. This procedure was performed on eight separate blood samples; and, the average value (for the eight samples at each fifteen minute interval) is plotted in figure 1.

J.S. Clegg : You refer to preliminary unpublished data on ATP release and suggest that ATP-Hb interactions may be involved. Do you know of any other data that suggest such interactions might occur?

Authors: We are not aware of any studies of potassium adsorption directly to purified hemoglobin. We do wish to mention two sources of information which indicate that ATP is involved in the binding (adsorption) of potassium to hemoglobin in particular, and proteins in general. First, the potassium content of "red blood cell ghosts" appears to be directly proportional to the protein (and hence, hemoglobin) content of the preparation (for a review, see reference 20, pages 128-131). Second, the role of ATP and even its non-hydrolyzable analogues in mediating protein assembly is beginning to be discovered. On one hand, its importance is described in nuclear processes [e.g., Mastrangelo IA, et al. (1989): ATP-dependent assembly of double hexamers of SV40 T antigen at the viral origin of DNA replication. Nature 338, 658-662]; on the other hand, dynamic protein associations between cytoskeletal elements are also dependent on ATP or its non-hydrolyzable analogues [Mandelkow EM, et al. (1988): Dynamics of the microtubule oscillator: role of nucleotides and tubulin-MAP interactions. EMBO J. 7, 357-365].

P.B. Bell: This paper reports that potassium and hemoglobin are released with similar kinetics when red blood cells are treated with Triton X-100 at a concentration below its critical micellar concentration. This result is said: (1) **to suggest** a cooperative process; (2) to support the **possibilty** that potassium **may** be selectrively adsorbed to hemoglobin; and (3) to provide evidence for **possible** hemoglobin-cytoskeletal and hemoglobin-hemoglobin interactions. The emphasis is mine but the words are the authors'. Therefore, as this paper is written, the authors provide jumping-off point for a lot of speculation about possibilities. But could the results not suggest some other explanation? Could the similar behavior of potassium and hemoglobin be merely coincidental?

R.L. Post: The authors note that the release kinetics was sigmoid. They relate this sigmodicity vaguely to "cooperative interaction between cellular constituents", adsorption of potassium to cellular proteins hemoglobin-cytoskeletal interactions, and a multiple protein interaction. No supporting evidence is offered. The kinetics was sigmoid with respect to

time, not with respect to concentration. Sigmodicity with respect to time, not concentration, does not require cooperativity; a precursor-product relationship is sufficient.

Authors: The reviewers' points are well taken. It is correct that we are suggesting that the interaction between potassium and hemoglobin is cooperative. This postulation evolves primarily from a concept and three sets of data. The concept is that all cells come from a unicellular origin--the fertilized egg--thus, the red blood should exhibit phenomena similar to other cells. The data are: (1) the cooperative uptake of potassium has been demonstrated in three smooth muscle types (arterial, intestinal, and uterine), skeletal muscle, and lymphocytes (for a review on this subject, please see chapter 11, pages 319-376, reference 20). (2) It is reported (Ling et al., Physiol. Chem. & Phys. & Med. NMR 16: 381, 1984) that the potassium concentration is directly proportional to the protein concentration of red blood cell ghosts. (3) The selective adsorption of sodium to hemoglobin as well as the selectivity decreasing in the order sodium, lithium, potassium, rubidium, and cesium has been demonstrated (Ling and Zhang, Physiol. Chem. & Phys. & Med. NMR 16: 221, 1984).

Lacking direct evidence for the cooperative uptake of potassium in the red blood cell, we can only agree with the reviewers that other explanations are possible.

Biophysical Journal Volume 69 December 1995 2563–2568

Effect of Non-Lytic Concentrations of Brij Series Detergents on the Metabolism-Independent Ion Permeability Properties of Human Erythrocytes

Attila Miseta,* Péter Bogner,‡ Ágnes Szarka,§ Miklós Kellermayer,* Csaba Galambos,* Denys N. Wheatley,¶ and Ivan L. Cameron‖

*Department of Clinical Chemistry, University Medical School Pécs, Pécs, Ifjuság u. 13. H-7643, Hungary; ‡Diagnostic Center, Pannon University of Agriculture, Faculty of Animal Production, Kaposvár, Dénesmajor 2, H-7400, Hungary; §Department of Biophysics, University Medical School Pécs, Pécs, Szigeti u. 12. H-7642, Hungary; ¶Department of Pathology, University of Aberdeen, Foresterhill, Aberdeen, AB9 2ZD, Scotland; and ‖Department of Cellular and Structural Biology, The University of Texas, Health Science Center at San Antonio, San Antonio, Texas 78284 USA

ABSTRACT Subcritical micellar concentrations (sub-CMC) of Brij-series detergents alter ion movements between human erythrocytes and their environment when metabolism has been slowed down by incubation at zero degrees centigrade. The effect of nonhemolytic concentrations of detergents on the erythrocyte K^+ and Na^+ movements is described. Results indicate a significant difference in monovalent cation movements, depending on the number of hydrophilic polyoxyethylene units (n). There is an increasing loss of K^+ and gain of Na^+ as n increases from 4 to 20. Where $n \geq 21$, ion movements are not significantly different from those found in erythrocytes not exposed to detergents. The carbon chain length of the detergent fatty acid residue (10–18 carbons) appears to be relatively unimportant, but detergents with unsaturated (oleic acid) hydrophobic regions potentiate K^+ release and Na^+ uptake when compared to the corresponding saturated fatty acid (stearic acid). The erythrocyte stabilizing effect of detergents against hypo-osmotic shock correlates well with the increase of monovalent ion traffic and the mobility of membrane lipids revealed by fluorescence anisotropy measurements.

INTRODUCTION

During the past three decades, a large number of non-ionic detergents have become available for the purification of membrane proteins. Among these the polyoxyethylene-octylphenols (NP-40, Triton X-100, and Triton X-114), the polyoxyethylene-sorbitans (Tween series detergents), the polyoxyethylene-alcohols (Lubrol series), and the polyoxyethylene fatty acid ether (Brij series) detergents are particularly popular (Fig. 1). The hydrophile-lipophile balance (HLB) values of the most frequently used members of these detergent groups fall between 12 and 18. Detergents in the low HLB region (HLB, 12–15) tend to be more efficient in extracting intrinsic membrane proteins, whereas those in the higher HLB range are frequently used for the extraction of external membrane proteins (Gennis, 1989). Because the membrane proteins must be transferred into detergent micelles during isolation, detergent concentrations are maintained at or above the critical micellar concentration (CMC). Owing to their practical application, detergent interactions with the lipid and protein components of plasma membranes have been studied in considerable detail (Jacobs et al., 1966; Jørgensen and Skou, 1971; Umbreit and Strominger, 1973; Kirkpatrick and Gordesky, 1974; Slinde and Flatmark, 1976; Gennis, 1989).

The transfer of plasma membrane constituents into detergent micelles is often associated with the apparently simultaneous collapse of cytoplasmic architecture. Milder detergent action removes lipid components and some of the membrane proteins, but the cytoplasmic architecture is maintained for a relatively long period of time (Schliwa et al., 1981, 1987; Kellermayer et al., 1986). Unexpectedly, plasma membrane fenestration does not result in immediate monovalent ion equilibration between the cell and its environment. All cell types treated with Brij 58 maintained high cytoplasmic K^+ and low Na^+ concentrations for much longer periods of time than would theoretically be required for total equilibration of an aqueous compartment of the size of the cell with its environment (Kellermayer et al., 1984, 1986, 1994; Cameron et al., 1988; Miseta et al., 1991). Ion equilibration appeared to be correlated with the collapse of cytoplasmic architecture and the release of cytoplasmic components, as shown by x-ray microprobe data for nucleated chicken erythrocytes (Cameron et al., 1988). Similar results were obtained when the density distribution of Brij 58-treated L929 cells was studied (Ridsdale and Clegg, 1991).

Despite these earlier observations, the ion equilibration process between detergent-treated cells and their environment remains a complex and largely unexplored phenomenon and depends on the detergent properties and on the blood group characteristics of the erythrocytes (Pazos-Sanou and Mata-Segrada, 1985; Rao et al., 1987; Isomaa et al., 1987; Isomaa and Hagerstrand, 1988). In theory ion equilibration in the presence of detergent can proceed in three ways: i) beyond their CMC values, the most frequently

for publication 30 May 1995 and in final form 23 August 1995.

reprint requests to Dr. Attila Miseta, Department of Clinical y, University Medical School Pécs, Pécs, Ifjuság u. 13, H-7643, Tel.: 36-72-324-122, ext. 2310 or 1425; Fax: 36-72-332-256; iseta@apacs.pote.hu.

the Biophysical Society

95/12/2563/06 $2.00

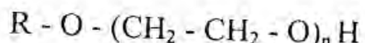
$$R - O - (CH_2 - CH_2 - O)_n H$$

FIGURE 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.

used detergents may cause erythrocyte disintegration, i.e. hemolysis ensues; ii) detergent-mediated opening of the plasma membranes occurs before lysis and presumably leads to the start of accelerated ion traffic between the cell and its environment; and iii) detergent molecules may themselves incorporate into plasma membranes, changing plasma membrane permeability without necessarily removing lipids. This third way is best studied when detergents are applied at nonlytic concentrations (Tragner and Csordás, 1987; Isomaa and Hagerstrand, 1988; Bogner et al., 1989; Bielawski, 1990). In addition, detergents may alter erythrocyte metabolism and/or interfere with membrane proteins involved in ion regulation (Jörgensen and Skou, 1971; Gennis, 1989).

Ways i) and ii) are largely eliminated, if sub-CMC, nonhemolytic concentrations of detergents are used. Although it is known that detergents facilitate K^+ loss and Na^+ uptake in sub-CMC, nonhemolytic concentrations, only a limited number of studies, in part with different aims, have been carried out on the detergent properties, which are involved in this process.

In the present report, we describe the dependency of detergent-mediated ion permeability changes on a) the size of the hydrophilic polyoxyethylene chain, and on b) the nature of the hydrophobic fatty acid residue of Brij series detergents (Fig. 1). Our results indicate that surprisingly small differences in either the hydrophobic or the hydrophilic residue can dramatically change the monovalent cation permeation properties of the erythrocyte. We also demonstrate a significant correlation between erythrocyte membrane permeability changes, the mobility of membrane lipids, and stabilization against osmotic lysis caused by detergent treatment.



MATERIALS AND METHODS

Materials

...inized blood was obtained from healthy volunteers, samples being ...d to wet ice immediately after collection.

...yoxyethylene 2-stearyl ether (Brij 72), polyoxyethylene 4-stearyl ..., polyoxyethylene 10-stearyl ether (Brij 76), polyoxyethylene 20-...l ether (Brij 78), polyoxyethylene 21-stearyl ether (Brij 721), ...ethylene 100-stearyl ether (Brij 700), and polyoxyethylene 10-...er (Brij 96), 1,6-diphenyl-1,3,5,-hexatriene (DPH), and trimethyl-...m DPH (TMA-DPH) were purchased from Sigma (St. Louis, ... All other chemicals were purchased from Reanal (Hungary). Deter...cks (10 mM/L) were prepared in a 50% water, 50% ethanol ... except for Brij 72, which was dissolved in ethanol.

Measurements of erythrocyte K^+, Na^+, and water levels

Blood samples were centrifuged at 3000 × g for 10 min, and the plasma and buffy coat were removed. The erythrocyte pellets were resuspended in ice-cold 0.15 M NaCl solution and centrifuged as described above, and the supernatant was removed. The washing procedure was repeated twice. For experimental purposes, erythrocytes were resuspended to give a hematocrit (Htc) of 4% in ice-cold TBSR solution (10 mM Tris-HCl, pH 7.4, 148 mM NaCl, and 2 mM RbCl). This solution served as the control. In other cases, TBSR was complemented with one of the above-described detergents. The final concentration was usually 40 μM, a concentration at which none of the above-mentioned detergents proved hemolytic. Ten-milliliter 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 were removed. The erythrocytes were transferred into microcentrifuge tubes of known weights and centrifuged at 16,000 × g for 10 min. The supernatants were removed, and residual pellets were weighed before being freeze-dried in a Savant speed vacuum (SC-110) system. Dry weights were subsequently measured, a 0.5 to 1 ml of 1 M HCl was added to each sample, and the sample was then incubated at room temperature on a rocker table for not less than 24 h.

K^+ and Na^+ levels were measured in a flame photometer (Eppendorf EFOX 5070). Actual ion concentrations were calculated after correction for dilution factors and were based on sample weight data. Each data point represents the mean value of five measurements. Owing to variations in the detergent resistance of different blood samples (Pazos-Sanou and Mata-Segrada, 1985), variations of data increased significantly if experiments were repeated with blood samples obtained from different donors. Nevertheless, we repeated each experiment at least three times. Results of representative experiments are presented in this report.

ATP measurements

Erythrocyte ATP levels were measured with a bioluminescent firefly luciferin/luciferase kit obtained from Boehringer, and a Berthold Biolumat LB-9505, as described by Köszegi (1988).

Osmotic resistance investigations

Classic osmotic resistance experiments were carried out on the erythrocytes. A 0.15 M (300 mOsm/L) stock solution of NaCl was prepared and diluted serially with distilled water. The osmolarity of the 15 solutions prepared fell between 270 and 88 mOsm/L as measured by a Wescor 5500 vapor pressure osmometer. The total volume of the individual samples was 4.4 ml. Equimolar sub-CMC concentrations of different Brij series detergents were added to each series respectively. Aliquots (150 μl) of isotonic saline-washed blood samples (Htc 50%) were added to each sample and incubated at 4°C for 2 h. After incubation the samples were centrifuged at 4000 × g for 5 min, and 3-ml supernatants were removed for photometric measurements, which was carried out by a Perkin-Elmer Lambda 2 spectrophotometer at 540 nm.

Erythrocyte ghost preparation and fluorescence emission anisotropy measurements

Erythrocyte ghosts were prepared by the method of Dodge et al. (1962). Ghosts were labeled with DPH or with its cationic derivative TMA-DPH in phosphate-buffered saline at 25°C for 30 min. Various detergent molecules were added in equimolar (20–40 μM) concentrations simultaneously, and the ghosts were shifted to ice for at least 1 h. The protein concentration of ghosts was ~100 μg/ml, and the final concentration of DPH or TMA-DPH was 1 μM. The protein concentration of ghost preparations was measured by Lowry's method (Lowry et al., 1951). Measurements were carried out with a Hitachi MPF-4 spectrofluorimeter equipped with polarization ac-

cessories at 4°C. Wavelengths were 360 nm and 425 nm for excitation and detection, respectively. Fluorescence anisotropy was calculated according to the equation: $r = (I_{vv} - GI_{vh})/(I_{vv} + 2GI_{vh})$, where I_{vv} and I_{vh} are the fluorescence intensities measured with a vertical polarizer, and a vertically or horizontally mounted analyzer, respectively ($G = I_{hv}/I_{hh}$) (Donner and Stoltz, 1985). The fluorescent intensities of dyes were always measured in the presence of detergents, but without ghosts. Results obtained were subtracted from those obtained in the presence of labeled ghosts. For each sample, fluorescence was corrected for the scattering effect of unlabeled ghosts.

RESULTS

K^+ release from detergent-treated human erythrocytes

Human erythrocytes were incubated in the presence of 40 μM of each detergent at a Htc of 4% at 0°C for a maximum of 4 h. None of the detergents induced significant hemolysis at these concentrations (<5% by the end of treatment). Erythrocyte ATP concentrations remained remarkably constant and similar to those of the native blood during these treatments (data not shown). Fig. 2 illustrates results from an experiment with polyoxyethylene-(n)-stearyl ethers where the number (n) of oxyethylene units varied from 4 to 100. The results in Fig. 2, *a* and *b*, indicate that polyoxythcylene-4-stearyl ether had little effect on K^+ release and on Na^+ uptake of erythrocytes, but K^+ release increased with increase of the oxyethylene chain length until n = 20. Particularly striking was the finding that polyoxyethylene-4-stearyl ether induced changes in erythrocyte K^+ and Na^+ concentrations that resembled the changes induced by polyoxyethylene-4-stearyl ether or in control (untreated) erythrocytes. Further increase of n does not cause a significant difference. Similar results were obtained when polyoxythylene-(n)-lauryl ethers were used. Because the experiments were carried out at low temperature, there is no active transport, and therefore rubidium uptake was parallel to Na^+ uptake (data not shown).

Erythrocyte water content remained remarkably constant, being similar to that of normal untreated (control) human erythrocytes (67–69%) after incubation.

In a similar set of experiments, the number of polyoxyethylene residues was kept constant (n = 10). Compared with untreated erythrocytes polyoxyethylene-10-stearyl ether treatment caused a modest acceleration of K^+ release and Na^+ uptake compared with untreated erythrocytes (Fig. 3). The results clearly demonstrate that the chain length of saturated fatty acids had little effect on electrolyte movements (Fig. 3). Likewise, incubation of erythrocytes with stearic (C = 18), cetyl (C = 17), or lauric ethers (C = 12) caused effects similar to that of polyoxyethylene-10-stearyl ether. However, a striking difference in monovalent cation movements occurred where polyoxyethylene (10)-oleyl ether (C = 18) was used. The loss of K^+ and the gain in Na^+ ions was dramatically accelerated.

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

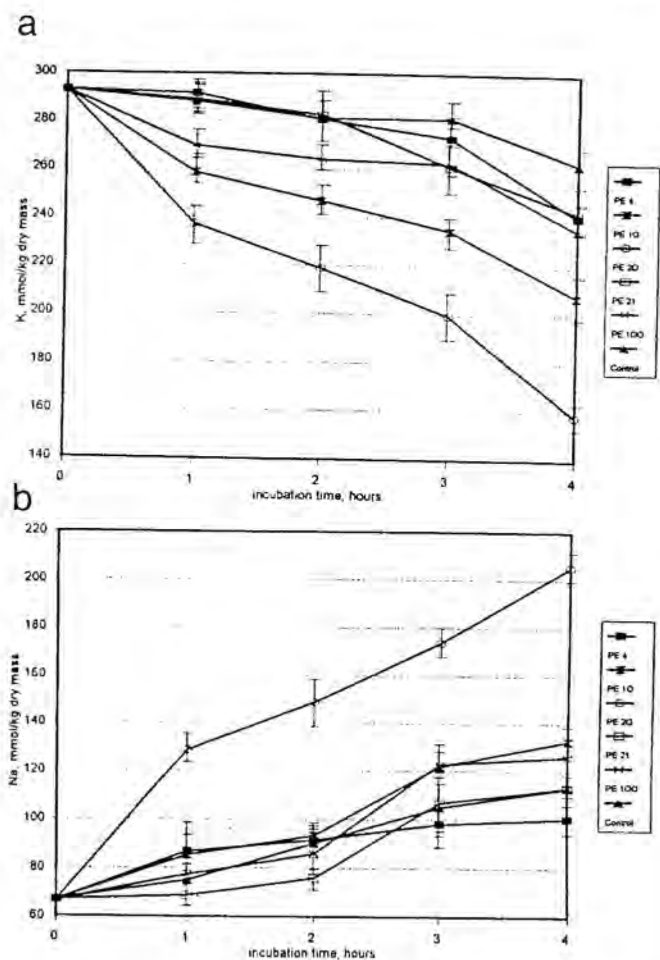


FIGURE 2 Incubation of human erythrocytes in the presence of n-stearyl ethers. Variations in the number of polyoxyethylene residues (n) in stearic-acid-ether type Brij series detergents alter the release of potassium ions from human erythrocytes incubated on wet ice. Equimolar, 40 μM concentrations of detergents were used at an Htc of 4%. Increasing potassium losses were observed where n increased from 4 to 20. A sudden reversal of the effect took place if $n \geq 21$. Complementary changes are seen in erythrocyte Na^+ concentrations (*b*). Each data point represents the mean of five measurements.

The osmotic resistance of detergent-treated erythrocytes

Classic osmotic resistance experiments were carried out with iso-osmotic saline-washed human erythrocytes at 4°C. Solutions (except controls) were complemented with equimolar concentrations of various Brij series detergents. The detergent concentrations were 40 μM. The results (Fig. 4) indicate that the addition of a 4-stearyl ether cause little effect on hemolysis compared to the untreated erythrocytes. The shift of the lysis curve to the right in Fig. 4. suggests that there was protection against osmotic lysis in 10-stearyl ether, and 20-stearyl ether-treated erythrocytes, the latter being the more effective. 21-Stearyl ether also protected erythrocytes against osmotic lysis, but it was a less effective protector against hemolysis than 20- and 10-stearyl ethers, respectively. Although some hemolysis occurred in mildly hypo-osmotic mediums complemented with polyoxyethyl-

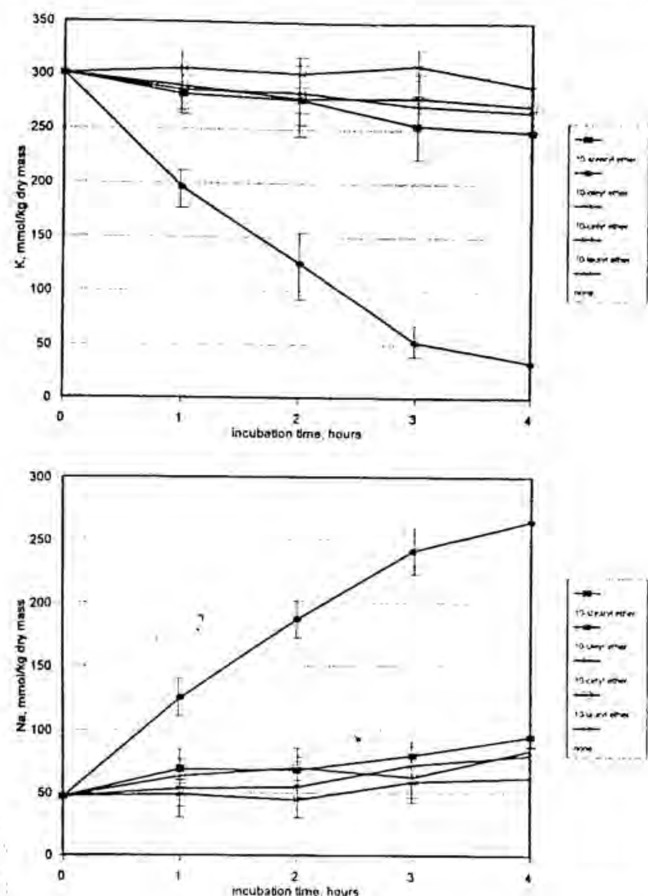


FIGURE 3 Incubation of human erythrocytes in the presence of 10-fatty-acyl ethers. The size of the saturated fatty acid residue did not play a significant role in the loss of potassium ions in various Brij series detergents, if the number of polyoxyethylene repeats was $n = 10$. Equimolar 40 μM concentrations of detergents were used at a Htc of 4%. Nevertheless, the substitution of the saturated stearic acid residue with an unsaturated oleic acid residue resulted in significant acceleration of the equilibration processes. Complementary changes are seen in erythrocyte Na^+ concentrations (*b*). Each data point represents the mean value of five measurements.

ene 10-oleyl ether, it protected the erythrocytes against osmotic lysis better than any of the stearyl ether derivatives. Note especially that the above rank order of polyoxyethylene adduct detergents in protecting erythrocytes against hemolysis is very similar to their effect in accelerating monovalent cation traffic between the erythrocyte and its environment.

...orescence anisotropy in detergent-treated ...throcyte ghosts

...throcyte ghosts were isolated and labeled with DPH or ...-DPH as described in Materials and Methods. Changes ...ed by short-chain ($n = 2$–4) hydrophobic detergents are ...gible, but 10- and 20-stearic ethers caused a signif-... decrease in fluorescent anisotropy ($p < 0.01$), ...as longer stearic acid ethers did not significantly ...sults (Table 1). The 10-oleic acid ether-treated

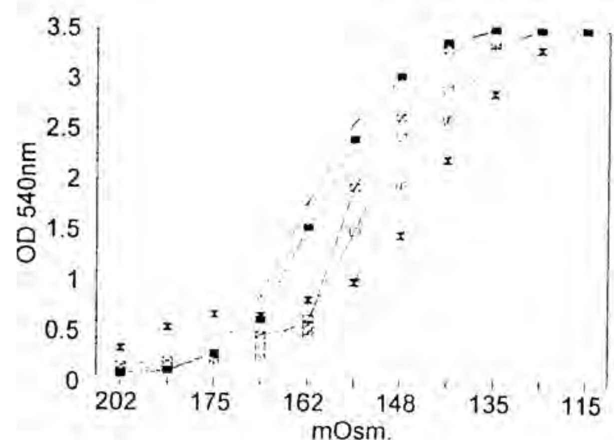


FIGURE 4 Osmotic lysis of human erythrocytes in serial dilutions of 0.89% NaCl solutions at 0 C. The shift of the lysis curves toward the right indicates increased osmotic resistance. All detergents used cause increased resistance. The rank order agrees well with the detergent potential to increase erythrocyte leakiness.

erythrocyte ghosts displayed the largest decrease in fluorescence anisotropy.

Note that the rank order of changes agrees with those seen in case of detergent-mediated increase in ion traffic and in protection against osmotic lysis.

DISCUSSION

The action of detergents on plasma membrane ion permeability properties is surprisingly complex where detergents are applied beyond their CMC values.

Monomer detergent molecules interact exclusively with plasma membranes when detergents are applied in sub-CMC concentrations. Here the complexities in ion equilibration seen above CMC values are largely eliminated as there is little or no lysis of cells, and the effects of membrane-incorporated detergent molecules can be more precisely studied. In previous studies a number of chemically unrelated amphiphiles have been shown to protect erythrocytes against hypotonic lysis (Seeman, 1972; Csordás and Schauenstein, 1984; Isomaa et al., 1986; Isomaa and Hagerstrand, 1988). It was generally assumed that the protecting effect depended upon the concentration of amphi-

TABLE 1 Fluorescence anisotropy in normal and in detergent-treated erythrocyte ghosts

Probe: Detergent	n	$\langle r \rangle$ DPH (mean ± SD)	$\langle r \rangle$ TMA-DPH (mean ± SD)
None	6	0.241 ± 0.001	0.296 ± 0.002
2-Stearyl ether	6	0.243 ± 0.002	0.290 ± 0.004
4-Stearyl ether	6	0.244 ± 0.001	0.289 ± 0.005
10-Stearyl ether	6	0.231 ± 0.007	0.263 ± 0.009
20-Stearyl ether	6	0.229 ± 0.004	0.257 ± 0.006
21-Stearyl ether	6	0.241 ± 0.003	0.281 ± 0.005
10-Oleyl ether	6	0.201 ± 0.003	0.247 ± 0.012

philes incorporated into the plasma membrane. Therefore a direct expansion of the plasma membranes was postulated to be the reason behind increased osmotic resistance of amphiphile-treated erythrocytes. In their excellent studies, Isomaa and Hagerstrand (1988) and Tragner and Csordas (1987) tested several detergents at 37°C but did not elucidate a relationship between increased osmotic resistance and increased monovalent cation permeabilities.

To eliminate complexities arising from possible alterations of erythrocyte metabolism and ion transport properties, we studied the effects of sub-CMC, nonhemolytic concentrations of Brij series detergents at 0°C. Polyoxyethylene fatty-acid ethers (Brij series detergents) added to normal human erythrocytes in nonhemolytic concentrations facilitate the loss of K^+ and the uptake of Na^+ and at 0°C (Fig. 2). The number of polyoxyethylene units (n) seems to be critical in the equilibration process. Small detergent molecules ($n = 2$–4) with low HLB values (HLB $\cong$ 5.4–10) did not significantly facilitate K^+ loss and Na^+ uptake. Increasing oxyethylene unit numbers are associated with increased ion permeability, but there is a sharp reversal of the effect of $n \geq 21$. This same effect had been observed in previous (unreported) studies with Brij 58 ($n = 20$) and Brij 35 ($n = 23$) detergents.

The length of the fatty acid residue appears to be relatively unimportant in ion movements in the size range investigated ($12 \leq C \leq 18$). This is the size range for the alkyl chain of most abundant lipids in the cell membranes (Gennis, 1989). We found that 10-stearic acid ether behaves in a manner similar to that of 10-lauric acid ether in facilitating ion release (Fig. 3). This observation was somewhat surprising because the hydrophobic tail region of detergents is known to be intercalated between membrane lipids (McIntosh et al., 1980; Gennis, 1989; Bressauer et al., 1985). Isomaa and Hagerstrand (1988) studied the effects of a variety of octa-ethyleneglycol-alkylethers (C_{10}-C_{16}) on the ion-exchange properties of human erythrocytes. They found that these derivatives potentiated K^+ release in the rank order $C_{14} > C_{12} > C_{10} > C_{16}$. However, they also noted "that passive fluxes of potassium were only slightly increased below CAH_{75}, but markedly increased at CAH_{max}." (CAH_x is a percentage expression of the antihemolytic concentrations of detergents.) Consequently they compared different concentrations of detergents, based on the assumption that at equiprotecting concentrations the number of detergent molecules in the lipid phase is similar, because of the different oil/water partitioning of detergents. However, the HLBs of 10-stearyl and 10-oleyl ethers are virtually identical, yet these two derivatives show a marked difference in their abilities to potentiate monovalent cation exchanges (Fig. 3).

A further observation is that equimolar concentrations of detergents cause protection against hemolysis in a rank order which agrees well with detergent capabilities in facilitating ion exchange between the cell and its environment (Fig. 4). This observation supports the idea that an accelerated equilibration between the detergent-treated erythrocytes and their environment could be important in protection against hypotonic hemolysis. 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. A direct expansion of the plasma membranes is unlikely to play a role in this phenomenon, because the oil/water partition of detergents follows an inverse order. Consequently, hydrophobic (low HLB) detergent molecules are more abundant in the plasma membranes than hydrophilic (high HLB) ones, yet the former offer little if any protection against hypotonic lysis (Fig. 4).

Unfortunately, a more direct comparison between the findings of others (Tragner and Csordás, 1987; Isomaa and Hagerstrand, 1988) and our data is not possible because their experiments were carried out at higher temperatures, and their detergents, although similar, were not identical to ours.

Further correlations were explored, when we used two fluorescent probes to follow alterations of membrane anisotropy caused by non-ionic detergents. DPH is assumed to be located in the hydrocarbon region of membranes, and there is evidence that its cationic derivative TMA-DPH is located near the phospholipid polar headgroups (Donner and Stoltz, 1985). Nevertheless, DPH and TMA-DPH signals changed similarly within our experimental setup (Table 1).

Results indicate that hydrophobic ($n = 2$–4) stearic acid ethers cause little alteration in fluorescent anisotropy, but 10- and 20-stearic acid ethers cause decreased fluorescent anisotropy in the case of detergent-treated red blood cell ghosts. Although the equilibrium distribution ratio of 10-oleyl ether must be similar to its 10-stearyl sibling, the former caused a more pronounced decrease in fluorescent anisotropy than the latter, indicating that the physicochemical nature of the interacting hydrophobic tail group may be more important than considered previously by many membrane researchers.

In conclusion, by using cold incubation and non-lytic concentrations of Brij series detergents we explored a relationship between detergent properties and accelerated ion traffic. A significant correlation was demonstrated between the increased osmotic resistance of detergent-treated erythrocytes, the detergent-mediated acceleration of ion traffic, and the detergent-dependent increased mobility of membrane lipids.

Although the mechanism of action of various detergents is considered in the light of their physicochemical properties, the fact that minor changes in detergent properties may cause dramatic alterations of ion traffic supports the idea that specific detergent-lipid and detergent-protein interactions should also be considered (Kirkpatrick et al., 1974; Gennis, 1989; Sun et al., 1968; Moore et al., 1989; Broring et al., 1989).

We thank Dr. Béla Somogyi for his helpful comments and Pappné Bácskai Sarolta and Peter Csutora for their excellent technical assistance.

The work was supported by Hungarian National Science Foundation grants OTKA T-016281, OTKA 1448, and OTKA F-016343.

REFERENCES

Bielawski, J. 1990. Two types of haemolytic activity of detergents. *Biochim. Biophys. Acta.* 1035:214–217.

Bogner, P., M. Kellermayer, and C. F. Hazlewood. 1989. Release of hemoglobin and potassium from human red blood cells treated with Triton-X 100 under the critical micellar concentration. *Scanning Microsc.* 3:1241–1245.

Bressauer, R., P. Chatelgin, E. Goormagtigh, and J. M. Ruysschaert. 1985. A semi-empirical conformational analysis of the interaction of n-alkanols with dipalmitoylphosphatidilcholine. *Biochim. Biophys. Acta.* 814:227–236.

Broring, K., C. W. Haest, and B. Deuticke. 1989. Translocation of oleic acid across the erythrocyte membrane. Evidence for a fast process. *Biochim. Biophys. Acta.* 986:321–331.

Cameron, I. L., K. E. Hunter, N. K. R. Smith, C. F. Hazlewood, A. Ludány, and M. Kellermayer. 1988. Role of plasma membrane and cytomatrix in maintenanance of intracellular to extracellular ion gradients in chicken erythrocytes. *J. Cell. Physiol.* 137:299–304.

Csordas, A., and K. Schauenstein. 1984. Structure and configuration-dependent effects of C18 unsaturated fatty acids on the chicken and sheep erythrocyte membrane. *Biochim. Biophys. Acta.* 769:571–577.

Dodge, J. T., M. Mitchell, and D. J. Hanahan. 1962. The preparation and chemical characteristics of Hb-free ghosts of human erythrocytes. *J. Biol. Chem.* 34:119–130.

Donner, M., and J. F. Stoltz. 1985. Comparative study on fluorescent probes distributed in human erythrocytes and platelets. *Biorheology.* 22:385–397.

Gennis, R. B. 1989. Characterization and structural priniceples of membrane proteins. *In* Biomembranes: Molecular Structure and Function. Springer Verlag, New York, Berlin. 87–91.

Isomaa, B., and H. Hagerstrand. 1988. Effects of nonionic amphiphiles at sublytic concentrations on the erythrocyte membrane. *Cell. Biochem. Funct.* 6:183–190.

Isomaa, B., H. Hagerstrand, and G. Paatero. 1987. Shape transformations induced by amphiphiles in erythrocytes. *Biochim. Biophys. Acta.* 899: 93–103.

Isomaa, B., H. Hagerstrand, G. Paatero, and A. C. Engblom. 1986. Permeability alterations and antihaemolysis induced by amphiphiles in human erythrocytes. *Biochim. Biophys. Acta.* 860:510–524.

Jacobs, E. E., F. H. Kirkpatrick, E. E. Andrews, W. Cunningham, and F. L. Crane. 1966. Lipid-free soluble cytochrome oxydase purification, properties and reaction characteristics. *Biochem. Biophys. Res. Commun.* 25:96–104.

Jørgensen, P. L., and J. C. H. R. Skou. 1971. Purification and characterization of $(Na^+ + K^+)$-ATPase. I. The influence of detergents on the activity of $(Na^+ + K^+)$-ATPase in preparations from the outer medulla of rabbit kidney. *Biochim. Biophys. Acta.* 233:366–380.

Kellermayer, M., A. Ludány, K. Jobst, Gy. Szücs, K. Trombitás, and C. F. Hazlewood. 1986. Cocompartmentation of proteins and K within the living cell. *Proc. Natl. Acad. Sci. USA.* 83:1011–1015.

Kellermayer, M., A. Ludány, A. Miseta, T. Kőszegi, G. Berta, P. Bogner, C. F. Hazlewood, I. L. Cameron, and D. N. Wheatley. 1994. Release of potassium, lipids, and proteins from nonionic detergent treated chicken red blood cells. *J. Cell. Physiol.* 159:197–204.

Kellermayer, M., D. Rouse, F. Györkey, and C. F. Hazlewood. 1984. Potassium retention in membraneless thymus lymphocyte nuclei. *Physiol. Chem. Phys. Med. NMR.* 16:503–511.

Kirkpatrick, F. H., S. E. Gordesky, and G. V. Marinetti. 1974. Differential solubilization of proteins, phospholipids, and cholesterol of erythrocyte membranes by detergents. *Biochim. Biophys. Acta.* 345:154–161.

Kőszegi, T. 1988. Rapid bioluminescent measurement of human erythrocyte ATP content. *Clin. Chim. Acta.* 34:2578–2580.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.

McIntosh, T. J., S. A. Simon, and R. C. McDonald. 1980. The organization of n alkanes in lipid bilayers. *Biochim. Biophys. Acta.* 597:445–463.

Miseta, A., M. Kellermayer, A. Ludany, I. L. Cameron, and C. F. Hazlewood. 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. *J. Cell. Physiol.* 146:394–398.

Moore, R. B., J. F. Manery, J. Still, and V. N. Mankad. 1989. The inhibitory effects of polyoxyethylene detergents on human erythrocyte acethylcholinesterase and Ca^{2+} + Mg^{2+} ATPase. *Biochem. Cell. Biol.* 67:137–146.

Pazos-Sanou, L., and J. F. Mata-Segrada. 1985. Relationship between the rate of detergent induced hemolysis and blood type. *Physiol. Pharmacol. Latinoam.* 35:105–108.

Rao, A., C. U. Onuora, and A. Cherian. 1987. Detergent induced lysis of erythrocytes in kwashiorkor. *Clin. Chim. Acta.* 168:1–5.

Ridsdale, J. A., and J. S. Clegg. 1991. Evidence for cooperativity of protein dissolution in Brij 58 permeabilized L929 cells. *J. Cell. Physiol.* 147: 242–247.

Seeman, P. 1972. The membrane actions of anesthetics and tranquilizers. *Pharmacol. Rev.* 24:583–655.

Schliwa, M., U. Euteneuer, and K. R. Porter. 1987. Release of enzymes of intermediary metabolism from permeabilized cells: further evidence in support of a structural organization of the cytoplasic matrix. *Eur. J. Cell. Biol.* 44:214–218.

Schliwa, M., J. van Blerkom, and K. R. Porter. 1981. Stabilization of the cytoplasmic ground substance in detergent opened cells and a structural and biochemical analysis of its composition. *Proc. Natl. Acad. Sci. USA.* 78:4329–4333.

Slinde, E., and T. Flatmark. 1976. Effect of the hydrophile-lipophile balance of non-ionic detergents (Triton X-series) on the solubilization of biological membranes and their integral b-type cytochrome. *Biochim. Biophys. Acta.* 445:796–805.

Sun, F. F., K. S. Prezbindowski, F. L. Crane, and E. E. Jacobs. 1968. Physical state of cytochrome oxydase. Relationship between membrane formation and ionic strength. *Biochim. Biophys. Acta.* 153:804–818.

Tragner, D., and A. Csordas. 1987. Biphasic interaction of Triton detergents with the erythrocyte membrane. *Biochem. J.* 244:605–609.

Umbreit, J. N., and J. L. Strominger. 1973. Relation of detergent HLB number to solubilization and stabilization of D-alanine carboxypeptidase from *Bacillus subtilis* membranes. *Proc. Natl. Acad. Sci. USA.* 70: 2997–3001.

Albumin-mediated Reversal of Potassium Depletion in Human Erythrocytes Treated with the Non-Ionic Detergent, Brij 58

Authors.: Peter Bogner M.D. #, Denys N. Wheatley Ph.D. D.Sc. ^,
Csaba Borbély M.D.* and Attila Miseta M.D., Ph.D.*

Laboratory.: Department of Clinical Chemistry, University Medical School
Pécs, Pécs , Ifjuság u. 13. H-7643 (HUNGARY)

Diagnostic Center, Pannon University of Agriculture, Faculty of Animal Production, Kaposvár, Dénesmajor 2, H-7400 HUNGARY

^ Department of Pathology, University of Aberdeen, Foresterhill, Aberdeen, AB9 2ZD, Scotland, UK

Running head: K+ in detergent-treated erythrocytes

Key words: Human erythrocyte - Potassium - Detergents - Albumins - Metabolism

*Corresponding author, Dr. Attila Miseta
Department of Clinical Chemistry,
University Medical School,
Pécs, Ifjuság u. 13.
H-7643

Present address, Department of Microbiology,
The University of Alabama at Birmingham,
BBRB 338, Box 6.
Birmingham, AL 35294-2170
Tel: (205) 975 6585
Fax.: (205) 975 5479
E-mail: attila_miseta@micro.microbio.uab.edu

Abstract

Exchange of erythrocyte intracellular (i/c) K+ for extracellular (e/c) Na+ in human erythrocytes treated with sub-CMC concentrations of the non-ionic detergent Brij 58 can be stopped by re-incubation in serum or albumin containing solutions. The 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 term of K+/kg water proceeds in two ways: (i) a metabolism-dependent net accumulation of K+ ions, and (ii) a metabolism-independent shrinkage of erythrocytes, this being the more significant factor.

Introduction

Detergents used in cell fractionation procedures, and for the permeabilization of various cell types show remarkable physico-chemical and physiological similarities to a variety of pharmaceutically active compounds, especially to anesthetics [1]. Among the widely used detergent molecules, the Brij series (polyoxyethylene fatty acid ethers) non-ionic detergents are particularly popular.

Earlier we showed that the erythrocyte stabilizing effect of Brij series detergents correlates well with increased ion traffic, and the mobility of membrane lipids revealed by fluorescence anisotropy [2].

Brij 58 (polyoxyethylene 20-cetyl ether), has also been used recently in different cell systems, with effective permeabilization occurring in bovine thymus lymphocytes [3], cultured mouse fibroblasts [4], L 929 cells [5], porcine lens epithelium [6], human erythrocytes [7] and nucleated chicken erythrocytes [8,9]. This occurs provided the detergent concentration exceeds the critical micellar concentration (CMC), as judged by EM evidence of plasma membranes fenestration [4,7-9,], removal of plasma membrane lipids [9], and uptake of dyes [5,9]. 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 cell with its environment. Loss of K+ from individual chicken erythrocytes - investigated by electron probe X-ray microanalysis [8] - 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 [5] 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 [10,11]. 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 [2].

Apart from corroborating and extending these findings, in our further studies described below, we will show that detergent treated cells destined to lysis may be rescued if treated with albumin-containing solutions. The present report deals with the reversibility of the detergent-mediated exchange of erythrocyte K^+ with extracellular Na^+ in human erythrocytes treated with sub-CMC concentrations of Brij 58.

Materials and Methods

Materials and blood specimens

Brij 58 (polyoxyethylene-20-cetyl-ether), Brij 78(polyoxyethylene-20-stearyl-ether), Brij 99 (polyoxyethylene- 20-oleyl-ether), cytochalasin B, and ouabain were purchased from Sigma (USA). 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 °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 pH, but checked the pH of serum prior to experiments.

The quantity of blood obtained from each donor was usually 15-20 ml, which limited us to no more than 2 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 [12], 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, 10-20 mM NaF, 10-20 mM Na-arsenate, and 10μM cytochalasin B.

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

10 ml samples were centrifuged at 3 000 g for 10 min. The supernatants were removed and stored at 4°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. No additional weight (water) loss was observed during longer drying periods. 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 mercuric thiocyanate method of Hamilton [13].

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°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 at 540nm, 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 [14].

Erythrocyte ghost preparation and fluorescence emission anisotropy measurements

Erythrocyte ghosts were prepared by the method of Dodge et al. [15]. Untreated ghosts, and Brij 58 treated ghosts (0.004% concentration) resuspended in PBS, were shifted to ice for one hour. Some of the samples were exposed briefly to 10g/l albumin complemented Hanks solution, centrifuged, the albumin containing supernatant removed and resuspended in ice-cold PBS. After that ghosts were labelled with 1,6-diphenyl-1,3,5,-hexatriene (DPH) in PBS at 25 °C for 30 min. The protein concentration of ghosts was ca. 100 µg/ml, and the final concentration of DPH 1 µM/l. The protein concentration of ghost preparations was measured by Lowry's method [16]. Fluorescence measurements were carried out with a Hitachi MPF-4 spectrofluorimeter (Japan) equipped with polarisation accessories at 4°C.

Wavelengths were 360 nm and 425 nm for excitation and detection respectively. Fluorescence anisotropy was calculated according to the equation: $r = (I_{vv} - GI_{vh})/(I_{vv} + 2GI_{vh})$, where I_{vv} and I_{vh} are the fluorescence intensities measured with a vertical polarizer, and vertically or horizontally mounted analyser respectively ($G = I_{hv}/I_{hh}$) [17]. The fluorescent intensities of dyes were always measured in the presence of detergents, but without ghosts. Results obtained were subtracted from those obtained in the presence of labelled ghosts. For each sample, fluorescence was corrected for the scattering effect of unlabelled ghosts.

Results

Release of monovalent cations from detergent-incubated erythrocytes at 0-4 ℃

Intact human erythrocytes released small quantities of K+ and took up similarly insignificant quantities of Na+ when incubated in iso-osmotic NaCl solution at 0°C for 4 h (Fig. 1). 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.

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 behave differently, and the results of these studies were reported elsewhere [2]. The reasons for choosing Brij 58 were that it caused significant changes in the electrolyte concentrations of erythrocytes within a relatively short period of time, and it was relatively easy to achieve this effect at non-hemolytic concentrations.

Detergent-treated cells accumulate K+ and release Na+ on reincubation in human plasma or serum

Reincubation of 0.004% Brij 58 treated human erythrocytes in detergent-free serum or plasma at 37oC resulted in an increased i/c [K+], and a decreased [Na+], albeit to a lesser extent (Fig. 2a). 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. 2a 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. 2b). 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 (Fig. 2a). However, the total monovalent cation content expressed in absolute terms was re-established (Fig. 2b).

Prolonged (pre-) 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

Before we have seen that 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). Although erythrocytes were incubated in iso-osmotic solutions a gradual swelling

accompanied the lack of extrusion of Na+. Multiple regression analysis indicated good relationships between the log of serum concentration and erythrocyte Na+, and water contents, **r** = **+ 0.9359** and **r** = **-0.9804** respectively ($p < 0.01$).

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 detergent-treated 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 serum or Hank's solution containing albumin, their tendency to equilibrate with the environment and lyse was diminished, in contrast to cells reincubated in protein-free Hank's solution, 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. Similarly, addition of 0.004% Brij 58 decreased the fluorescence anisotropy of erythrocyte ghosts from a value of

r = **0.232 ± 0.007** (n = 5) to **r** = **0.207 ± 0.018** (n = 5). Reincubation of the same detergent treated ghosts in the presence of 10 g/l albumin reversed this effect, and the fluorescence anisotropy was somewhat higher (**r** = **0.251 ± 0.007**; n = 5) than in the control cells.

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

There was a significant decrease of i/c [ATP] during exposure to detergent (Fig. 3). A transient increase and a subsequent gradual decrease of ATP occurred when erythrocytes were reincubated in serum at 37°C.

Brij 58 treated erythrocytes incubated in serum consumed glucose with a rate of **6.06 ± 0.11 mM / kg Hb / h**, about twice the rate of glucose consumption of control erythrocytes **2.56 ± 0.12 mM / kg Hb / h** (n = 6 in both cases). Similarly, Brij 58 incubated erythrocytes produced lactate with rates of **12.75 ± 1.11 mM / kg Hb / h** respectively, about twice as fast as normal plasma incubated erythrocytes (**5.01 ± 0.65 mM / kg Hb / h**) (n = 6 in both case). 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).

[Er]ythrocytes incubated on ice were unable to utilise measurable quantities of glucose, and [pro]duced 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 °C, just as at 0°C. Neither the addition of 0.5 mM ouabain nor 10 μM cytochalasin B prevented the elevation of K^+.

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. Treatment with the actin inhibitor cytochalasin B did not prevent the net accumulation of K^+.

Discussion

Polyoxyethylene adduct detergents, are routinely applied for the isolation of different membrane proteins [18,19]. Among these, the Brij series (n-oxyethylene fatty acid ethers) detergents are becoming increasingly popular. This is in part due to their relatively easy removability, which is frequently the last critical step during the isolation of functionally intact membrane proteins. It is now well established that the addition of albumin, a protein noted for its capacity to bind different hydrophobic molecular species including fatty acids, effectively and competitively removes these detergent molecules *in vitro*.

The major goal of the present work was to demonstrate that such removal of detergent molecules, and restoration of near normal cellular ion environment is possible *in vivo*. Several series of experiments on the ion equilibrium of detergent treated cells have been carried out in our laboratory during the past decade [2-4, 6,8-10,20].

Based on these, we assume that ion equilibration between the detergent treated erythrocytes and their environment may proceed in three ways: (i) beyond their CMC values, the most frequently used detergents cause erythrocyte disintegration, i.e. hemolysis ensues; (ii) detergent-mediated opening of the plasma membranes occurs prior to lysis, and presumably leads to accelerated ion traffic between the cell and its environment; (iii) detergent molecules themselves become incorporated in plasma membranes, changing their permeability without removing lipids.

In the present report, we demonstrate that erythrocytes treated with near CMC concentrations of Brij 58 remain remarkably stable at 0 °C for 1h, but are destined to disintegration and lysis during longer incubation and/or if they are incubated at 37 °C.

However, erythrocytes may be rescued from lysis by treatment with human serum or with albumin-containing solutions. The process involves *the re-establishment of near normal electrolyte levels* within the erythrocytes. This is remarkable because the detergent treatment results in significant alterations in both erythrocyte K^+ and Na^+ levels. This involves the release significant quantities of K^+ and the uptake of similar quantities of Na^+ from the environment (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). The swelling of the detergent-treated erythrocytes indicates that their lysis is coupled with an excessive water uptake.

The ion equilibration processes and swelling were quickly stopped and partially reversed by the reincubation of erythrocytes in human serum or plasma (Fig. 2 and Table 1). 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 (data not shown). Also, the effective removal of detergents from erythrocyte membranes was supported by our fluorescence anisotropy measurements. The detergent treatment of

erythrocyte ghosts results in a significant drop of fluorescence anisotropy, which was reversed by exposure to albumin containing solutions.

Our observations indicate that the apparent elevation of i/c K+ levels in serum involves two components: (i) one is an active component responsible for the modest net accumulation of K+ ions (Fig. 2b); (ii) and the other is associated with the loss of erythrocyte water and a relative increase of K+ levels in the decreased aqueous compartment.

ad (i) The first mechanism by which erythrocytes increased their [K+] was by a net uptake of ions at 37 °C (Fig. 2b). 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 [21]. Unlike most other cell types, the sole source of ATP in human erythrocytes comes from the Embden-Meyerhof pathway [22]. Indeed, a fall of ATP level has been demonstrated (Fig. 3), 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 significantly 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 °C. Neither erythrocyte metabolism nor the membrane "pump" is active at this temperature [22]. 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.

ad (ii) Shrinkage of erythrocytes in iso-osmotic environments may hypothetically occur if (a) the loss of solutes exceeds the uptake of solutes, (b) the cytoskeletal elements of erythrocytes undergo conformational changes or (c) the hydration potential of haemoglobin molecules changes.

ad (a) Indeed, the K+ + Na+ concentrations appear to be higher during reincubation (Fig. 2a.). However, this was due to the slightly basic pH of the plasma, and could therefore be accounted to a new electrostatic equilibrium. Supporting this, a stoichiometric decrease of Cl^- followed the elevation of monovalent cation levels (data not shown).

ad (b) Among the inorganic components of serum or Hank's solution, Ca2+ is probably the most important. This is best demonstrated if the effects of serums dialysed against 5mM KCl complemented 145mM NaCl solution and Hanks solution were compared. The readdition of Ca2+ to the former solution corrected for the difference. However, cytochalasin B, an inhibitor of actin did not prevent the shrinkage of erythrocytes, and elevation of K+ levels (Table 2.). In the absence of calcium-sequestering organelles, its total i/c concentration is low in human erythrocytes (albeit the free Ca2+ level is similar to that measured in other cell types), whereas Mg2+ is in the millimolar range [23]. The i/c Ca2+ level appears to be

higher in the denser (i.e. supposedly older) erythrocytes [24], which could be responsible for some differences in the behaviour of individual erythrocytes.

ad (c) The hydration of hemoglobin molecules changes significantly by pH, ionic strength etc. [25-27]. Although a straightforward interpretation of relationship between protein hydration and cellular volume regulation remains yet to be explored, there is little doubt that such relationship exists.

In conclusion, Brij 58-treated human erythrocytes can be rescued from lysis by *serum treatment*. Among serum components *albumin* effectively removes detergent molecules, and stops erythrocyte swelling, ion equilibration and disintegration. Erythrocyte K^+ concentration comes near to normal values during reincubation in serum. This is achieved in part by a metabolism linked process, which could be prevented by incubation in the cold, metabolic inhibitors, or by ouabain. More significantly, detergent-treated and serum-incubated erythrocytes *shrink*, and their electrolyte concentration elevates. Ca^{2+} ions appear to be involved in this process, but the mechanism of their action remains to be explored.

Acknowledgements:

We are grateful to Drs. Miklós Kellermayer and Ivan Cameron for helpful discussions. We thank Mrs. Pappné Sarolta Bácskai, Ágnes Szarka, and Dr. Norbert Süle for their excellent technical assistance. This work was supported by OTKA F-016343 and T-016281.

References:

1. Seeman P (1972) The membrane action of anesthetics and tranquilizers. Pharm Rev 24:583.
2. Miseta A, Bogner P, Szarka A, Kellermayer M, Galambos Cs, Wheatley DN, Cameron IL, (1995) Effect of non-lytic concentrations of Brij series detergents on the metabolism independent ion permeability properties of human erythrocytes. Biophys J 69:2563
3. Kellermayer M, Rouse D, Györkey F, Hazlewood, CF (1984) Potassium retention in membraneless thymus nuclei. Physiol Chem Phys Med NMR 16:503
4. Kellermayer M, Ludány A, Jobst K, Szücs Gy, Trombitás K, Hazlewood CF (1986) Cocompartmentation of protein and K^+ within the living cell. Proc Natl Acad Sci USA. 83:1011
5. Ridsdale JA, Clegg JS (1991) Evidence for the cooperativity of protein dissolution in Brij 58 permeabilized L929 cells. J Cell Physiol 147:242
6. Miseta A, Kellermayer M, Ludány A, Cameron IL, Hazlewood CF (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. J Cell Physiol 146:394
7. Cameron IL, Cox LA, Liu XR, Fullerton GD (1991) Maintenance and mobility of haemoglobin and water within the human erythrocyte after detergent disruption of the plasma membrane. J Cell Physiol 149:365
8. Cameron IL, Hunter KE, Smith NKR, Hazlewood CF, Ludány A, Kellermayer

M (1988) Role of plasma membrane and of cytomatrix in maintenance of intracellular to extracellular ion gradients in chicken erythrocytes. J Cell Physiol 137:299

9. Kellermayer M, Ludány A, Miseta A, Kôszegi T, Berta G, Bogner P, Hazlewood CF, Cameron IL, Wheatley DN (1994) Release of potassium, lipids and proteins from non-ionic detergent treated chicken red blood cells. J Cell Physiol 159:197
10. Bogner P, Kellermayer M, Hazlewood, CF (1989) Release of haemoglobin and potassium from human red blood cells treated with Triton X-100 under the critical micellar concentration. Scanning Microsc 3:1241
11. Isomaa B, Hagerstrand H (1988) Effects of non-ionic ampiphiles at sublytic concentrations on the erythrocyte membrane. Cell Biochem Function 6:183
12. Pazos-Sanou L, Mata-Segrada JF (1985) Relationship between the rate of detergent-induced hemolysis and blood type. Physiol. Pharmacol. Latinoam. 35:105
13. Hamilton RH (1966) Direct photometric method for chloride, employing mercuric thiocyanate. Clin. Chem. 12:1
14. Kõszegi T. (1988) Rapid bioluminescent measurement of human erythrocyte ATP content. Clin Chem 34:2578
15. Dodge JT, Mitchell M, Hanahan DJ (1962) The preparation and chemical characteristics of Hb-free ghosts of human erythrocytes. J Biol Chem 34:119
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with folin phenol reagent. J Biol Chem 193:265

17. Donner M, Stoltz JF (1985) Comparative study on fluorescent probes distributed in human erythrocytes and platelets. Biorheology 22:385

18. Gennis RB (1989) Biomembranes: molecular structure and function. Springer-Verlag, New York Inc. New York.

19. Tragner D, Csordás A (1987) Biphasic interaction of Triton detergents with erythrocyte membrane. Biochem J 244:605

20. Hazlewood CF, Kellermayer M (1988) Ion and water retention by permeabilized cells. Scanning Microsc. 2:267

21. Post RL (1989) Seeds of sodium, potassium ATPase. Ann. Rev. Physiol. 51:1

22. Agar NS, Board PG (1983) Red cell metabolism. In: Red Blood Cells of Domestic Mammals. Agar NS, Board PG, eds. pp. 227-251. Elsevier, Amsterdam, The Netherlands

23. Miseta A, Bogner P, Berényi E, Kellermayer M, Galambos Cs, Wheatley DN, Cameron IL (1993) Relationship between cellular ATP, potassium, sodium, and magnesium concentrations in mammalian and avian erythrocytes. Biochim Biophys Acta 1175:133

24. Cameron IL, Hardman WE, Smith NKR, Fullerton GD, Miseta A. (1993) Changes in the concentration of ions during senescence of the human erythrocyte. Cell Biol Int 17:93

25. Cameron IL, Fullerton GD, (1990) A model to explain the osmotic pressure behaviour of haemoglobin and serum albumin. Biochem Cell Biol 68:894

26. Kanal MK, Fullerton GD, Cameron IL (1994) A study of the molecular sources of nonideal osmotic pressure of bovine serum albumin solutions as a function of pH. Biophys J 66:153

27. Keener CR, Fullerton, GD, Cameron, IL, Xiong, J (1995) Solution nonideality related to solute molecular characteristics of amino acids. Biophys J 68:291

Figure legends:

Figure 1.

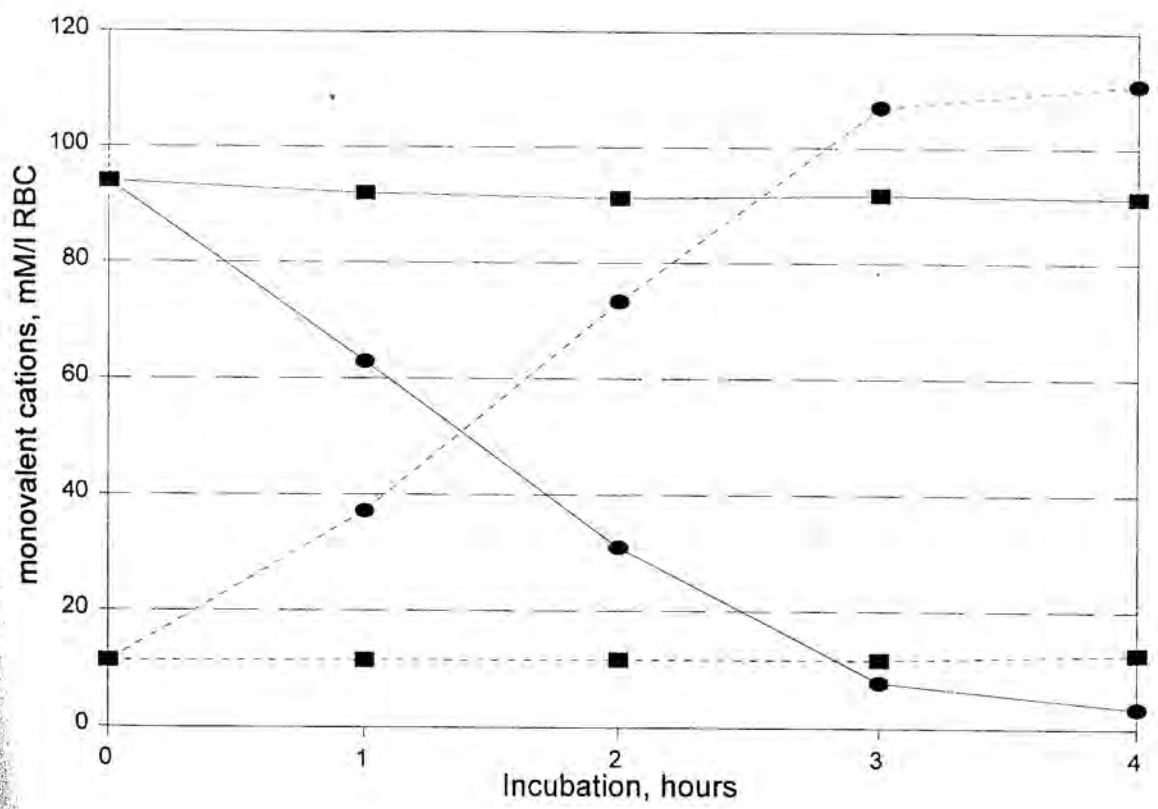
Changes of monovalent cation concentrations of erythrocytes incubated in TBS, and in 0.004% Brij 58 complemented TBS during incubation at 4 °C. Squares; Control RBCs, incubated in TBS. Circles; RBCs incubated in 0.004% Brij 58 complemented TBS. (Continuous lines indicate potassium levels, dotted lines indicate sodium levels.) Each point represents the mean value of 3 measurements.

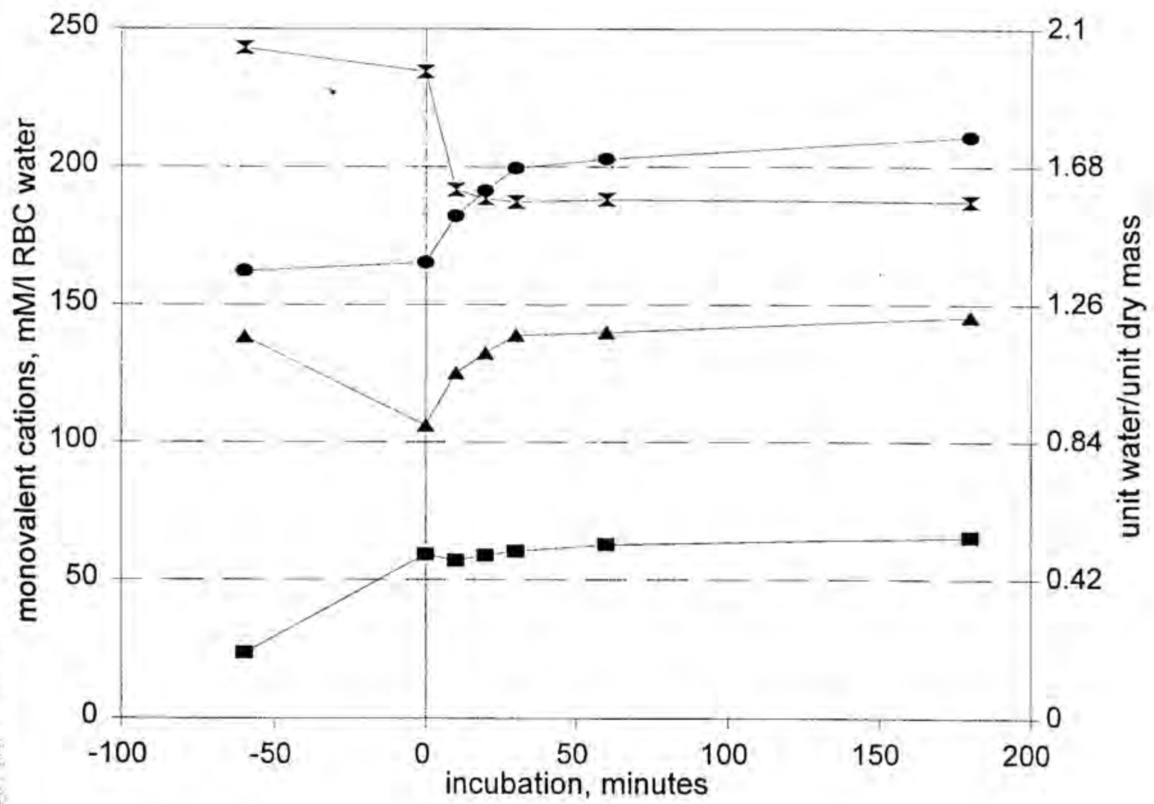
Figure 2.

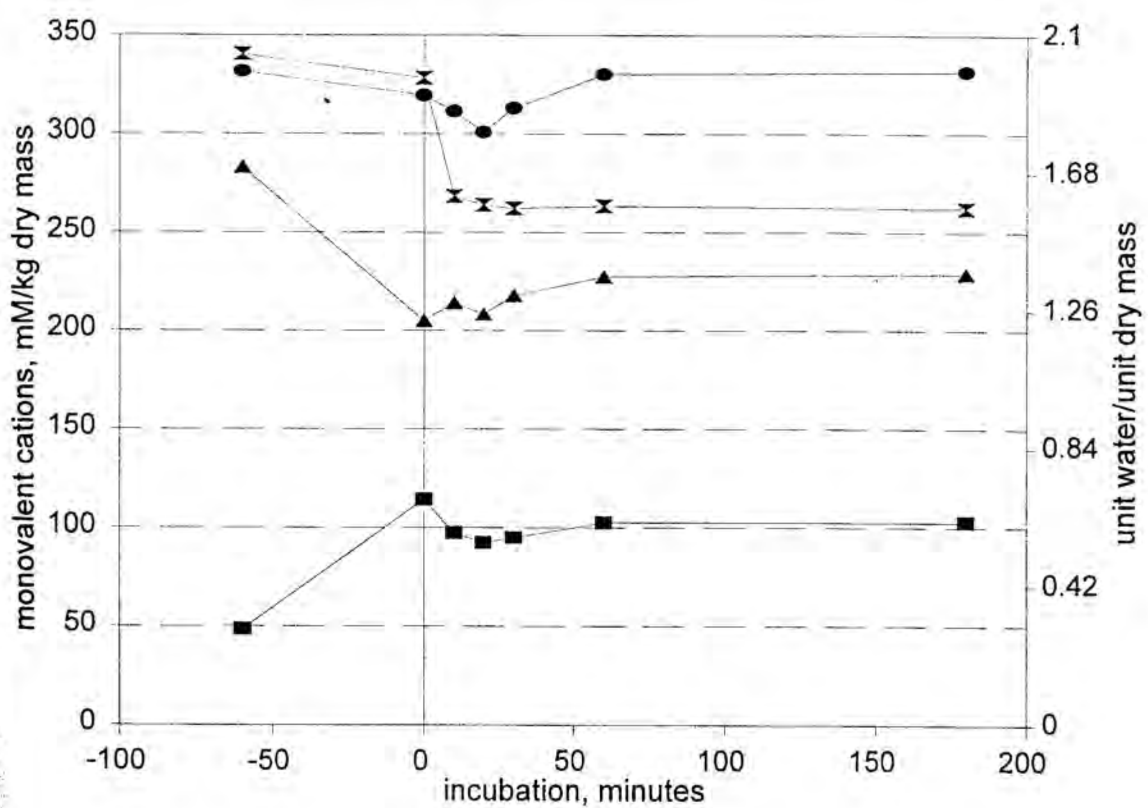
Changes in monovalent cation concentrations (primary Y axis) and water contents (secondary Y axis) of erythrocytes incubated in 0.004% Brij 58 complemented TBS at 4 °C for 1 h, and re-incubated in serum at 37 °C for 3 h. (Vertical line separates incubation in the presence of detergent from re-incubation in serum.) Triangles; K^+, squares; Na^+, circles; (K^+ + Na^+), hourglasses; water. Monovalent cation concentrations are shown in mmol/l RBC water (Fig. 2a.), or mmol/kg dry mass (Fig. 2b.) units (see text for explanation). Each point represent the mean value of 3 measurements.

Figure 3.

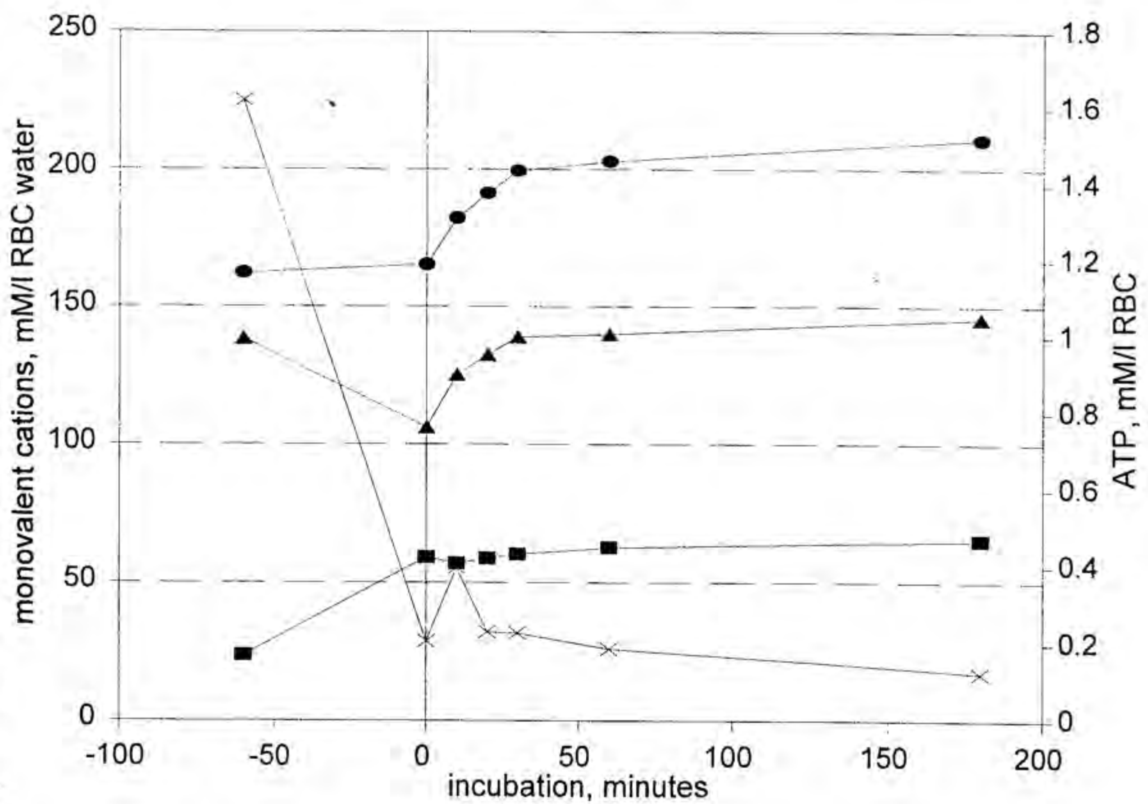
Changes in monovalent cation concentrations (primary Y axis) and ATP levels (secondary Y axis) of erythrocytes incubated in 0.004% Brij 58 complemented TBS at 4 °C for 1 h, and re incubated in serum at 37°C for 3 h. (Vertical lane separates incubation in the presence of detergent from re incubation in serum.) .) Triangles; K^+, squares; Na^+, circles; (K^+ + Na^+), hourglasses; water, X-es; ATP. Each point represents the mean value of 3 measurements.


120
100
80
60
40
20
0
monovalent cations, mM/l RBC
0
1
2
3
4
Incubation, hours


250
200
150
100
50
0
-100
-50
0
50
100
150
200
2.1
1.68
1.26
0.84
0.42
0
monovalent cations, mM/l RBC water
unit water/unit dry mass
incubation, minutes



monovalent cations, mM/kg dry mass
350
300
250
200
150
100
50
0
unit water/unit dry mass
2.1
1.68
1.26
0.84
0.42
0
-100
-50
0
50
100
150
200
incubation, minutes


monovalent cations, mM/l RBC water
ATP, mM/l RBC
incubation, minutes
250
200
150
100
50
0
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-100
-50
0
50
100
150
200

Sample	Incubation#	Reincubation 37 °C	K^+ mM/kg RBC	Na^+ mM/kg RBC	H_2O u.w./u.d.
Native	-	-	97.5	11.5	2.07
1.	+	-	68.0	28.2	2.13
2.	+	Serum	96.1	30.2	1.69
3.	+	10% Serum	97.0	29.3	1.90
4.	+	1% Serum	63.0	39.0	2.07
5.	+	0.1% Serum	44.0	61.1	2.41
6.	+	0.01% Serum	20.0	93.0	2.73
7.	+	Serum filtrate, M.W. < 50.000D	6.8	132.0	2.95
8.	+	Hank's	11.9	102.0	3.07
9.	+	Hank's + glucose	12.0	105.1	2.89
10.	+	Hank's + albumin	63.0	34.0	2.07
11.	+	Dialysed serum @ M.W. > 8.000D	65.0	39.2	2.39
12.	+	Dialysed serum $ M.W. > 8.000D	91.0	29.0	1.89

In TBS complemented with 0.004% Brij 58, at 0°C for 1h.
Serum dialysed against distilled water.
Serum dialysed against glucose free Hanks' solution.

Sample	Incubation #	Reincubation ##	K^+ mM/kg RBC	Na^+ mM/kg RBC	K^+ mM/kg dry mass	Na^+ mM/kg dry mass
Native	-	-	94.2	10.6	290.4	32.7
1.	+	-	64.9	33.1	205.2	104.7
2.	+	Serum	77.6	36.2	215.0	100.2
3.	+	Serum, 0 °C	71.8	43.1	194.9	117.0
4.	+	Serum, 10mM NaF	77.2	37.2	203.9	98.2
5.	+	Serum, 5mM Na-arsenate	87.9	37.9	198.0	100.9
6.	+	Serum, 0.5mM ouabaine	68.0	48.9	200.0	143.9
7.	+	Serum, 10μM cytochalasine B	76.0	37.2	214.1	104.2

In TBS complemented with 0.004% Brij 58, at 0°C for 1h.
The incubation temperature is 37°C, unless indicated otherwise.

Összefoglalás (tézisek)

A bemutatott kísérletekben az egyértékű ionok megoszlását vizsgáltam permeabilizált sejtekben. A permeabilizálást egy kísérletsorozatban izotóniás szacharózban végzett homogenizálással, a többi kísérletben nem-ionos detergensekkel végeztük.

I. Megállapítottam, hogy izotóniás szacharózban feltárt thymus limfocitákban a K^+ ionok mobilitása korlátozott, a sejtpreparátum ionmentes médiumban is K^+ ionokat tart vissza, míg a Na^+ ionok nagy része ezen körülmények között "elveszik". Tekintettel az ionmozgást "gátló" fizikai barrierekre ill. azok hiányára, arra a következtetésre jutottam, hogy a limfocita preparátumban, a visszamaradt K^+ ionok nem lehetnek szabadon, oldott állapotban.

II. Hasonló eredményre jutottam Brij 58, nem-ionos detergenssel végzett kísérleteinkkel, melyekben csirke vörösvérsejteket permeabilizáltunk. A sejtfelszín integritását a Brij 58 detergens két percen belül megbontja, mely morfológiailag igazolható elektronmikroszkópiával. A lipid analízis azt mutatta, hogy 5 percen belül a a plazmamembrán lipidjei a szolubilis fázisba kerülnek. Ennek ellenére a K^+ ionok és a citoplazmatikus fehérjék jelentős része a sejtekből csak percekkel később, egymással parallel szabadul fel.

III. A detergenshatás részletesebb vizsgálatára egy új kísérleti modellt dolgoztam ki, melynél alacsony (sub-CMC: a kritikus micellaképző koncentrációnál alacsonyabb) detergens koncentrációt alkalmazva, a K^+ ionok és fehérje kiáramlás időben elhúzódóvá válik. Sub-CMC detergens koncentráció esetén a "membranolitikus" hatás is lelassul, így a lízist megelőző molekuláris történésekbe is bepillantást nyerhetünk. Erre vonatkozólag fluoreszcens anizotrópia méréseket végeztünk.

IV. Összefüggést találtam a Brij detergenssor kémiai szerkezete, valamint a membránra kifejtett hatása között. Alacsony (sub-CMC) koncentrációt

alkalmazva a detergensek ionpermeabilitást fokozó hatása és a membránpreparátumokban mért fluoreszcens anizotrópia értékek ("membrán-fluiditás") szoros korrelációt mutatnak. A sub-CMC detergens koncentrációval előidézett K^+, Na^+ permeabilitás fokozódás albumin hozzáadásával megállítható, és a sejt eredeti ionviszonyai részlegesen visszaállíthatók. A folyamat részben metabolizmus-függő, másrészt attól független, Ca^{2+}-dependens mechanizmusokra épül.

V. A bemutatott kísérletek alapján arra a következtetésre jutottam, hogy az egyértékű K^+ ion intracelluláris mobilitása korlátozott. Permeabilizálást követően a K^+ ionok az intracelluláris fehérjékkel parallel távoznak az intracelluláris kompartmentből, mely indirekt bizonyítéknak tekinthető a K^+ ionok és a fehérjék asszociációját (ko-kompartmentalizáció) tekintve. A nem-ionos detergensek hatásmechanizmusának vizsgálata arra mutatott rá, hogy sejtfelszíni struktúrák a passzív "ion-transzportban" meghatározó szereppel bírnak.

Idézett közlemények

1. Baker, P.F. and Knight, D.E. 1989. Experimental control of intracellular environment. *Methods in Enzymol.* 171:817-828.

2. Biegel, D. and Pachter, J.S. 1991. "In situ" translation: use of the cytoskeletal framework to direct cell-free protein synthesis. *In Vitro Cell. Dev. Biol.* 27A: 75-85.

3. Bielawski, J. 1990. Two types of hemolytic activity of detergents. *Biochim. Biophys. Acta* 1035:214-217.

4. Bogner, P., Kellermayer, M., and Hazlewood, C.F. 1989. Release of hemoglobin from human red blood cells treated with Triton X-100 under the critical micellar concentration. *Scan. Micros.* 3:1241-1245.

5. Bogner, P., Berke, T. & Kellermayer, M. 1992. Motional characteristics of K^+ and Na^+ in intact and sucrose-permeabilized rat lymphocytes. *Physiol. Chem. Phys. Med. NMR.* 24: 281-288.

6. Bogner, P., Wheatley, D.N. Borbély, C. & Miseta, A. 1996. Albumin-mediated reversal of potassium depletion in human erythrocytes treated with the non-ionic detergent, Brij 58. *Cell Biol. Intl.* submitted

7. Bromberg, L.E. and Klibanov, A.M. 1994. Detergent-enabled transport of proteins and nucleic acids through hydrophobic solvents. *Proc. Natl. Acad. Sci. USA.* 91:143-147.

8. Cameron, I.L., Hunter, K.E., Smith, N.K.R., Hazlewood, C.F., Ludány, A., and Kellermayer, M. 1988. Role of plasma membrane and of cytomatrix in maintenance of intracellular to extracellular ion gradients in chicken

erythrocytes. *J. Cell. Physiol.* 137:299-304.

9. Cameron, I. L., Contreras, E., Fullerton, G. D. Kellermayer, M., Ludány, A., and Miseta A. 1988. Extent and properties of nonbulk "bound" water in crystalline lens cells. *J. Cell. Physiol.* 137:125-132.

10. Cameron, I.L., Cox, L.A., Liu, X.R., and Fullerton, G.D. 1991. Maintenance and mobility of hemoglobin and water within the human erythrocyte after detergent disruption of the plasma membrane. *J. Cell. Physiol.* 149:365-374.

11. Darnel, J., Lodish, H. and Baltimore, D. 1986. Molecular Cell biology. *Scientific American Books Inc. New York*

12. Dean, W.L. and Suarez, C.P. 1984. Binding, activation and solubilization of the Ca^{2+}-ATPase from sarcoplasmic reticulum by nonionic detergents. *Membr. Biochem.* 5:181-191.

13. del Río, E., Gonzáles-Manas, J. M., Gurtubay, J. G. and Goni, F. M. 1991. On the mechanism of bacteriorhodopsin solubilization by surfactants. *Arch. Biochem. Biophys.* 291:300-306.

14. Dodge, J.T., Mitchell, M. and Hanahan, D.J. 1962. The preparation and chemical characteristics of Hb-free ghosts of human erythrocytes. *J. Biol. Chem.* 34:119-130.

15. Gonzales-Manas, J.M., Virto, M.D., Gurtubay, J.G. and Goni, F.M. 1990. Interaction of Triton X-100 with purple membranes. *Eur. J. Biochem.* 188:673-678.

16. Helenius, A. and Simons, K. 1975. Solubilization of membranes by detergents. *Biochim. Biophys. Acta* 415:29-79.

17. Helenius, A., McCaslin, D.R., Fries, E., and Tanford C. 1979. Properties of detergents. *Methods in Enzymol.* 56:735-749.

18. Hisanaga, S., Endo, S., Hirokawa, N., Sakai, H. and Pudles, J. 1992. Ultrastructure of detergent-resistant cytoskeletons in the noncortical domain of sea urchin eggs as revealed by quick-freeze deep-etch technique. *Cell Struct. Funct.* 17:277-285.

19. Isomaa, B., Hågerstrand, H. 1988. Effects of non-ionic ampiphiles at sublytic concentrations on the erythrocyte membrane. *Cell. Biochem. Funct.* 6:183-190.

20. Johanson, F., Olbe, M., Sommarin, M. and Larsson, C. 1995. Brij 58, a polyoxyethylene acyl ether, creates membrane vesicles of uniform sidedness. A new tool to obtain inside-out (cytoplasmic side-out) plasma membrane vesicles. *Plant J.* 7:165-173.

21. Kellermayer, M. and Jobst, K. 1971. Isolation of cell nuclei in coverslip cultures. *Beitr. Path.* 142: 321-323.

22. Kellermayer, M., Rouse, D., Györkey, F. and Hazlewood, C.F. 1984. Potassium retention in membraneless thymus nuclei. *Physiol. Chem. Phys. Med. NMR* 16:503-511.

23. Kellermayer, M., Ludány, A., Jobst, K., Szűcs, Gy., Trombitás, K. and Hazlewood, C.F. 1986. Co-compartmentation of protein and K^+ within the living cell. *Proc. Natl. Acad. Sci. USA.* 83:1011-1015.

24. Kellermayer, M., Ludány, A., Miseta, A., Kőszegi, T., Berta, G., Bogner, P., Hazlewood, C.F. , Wheatley, D.N. and Cameron, I.L. 1994. Release of potassium, lipids and proteins from non-ionic detergent treated chicken red blood cells. *J. Cell. Physiol.* 159:197-204.

25. Kinne-Saffran, E. and Kinne, R.K.H. 1989. Membrane isolation: strategy, techniques, markers. *Methods in Enzymol.* 172:3-17.

26. Knull, H.R., Laursen, S.E. and Cottick, A.A. 1987. Cytomatrix - glycolytic enzyme interactions. *In:* Modern Cell Biology, Vol 5. Molecular Mechanisms in the Regulation of Cell Behaviour, pp. 157-161.

27. Kőszegi, T. 1988. Rapid bioluminescent measurement of human erythrocyte ATP content. *Clin. Chem.* 34:2578.

28. Kragh-Hansen, U., le Marie, M., Noel, J., Gulik-Krzywicki, T. and Moller, J. V. 1993. Transitional steps in the solubilization of protein-containing membranes and liposomes by nonionic detergent. *Biochemistry* 32:1648-1656.

29. Laemmli, U.K. 1970. Cleavege of structural proteins during the assembly of the head of the Bacteriophage T4. *Nature* 227:680-685.

30. Lasch, J., Hoffman, J., Omelyanenko, W.G., Klibanov, A.A., Torchilin, V.P., Binder, H. and Gawrisch, K. 1990. Interaction of Triton X-100 and octylglucoside with the liposomal membranes at sublytic and lytic concentrations. Spectroscopic studies. *Biochim. Biophys. Acta* 1022:171-180.

31. Lichtenberg, D., Robson, R.J., and Dennis, E.A. 1983. Solubilization of phospolipids by detrgents: structural and kinetic aspects. *Biochim.*

Biophys. Acta 737:285-304.

32. Ling, G.N. 1984. In search of the physical basis of life. *Plenum Press, New York*

33. Ling, G.N. 1992. A revolution in the physiology of the living cell. *Krieger Publishing Co., Malabar, USA*

34. Mathur, R.L., Reddy, M.C., Yee, S., Imbesi, R., Groth-Vasselli, B. and Farnsworth, P.N. 1992. Investigation of lens glycolytic enzymes: species distribution and interaction with supramolecular order. *Exp. Eye Res.* 54:253-260.

35. Miseta, A., Kellermayer, M., Ludány, A., Cameron, I.L. and Hazlewood, C.F. 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. *J. Cell. Physiol.* 146:394-398.

36. Miseta, A., Bogner, P., Berényi, E., Kellermayer, M., Galambos, Cs., Wheatley, D.N., Cameron, I.L., 1993. Relationship between cellular ATP, potassium, sodium, and magnesium concentrations in mammalian and avian erythrocytes. *Biochim. Biophys. Acta* 1175:133-139.

37. Miseta, A., Bogner, P., Szarka, Á., Kellermayer, M., Galambos, Cs., Wheatley, D.N., Cameron, I.L.: Effect of non-hemolytic concentrations of Brij-series detergents on the metabolism-independent ion-permeability properties of human erythrocytes. *Biophys. J.* 69:2563-68.

38. Molitoris, B.A., Geerdes, A. and McIntosh, J.R. 1991. Dissociation and redistribution of Na,K-ATPase from its surface membrane actin

cytoskeletal complex during cellular ATP depletion. *J. Clin. Invest.* 88:462-9.

39. Moni, R.W., Parsons, P.G., Quinn, R.J. and Willis, R.J. 1992. Critical micelle concentration and hemolytic activity - a correlation suggested by the marine sterol, halistanol trisulfate. *Biochem. Biophys. Res. Comm.* 182:115-120.

40. Negrutskii, B.S., Stapulionis, R. and Deutscher, M.P. 1994. Supramolecular organization of the mammalian translation system. *Proc. Natl. Acad. Sci. USA.* 91:964-968.

41. O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007-4021.

42. Partearroyo, M.A., Ostolaza, H., Goni, F.M. and Barberá-Guillem, E. 1990. Surfactant-induced cell toxicity and cell lysis. *Biochem. Pharmacol.* 40:1323-1328.

43. Pazos-Sanou, L., Mata-Segrada, J.F., 1985. Relationship between the rate of detergent-induced hemolysis and blood type. *Physiol. Pharmacol. Latin.* 35:105-108.

44. Porter, K.R. 1984. The cytomatrix: a history of its study. *J Cell. Biol.* 99:3s-12s.

45. Regen, S. L., Jayasuriya, N. and Fabianowski, W. 1989. Supramolecular surfactants: amphiphilic polymers designed to disrupt lipid membranes. *Biochem. Biophys. Res. Comm.* 159:566-571.

46. Ridsdale, J.A., and Clegg, J.S. 1991. Evidence for the cooperativity of protein dissolution in Brij 58 permeabilized L929 cells. *J. Cell. Physiol.* 147:242-247.

47. Salviati, G., Betto, R., Ceoldo, S., Biasia, E., Bonilla, E., Miranda, A.F. and Dimauro, S. 1989. Cell fractionation studies indicate that dystrophin is a protein of surface membranes of skeletal muscle. *Biochem. J.* 258:837-841.

48. Schliwa, M., van Blerkom, J. and Porter, K.R. 1981. Stabilization of the cytoplasmic ground substance in detergent opened cells and a structural and biochemical analysis of its comparison. *Proc. Natl. Acad. Sci. USA.* 78:4329-4333.

49. Schliwa, M., Euteneuer, U., and Porter, K.R. 1987. Release of enzymes of intermediary metabolism from permeabilized cells: further evidence in support of a structural organization of cytoplasmic matrix. *Eur. J. Cell Biol.* 44:214-218.

50. Shiao, Y., Chen, J. and Wang, C. 1989. The solubilization and morphological change of human platelets in various detergents. *Biochim. Biophys. Acta* 980:56-68.

51. Slinde, E. and Flatmark, T. 1976. Effect of the hydrophile-lipophile balance of non-ionic detergents (Triton X-series) on the solubilization of biological membranes and their integral *b*-type cytochromes. *Biochim. Biophys. Acta* 455:796-805.

52. Smith, P.R., Saccomani, G., Joe, E.H., Angelides, K.J. and Benos, D.J. 1991. Amiloride-sensitive sodium channel is linked to the cytoskeleton in renal epithelial cells. *Proc. Natl. Acad. Sci. USA* 88:6971-5.

53. Srere, P. 1987. Complexes of sequential metabolic enzymes. *Ann. Rev. Biochem.* 56:89-124.

54. Stocchi, V., Cuccharini, L., Magnani, M., and Fornaini, G. 1987. Adenine and pyridine nucleotides in the erythrocytes of different mammalian species. *Biochem. Internat.* 14:1043-1053.

55. Swezey, R.R. and Epel, D. 1986. Regulation of glucose-6-phosphate dehydrogenase activity in sea urchin eggs by reversible association with cell structural elements. *J. Cell Biol.* 103:1509-1515.

56. Traub, P., Perides, G., Schimmel, H. and Scherbarth, A. 1986. Interaction in vitro of nonepithelial intermediate filament proteins with total cellular lipids, individual phpspholipids and a phospholipid mixture. *J. Biol. Chem.* 262:13742-13748.

57. Tragner, D. and Csordás, A. 1987. Biphasic interaction of Triton detergents with erythrocyte membrane. *Biochem. J.* 244:605-609.

58. Tribout, M., Paredes, S., Gonzales-Manas, J.M. and Goni, F. M. 1991. Binding of Triton X-100 to bovine serum albumin as studied by surface tension measurements. *J. Biochem. Biophys. Methods* 22:129-133.

59. Umbreit, J.N. and Strominger, J.L. 1973. Relation of detergent HLB number to solubilization and stabilization of D-alanine carboxypeptidase from Bacillus subtilis membranes. *Proc. Natl. Acad. Sci. USA* 70:2997-3001.

60. Zulauf, M., Fürstenberger, U., Grabo, M., Jaggi, P., Regenass, M. and Rosenbuch, J.P. 1989. Critical micellar concentrations of detergents. *Methods in Enzymol.* 172:528-538.

A jelölt publikációi (eredeti közlemények)

1. Bogner, P., Kellermayer, M. & Hazlewood, C.F.(1989): Release of hemoglobin and potassium from human red blood cells treated under the critical micellar concentration. *Scanning Microscopy* 3:1241-1245.

2. Miseta, A., Kellermayer, M., Kollár, B. & Bogner, P.(1989): Paraproteins associated with low plasma sodium levels. *Physiolgical Chemistry and Physics and Medical NMR* 21:171-175.

3. Bogner, P., Skehan, P. Kenney, S. Sainz, E. Akeson, M.A. & Friedman, S.J. (1992): Stabilization of intercellular contacts in MDCK cells during Ca^{++} deprivation: selective effects of monocarboxylic acids on desmosomes. *Journal of Cell Science* 103:463-473.

4. Bogner, P., Berke, T. & Kellermayer, M. (1992): Motional characteristics of K+ and Na+ in intact and sucrose-permeabilized rat lymphocytes. *Physiological Chemistry and Physics & Medical NMR* 24:281-288.

5. Ting, S.M., Bogner, P. & Dignam, J.D. (1992): Isolation of prolyl-tRNA synthetase as a free form and as a form associated with glutamyl-tRNA synthetase. *Journal of Biological Chemistry* 267:17701-9.

6. Miseta, A., Bogner, P., Berényi, E., Kellermayer, M., Galambos C., Wheatley, D.N. & Cameron, I.L. (1993): Relationship between cellular ATP, potassium, sodium and magnesium concentrations in mammalian and avian erythrocytes. *Biochimica et Biophysica Acta* 1175:133-139.

7. Miseta, A., Bogner, P., Kellermayer, M., Wheatley, D.N., Takács, I., Nagy, G. & Cameron, I.L. (1993): Erythrocyte potassium and sodium

polymorphism in donkey (Equus asinus). *Comparative Biochemistry and Physiology* 106A:479-482.

8. Geraldes, C.F.G.C., Sherry, A.D., Lázár, I., Miseta, A., Bogner, P., Berényi, E., Sümegi, B., Kiefer, G.E., McMillan, K., Maton, F. & Muller, R.N. (1993): Relaxometry, animal biodistribution and magnetic resonance imaging studies of some new gadolinium (III) macrocyclic phosphinate and phosphonate monoester complexes. *Magnetic Resonance in Medicine* 30:696-703.

9. Wheatley, D.N., Miseta, A., Kellermayer, M., Galambos, C., Bogner, P., Berényi, E. & Cameron, I.L.(1994): Cation distribution in mammalian red blood cells: interspecies and intraspecies relationships between cellular ATP, potassium, sodium and magnesium concentrations. *Physiological Chemistry and Physics and Medical NMR* 26:111-118.

10. Berényi, E., Bogner, P., Miseta, A., Kövér, G., Horn, P. & Wheatley, D.N. (1994): Pre-slaughter characteristics of proton nmr relaxation parameters in malignant hyperthermia susceptible pigs. *Sciences des Aliments* 14:461-469.

11. Kellermayer, M., Ludány, A., Miseta, A., Kőszegi, T., Berta, G., Bogner, P., Hazlewood, C.F., Cameron, I.L. & Wheatley, D.N. (1994): Release of potassium, lipids and proteins from non-ionic detergent treated chicken red blood cells. *Journal of Cellular Physiology* 159:197-204.

12. Miseta, A., Bogner, P., Szarka, Á., Kellermayer, M., Galambos, Cs., Wheatley, D.N., Cameron, I.L. (1995): Effect of non-lytic concentrations of Brij-series detergents on the metabolism-independent ion permeability properties of human erythrocytes. *Biophysical Journal* 69:2563-2568.

13. Berényi, E., Szendrő, Zs., Rózsahegyi, P., Bogner, P. & Sulyok, E. (1996): Postnatal changes water content and proton magnetic resonance relaxation times in newborn rabbit tissues. *Pediatric Research* 39: 1091-1098.

14. Bogner, P., Berényi, E., Miseta, A., Horn, P., Kellermayer, M., Wheatley, D.N. & Jolesz, F.A.: Changes of nmr relaxation parameters of muscle tissue in malignant hyperthermia susceptible pigs. (in press, *Academic Radiology*)

15. Bogner, P., Wheatley, D.N. Borbély, C. & Miseta, A. (1996): Albumin-mediated reversal of potassium depletion in human erythrocytes treated with the non-ionic detergent, Brij 58. (submitted to *Cell Biology International*)

Köszönetnyilvánítás

Köszönetemet szeretném kifejezni minden munkatársamnak Pécsett és Kaposvárott, akik segítettek munkámban az elmúlt évek során.

Köszönettel tartozom dr. Kellermayer Miklósnak (a Mester), aki diákkörös éveim óta, 1983-tól tanított és segített.
Köszönök mindent dr. Ludány Andrea és dr. Kőszegi Tamás kollegáimnak, hogy úgy hallgatóként, mint munkatársként tudásukkal, tanácsaikkal mindig segítségemre voltak.
Örömmel dolgoztam/dolgozom együtt dr. Miseta Attila barátommal, akivel úgy gondolom jó dolgokat csináltunk, és remélem fogunk is.
A nagy csapatból, amit a jelenlegi és régebbi munkatársak és tanítványok alkotnak, ki kell emelnem néhány nevet, akiket szintén köszönet illet, dr. Nagy Esztert, dr. Csutora Pétert, dr. Borbély Csabát, dr. Szűcs Györgyöt, dr. Berke Tamást, dr. Galambos Csabát, dr. Süle Norbertet, Szarka Ágnest, dr. Henics Tamást, dr. Jilling Tamást.

Nagyon sokat köszönhetek dr. Repa Imre és dr. Berényi Ervin barátaimnak, akik tudományos munkámat önzetlenül támogatták és segítették. Bízom benne, hogy közös munkánk még sokáig folytatódhat.

Külföldi tanulmányutam során és itthon jónéhány kiváló kutatóval találkoztam, akik közül feltétlenül szeretném megemlíteni a következőket: dr. David Dignam, dr. Susan Friedman, dr. Gilbert Ling, dr. Denys Wheatley, dr. Carlton Hazlewood, dr. Jolesz Ferenc, dr. Ivan Cameron, dr. Jim Clegg, dr. Sümegi Balázs, dr. Somogyi Béla.

A kísérletes munkában rengeteg segítséget kaptam Pappné Bácskai Saroltától, Fábián Jánosnétől és Stocker Lajosnétől, amit ezúton köszönök.