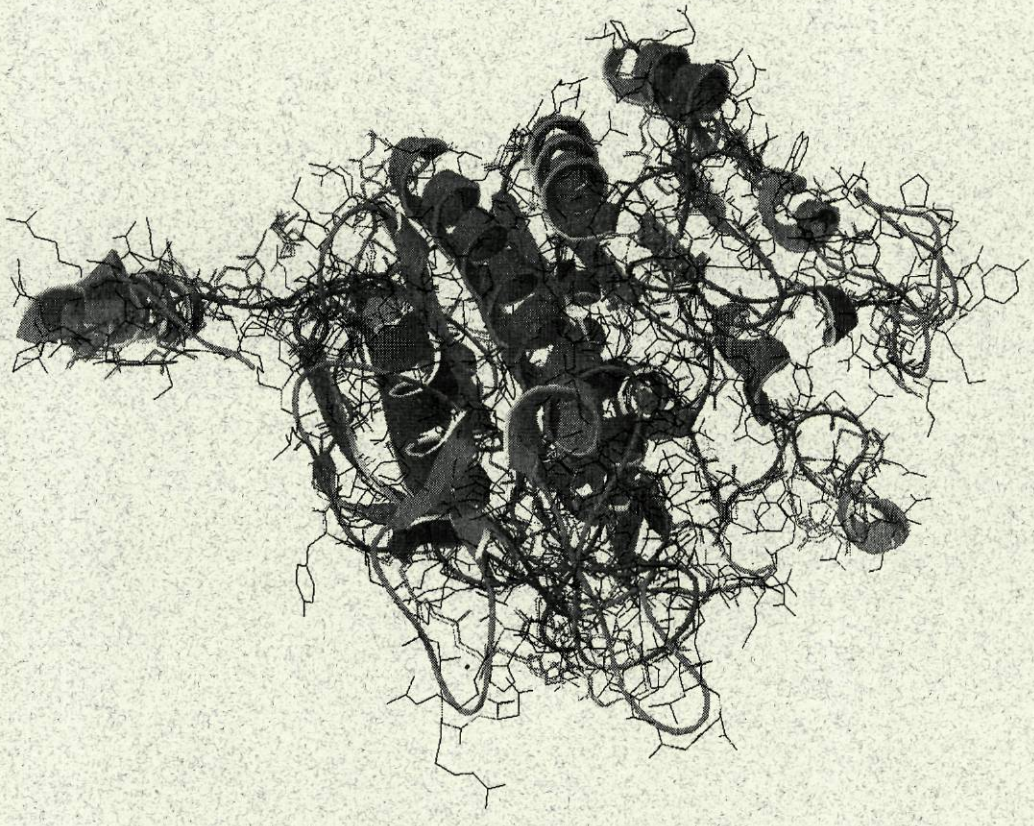
Maria João dos Reis Conceição Martins



Fosfátase Alcalina

Actividades em diversas situações fisiológicas e patológicas
Relação com sistemas de transporte transmembranar

Outubro de 2001

Dissertação de candidatura ao grau de doutora apresentada
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ABREVIATURAS

AMP:	Adenosina-5'-monofosfato
2A2M1P:	2-Amino-2-metil-1-propanol
ARN:	Ácido ribonucleico
CFTR:	"Cystic fibrosis transmembrane conductance regulator"
Edta:	Ácido etilenodiaminotetracético
FA:	Fosfátase alcalina
FAtne:	Fosfátase alcalina tecidual não específica
FAint:	Fosfátase alcalina intestinal
FApl:	Fosfátase alcalina placentária
FAcg:	Fosfátase alcalina das células germinais
hArg-L:	Homoarginina-L
Leu-L:	Leucina-L
IBMX:	3-Isobutil-1-metilxantina
MRP:	"Multidrug resistance protein"
Phe-L:	Fenilalanina-L
Pgp:	Glicoproteína-P
Trp-L:	Triptofano-L
Tris:	Tris-(hidroximetil)-aminometano

SUMÁRIO

1 – INTRODUÇÃO

O trabalho apresentado nesta dissertação engloba o estudo e o desenvolvimento de métodos simultaneamente adequados à sua utilização quer no doseamento da actividade da fosfátase alcalina sérica total quer na revelação e doseamento das fracções electroforéticas da enzima. Utilizando os métodos apresentados, aprofundámos o conhecimento da modulação da actividade sérica total da fosfátase alcalina e da actividade das fracções electroforéticas da enzima em diferentes condições de nutrição. Este trabalho de dissertação compreende também o estudo, no soro e/ou em homogeneizados de tecidos, da modulação da referida actividade enzímica por compostos endógenos e fármacos (substratos e moduladores não só da enzima como também de diferentes sistemas de transporte), mas utilizando um método experimental distinto dos apresentados por nós. A selecção dos compostos endógenos esteve, em parte, relacionada com os resultados respeitantes à modulação da actividade da enzima nas condições de nutrição analisadas. A escolha dos fármacos incluídos no referido estudo baseou-se, parcialmente, nos resultados obtidos com os compostos endógenos. Adicionalmente, modulando-se a actividade da fosfátase alcalina (por alteração do valor de pH do meio de incubação e pela presença de inibidores e activadores da enzima – seleccionados, em parte, de entre os fármacos anteriormente usados) investigou-se, em hepatócitos isolados, a possível relação entre a fosfátase alcalina e o transporte de um dos compostos endógenos testados anteriormente.

A designação da enzima (FA; EC 3.1.3.1) foi atribuída, em 1961, pelo Comité de Nomenclatura da União Internacional de Bioquímica e Biologia Molecular (IUBMB) e tem como base a sua actividade catalítica. Na classe EC 3 estão incluídas as enzimas que foram classificadas como hidrólases; na subclasse EC 3.1 as hidrólases que actuam sobre ligações éster e na sub-subclasse EC 3.1.1 as hidrólases que actuam sobre mono-estéres fosfóricos[1]. As propriedades que conferem

[1] Ver: a) IUBMB (2001). Enzyme nomenclature [Internet]. Queen Mary, University of London. Disponível em: http://www.chem.qmw.ac.uk/iubmb/enzyme/ [19 Setembro 2001]; b) Schomburg D (2001). BRENDA - The comprehensive enzyme information system [Internet]. Institute of Biochemistry, University of Cologne. Disponível em: http://brenda.bc.uni-koeln.de/ [2 Outubro 2001]; e c) Proteome informatics group, SWISS-PROT group, GlaxoSmithKline R&D S.A., Two-dimensional gel electrophoresis laboratory of the Geneva University Hospital (2001). ExPASy – Enzyme [Internet]. Swiss Institute of Bioinformatics, Geneva. Disponível em: http://www.expasy.ch/enzyme/ [9 Outubro 2001].

individualidade à FA foram caracterizadas *in vitro* e são diferentes das actividades de outras fosfátases como, por exemplo, a fosfátase do fosfato 6 de glicose e a pirofosfátase inorgânica. Caracteristicamente, os valores máximos de actividade, quando se usam concentrações de ordem milimolar de certos substratos, são obtidos em meios de ensaio com valores de pH muito superiores ao pH fisiológico. Todos os substratos conhecidos contêm um resíduo fosfato numa posição terminal cuja ligação à restante molécula sofre rotura hidrolítica durante o processo catalítico. A FA pode também funcionar como transférase: em determinados sistemas de ensaio, o resíduo fosfato em posição terminal na molécula do substrato dador é transferido para um substrato aceitador. A FA constitui um sistema de múltiplas formas enzímicas, em concreto isozimas e isoformas, cuja heterogeneidade se deve, respectivamente, a factores genéticos e a modificações ocorridas após a síntese proteica. As diferentes isozimas são codificadas por diferentes genes e modificações pós-tradução em cada uma delas originam as várias isoformas (1 - 60).

Nos procariontes a FA é segregada para o espaço periplasmático. Nos eucariontes a FA encontra-se ligada ao exterior da superfície celular sendo, por isso, classificada como uma ecto-enzima. Esta característica está na base do uso da FA como marcador enzímico no processo de isolamento e purificação de membranas celulares. O glicosilfosfatidilinositol funciona como âncora glicosídica, estabelecendo a ligação entre o carboxilo terminal da enzima e a membrana plasmática. A FA apresenta-se organizada em agregados (14, 18, 20 - 23, 28, 30, 34 - 38, 40 - 43, 45 - 48, 51 - 58, 60 - 85).

No Homem existem várias isozimas da FA: a FAtne, a FAint, a FApl e a FAcg. Funcionalmente podem distinguir-se na estabilidade ao calor, no pH óptimo, na afinidade relativamente a substratos e na inibição (ou activação, ver discussão) por determinados compostos (3, 7 - 14, 16, 20, 24, 25, 27 - 29, 34 - 37, 39, 42, 43, 45, 49, 50, 55, 58, 59, 62, 64, 67, 68, 70, 73, 74, 77, 80 - 82, 85 - 117).

A FAtne encontra-se em quase todos os tecidos sendo muito mais abundante no osso e rim e, um pouco menos, no fígado. No caso dos outros três proteídos, embora a especificidade relativamente ao tecido que lhes confere o nome não seja absoluta, eles são conhecidos como isozimas teciduais específicas, e a designação de cada um deles reflecte o local de expressão mais elevada. A existência de uma FAint com propriedades únicas e uma elevada actividade específica é uma característica dos mamíferos; o proteído responsável pela actividade fosfatásica alcalina no intestino de

peixes e répteis é comum a outros tecidos e apresenta características enzímicas semelhantes às da FAtne. No Rato existem duas isozimas intestinais distintas e a FAtne expressa-se em quantidades elevadas na placenta. A FApl só é expressa no Homem e noutros primatas (11, 16, 18, 20 - 22, 24, 26 - 28, 30, 31, 33, 36, 37, 42 - 45, 47, 49, 50, 55, 58, 60, 64, 67, 69 - 71, 74 - 76, 78, 85, 96, 101, 118 - 130).

A expressão do gene da FAtne na placenta humana diminui, desaparecendo totalmente, ao longo do primeiro trimestre da gravidez, sendo acompanhada pelo surgimento da expressão do gene da FApl. Esta isozima começa a aumentar na corrente sanguínea entre o primeiro e o segundo trimestres observando-se um aumento mais intenso no terceiro trimestre. Este aumento é, por exemplo, frequentemente, ainda mais marcado em situações de pré-eclâmpsia e eclâmpsia. Na gravidez humana, entre o segundo e terceiro trimestres, é comum observar-se um aumento da FAtne óssea na corrente sanguínea que é, no entanto, inferior ao da FApl (8, 10, 11, 25, 26, 32, 42, 45, 47, 55, 62, 64, 67, 71, 86, 92, 131). A FAtne sérica de origem óssea aumenta na osteoporose, no raquitismo, na osteomalácia e na doença de Paget (25, 33, 42, 43, 45, 87, 88, 132).

A FApl, foi uma das primeiras enzimas a ser reconhecida como um proteído oncofetal, tendo sido originalmente identificada num doente (Regan) com carcinoma pulmonar de células pavimentosas. Posteriormente, foram descobertas a FAcg ("placental alkaline phosphatase like") num doente (Nagao) com carcinoma pleural e a FAint fetal ("intestinal alkaline phosphatase like"), uma isoforma da FAint do adulto, num doente (Kasahara) com hepatoma. A expressão eutópica ou ectópica da FA pode ocorrer em tumores, metástases, lesões pré-cancerosas e linhas celulares relacionadas. Em doentes com seminoma tem-se observado, invariavelmente, produção muito elevada de FAcg. No entanto, observou-se que a FA estava aumentada somente em parte dos doentes com um determinado tipo de tumor (com a excepção do seminoma), que um mesmo tipo de tumor podia estar associado à expressão de mais do que uma isozima e que a expressão de uma dada isozima podia estar associada a mais do que um tipo tumoral. A lesão dos tecidos produtores de FA induz alterações na sua libertação para a corrente sanguínea, tendo sido possível mostrar uma correlação entre desenvolvimento tumoral e níveis séricos de isozimas ou isoformas da FA. Por exemplo, em doentes com cancro da próstata ou da mama, os níveis séricos da FAtne óssea correlacionam-se com a presença de metástases ósseas, podendo o seu doseamento ser útil na avaliação clínica daqueles doentes. A

FAtne hepática associada a fragmentos de membrana plasmática ("membrane bound liver alkaline phosphatase: mem-LiALP"), uma das isoformas da FAtne, embora também possa estar presente no soro de adultos saudáveis em muito pequenas quantidades, aumenta em doentes com metástases hepáticas ou colestase. O aumento da FAtne hepática sérica que se observa nestas situações patológicas pode ser uma consequência do aumento, mais ou menos proeminente, de todas as isoformas da FAtne hepática (10, 11, 15 - 17, 22, 24 - 28, 31, 32, 35 - 37, 40, 42, 43, 45, 47, 48, 55, 56, 62, 64, 67, 70, 72, 74, 85, 87, 88, 92, 100, 101, 129, 130, 132 - 148).

A libertação de cada uma das isozimas e isoformas da FA para a corrente sanguínea, o lúmen intestinal ou a bile está na dependência de situações fisiológicas e patológicas. A estrutura e composição de cada uma das isozimas após libertação também contribui para a variabilidade das isoformas associadas a cada isozima. Na prática clínica corrente efectua-se com frequência o doseamento da actividade fosfatásica alcalina total no soro. O mecanismo e a velocidade com que as diversas isozimas da FA são eliminadas da circulação sanguínea também condicionam os seus níveis séricos, sendo que o conhecimento do modo de eliminação de cada uma delas é importante para uma correcta interpretação desses níveis e consequentemente do valor da actividade fosfatásica alcalina total (1, 8, 10 - 13, 15 - 18, 21 - 28, 31 - 37, 40 - 45, 47 - 49, 51, 53, 55, 56, 60, 62 - 65, 67, 69, 70, 72 - 78, 86 - 88, 92, 95 - 98, 100 - 102, 105, 118, 128 -144, 146 - 158).

O doseamento espectrofotométrico ou espectrofluorimétrico da actividade sérica total da FA e das respectivas isozimas e isoformas assim como da actividade em tecidos ou noutras amostras biológicas tem sido efectuado a diferentes valores de pH e com recurso a diferentes amortecedores de pH [como por exemplo glicina, carbonato/bicarbonato, 2A2M1P, dietanolamina (com ou sem succinato), N-metil-D-glucamina e Tris] e a diferentes substratos (como por exemplo α- e β-naftilfosfatos, β-glicerolfosfato, p-nitrofenilfosfato, fenolftaleínamonofosfato, fenolftaleínadifosfato, fenilfosfato e o-carboxifenilfosfato) (1 - 10, 12 - 14, 16, 18 - 21, 23 - 25, 28, 29, 31 - 35, 39, 41, 42, 46 - 49, 52, 53, 58, 59, 62, 63, 66, 68, 73 - 81, 83, 85 - 95, 97 - 103, 105, 106, 108 - 110, 112 - 115, 118, 128, 130 - 134, 141, 142, 149, 152 - 154, 157, 159 - 169). A grande variabilidade nos valores de actividade obtidos com diferentes métodos, associada à variabilidade dos próprios métodos, dificulta a comparação (e interpretação) entre os dados de diferentes laboratórios e/ou disponíveis na literatura científica. A conveniência do uso, em Bioquímica Clínica, de condições iguais no doseamento da FA sérica total

justifica que, internacionalmente, tenham sido recomendados determinados métodos (5, 6, 8, 12, 13, 170).

A variedade de metodologias também é elevada nos estudos de identificação e doseamento espectrofotométrico ou espectrofluorimétrico da actividade das isozimas e isoformas da FA separadas por electroforese. Refira-se a título explicativo que as fracções enzímicas obtidas após separação electroforética da FA sérica total poderão ou não representar mais do que uma isozima ou isoforma da FA. Para além de outras variantes técnicas, como o suporte da electroforese, o campo eléctrico aplicado e o tratamento da amostra, têm sido usados na revelação da electroforese diferentes substratos (como por exemplo α- e β-naftilfosfatos, p-nitrofenilfosfato, 4-metilumbeliferilofosfato e 5-bromo-4-cloro-3-indolilfosfato), diferentes amortecedores de pH (como por exemplo 2-amino-2-metil-1,3-propanediol, 2A2M1P, Tris-borato e etilaminoetanol) e diferentes valores de pH (9, 16, 18 - 20, 23, 24, 27, 31, 33, 35, 42, 48, 49, 52, 62, 63, 66, 69, 73, 83, 88, 91, 92, 94, 95, 97, 98, 100, 102, 118, 132 - 134, 141 - 143, 149, 152, 157, 163, 164, 167).

Na maior parte dos estudos que de algum modo comparam (ou interpretam em conjunto) a actividade sérica total da FA com a actividade das fracções obtidas por separação electroforética, é frequente usarem-se condições de ensaio distintas nos dois tipos de doseamento referidos (9, 10, 33, 49, 73, 83, 88, 91, 92, 95, 97, 102, 132, 142, 152) o que, na nossa opinião, pode ser fonte de equívocos.

A actividade sérica total da FA corresponde ao somatório da actividade de cada uma das isozimas séricas e a actividade de cada uma dessas isozimas pode ser distintamente influenciada pelo substrato, amortecedor de pH e valor de pH usados na quantificação. Por este motivo, o valor da actividade enzímica total medida no soro depende não só da proporção das diferentes isozimas na amostra em estudo mas também da influência diferencial que as condições de ensaio têm na actividade de cada uma delas. Assim, quando se define como objectivo determinar, por electroforese, o contributo relativo de cada uma das isozimas para a actividade sérica total em determinadas condições experimentais, deverão ser utilizadas essas mesmas condições na quantificação da actividade sérica total e das suas fracções electroforéticas. Porque as condições de ensaio influenciam de modo diferente as distintas isozimas, as percentagens de actividade das fracções medidas em determinadas condições são válidas para essas condições e não podem, em princípio, ser extrapoladas para outras. No entanto, pouca atenção se tem dado a esta

problemática e ao desenvolvimento de técnicas em que se usem as mesmas condições de ensaio nos dois tipos de doseamento referidos (3, 7 - 10, 12, 13, 99, 131, 141, 149, 163, 164, 166, 167).

A FA existe em muitos tecidos, como já mencionado, e num amplo leque de seres vivos: desde o *Dictyostelium discoideum* até ao Homem. Apesar de esta distribuição indiciar que a FA tem uma função fundamental nos seres vivos, o seu papel fisiológico e os seus substratos *in vivo* não estão ainda totalmente esclarecidos (9, 10, 20, 30, 37, 38, 42, 43, 45, 47, 53 - 55, 57 - 59, 68, 79 - 82, 85, 93, 106, 111, 112, 114, 130, 158, 171 - 175).

É possível que a FA tenha um papel importante na meiose. A FA foi também associada ao desenvolvimento embrionário, ao processo mitótico e à apoptose. A FAtne poderá também estar envolvida em actividades relacionadas com a diferenciação (tais como interacções célula-célula ou célula-substrato), proliferação e migração celulares (37, 38, 68, 71, 79, 82, 106, 175). A FAtne poderá desempenhar um papel importante nos sistemas de sinalização mediada por receptores purinérgicos, quer terminando a resposta dos receptores P2 quer provocando a formação de adenosina, o agonista fisiológico dos receptores P1, hidrolisando o AMP (59). Vários dados experimentais apoiam a possibilidade das FAint e FAtne terem uma função importante na defesa imunológica (53, 54). A FAtne é considerada como um marcador da barreira hemato-encefálica, embora pouco se saiba sobre o papel funcional desta enzima no sistema nervoso (57). Foi recentemente proposto que a FA possa estar envolvida na regulação de sistemas de transporte na barreira hemato-encefálica, pois a modulação da sua actividade está associada à modulação do transporte de catiões orgânicos e à modulação da internalização da insulina na linha celular RBE4 (derivada de células endoteliais de microvasos do cérebro do Rato) (111, 112, 114).

A identificação, no gene da FAtne, de mutações associadas à hipofosfatasemia humana, e a demonstração que ratinhos "knock-out" para a FAtne são um bom modelo da forma infantil daquela doença, permitiram estabelecer que esta enfermidade resulta de um erro inato do metabolismo e que a FAtne possui, *in vivo*, um papel fundamental na mineralização óssea, formação da dentição e desenvolvimento pós-natal. As mutações referidas são responsáveis pela diminuição ou ausência da actividade catalítica da FAtne que, em certos casos, poderá ter origem na diminuição da estabilidade do ARN mensageiro ou na degradação da própria isozima em consequência de uma estrutura alterada. Além disso, também se observou

que os fibroblastos de doentes com hipofosfatasemia têm baixa actividade ecto-fosfatásica relativamente ao fosfato de piridoxal e à fosfoetanolamina. O facto de estes dois compostos, e também o pirofosfato inorgânico, se acumularem endogenamente na hipofosfatasemia, indica que estas substâncias são substratos naturais da FAtne. Em consonância com a ideia daqueles compostos poderem ser substratos de mais do que uma isozima da FA está a observação que, reflectindo a expressão da FApl na placenta, na mulher com hipofosfatasemia as concentrações do fosfato de piridoxal, da fosfoetanolamina e do pirofosfato inorgânico se normalizam durante a gravidez. Possivelmente, não só a actividade enzímica da FAtne como também a sua ligação ao colagéneo contribuem para o seu mecanismo de acção na mineralização óssea. Esta mineralização extracelular mediada pela FAtne é consistente com um hipotético papel da enzima em processos de mineralização patológica como a que pode ocorrer no endotélio vascular de arteríolas (10, 11, 20, 28, 30, 36, 42, 43, 45, 47, 53, 55, 58, 70, 80, 81, 85, 93, 106, 130, 158, 172 - 174, 176; E Mornet 2001, comunicação pessoal).

A concentração da FA em superfícies de troca entre o meio externo e o meio interno, ou entre dois compartimentos bem diferenciados, é compatível com uma função associada ao transporte transmembranar. Tal como no caso de múltiplas enzimas intracelulares, também a regulação da actividade de sistemas de transporte transmembranar por mecanismos de fosforilação/desfosforilação parece ser um fenómeno frequente. Dada a capacidade da FA para catalisar a hidrólise de resíduos fosfato, incluindo os ligados a proteídos, é possível que a FA tenha um papel na regulação da actividade de sistemas de transporte transmembranar: ou por desfosforilação directa dos mesmos ou por desfosforilação de moléculas que modulem a actividade dos transportadores (ver discussão).

2 – CONTRIBUIÇÃO EXPERIMENTAL PESSOAL

Nos artigos a seguir apresentados, realizados em colaboração[2], mostra-se um conjunto de resultados experimentais originais que permitiram:

(i) desenvolver metodologias para calcular a actividade sérica total da FA e a das suas fracções electroforéticas, evitando a interferência do efeito diferencial das condições de ensaio em cada uma das isozimas da FA (Artigos I, II e III);

(ii) sugerir o aproveitamento das condições de ensaio para a individualização e/ou conhecimento bioquímico e semiológico das fracções electroforéticas da FA (Artigos I, II e III);

(iii) mostrar que a ingestão de fibras de celulose, nomeadamente serrim, sem acesso a alimentos, aumentava, tal como os alimentos, a FAint sérica (Artigo II);

(iv) sugerir que os sais biliares conjugados sejam usados na discriminação *in vitro* entre a FAtne hepática e a FAint, por exemplo na identificação de situações fisiológicas ou patológicas em que se registe uma alteração das quantidades séricas relativas destas isozimas (Artigo IV);

(v) mostrar que não era indiferente o uso de isoformas da FAtne em estudos de modulação da actividade da FAtne (Artigo V);

(vi) sugerir que o levamisole, a teofilina, o IBMX, a lidocaína, a quinidina, a bupivacaína, o verapamil, o "kaempferol" e a genisteína possam ser usados na discriminação *in vitro* entre as isoformas hepática e renal da FAtne, por exemplo na identificação de situações patológicas em que se registe uma alteração das quantidades séricas relativas destas isoformas (Artigo V);

(vii) formular a hipótese da FAtne poder ser farmacologicamente manipulada *in vivo* (Artigo V);

(viii) formular a hipótese das FAtne hepática e renal poderem estar envolvidas na modulação de sistemas de transporte (Artigo V); e

(ix) sugerir que a FAtne hepática possa estar associada ao transporte transmembranar, nomeadamente captação, do taurocolato (Artigo VI).

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[2]A autora (com o nome Maria João Martins) teve uma contribuição determinante no tratamento, interpretação e discussão dos resultados apresentados nos Artigos I-VI, na realização da parte experimental descrita nos Artigos I, IV, V e VI e na redacção dos Artigos I, III, IV e V. Teve uma participação menos intensa na realização experimental descrita nos Artigos II e III e na redacção dos Artigos II e VI.

2.1 - Quantificação de actividades fosfatásicas alcalinas

2.1.a - Fosfátase alcalina sérica do Rato: uso do α- e do β-naftilfosfato na quantificação da sua actividade total e na sua caracterização electroforética (Artigo I).

2.1.b - Fracções electroforéticas da fosfátase alcalina sérica do Rato: variações com a alimentação, o jejum e a ingestão de fibras de celulose (Artigo II).

2.1.c - Fracções electroforéticas da fosfátase alcalina sérica do Rato: importância das condições de ensaio na sua revelação e na sua quantificação (Artigo III).

Artigo I

Use of α- and β-naphthyl phosphates in total serum alkaline phosphatase activity quantitation and electrophoretic characterization.

Maria J. Martins, Manuel N.M.P. Alçada, Paulo O. Dias, Cândido Hipólito-Reis.

Ital Biochem Soc Trans 1999; 13: 124.

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USE OF α- AND β-NAPHTHYL PHOSPHATES IN TOTAL SERUM ALKALINE PHOSPHATASE ACTIVITY QUANTITATION AND ELECTROPHORETIC CHARACTERIZATION

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Assay of total serum alkaline phosphatase (orthophosphoric-monoester phosphohydrolase, alkaline optimum, E.C. 3.1.3.1 ALP), which reflects individual isoenzymic activities, and electrophoretic characterization of serum ALP isoenzymic pattern (visualization and activity calculation) are frequently used in medical diagnosis. A given set of experimental conditions separately and independently affects each isoenzyme activity because each one possesses its own particular kinetic characteristics.

So results from both procedures will be dependent on initial serum isoenzymes proportions and on serum isoenzymes activity values modulation by assay conditions. Only if both techniques are equally influenced by experimental conditions a close and accurate comparison between activity values provided by them is possible. This fact has not been taken extensively into account.

In the present work, using serum from Wistar rats (starved for 24 h, 450-615 g, n=15), is described the development and/or adaptation and use of the same experimental conditions (meaning incubation medium) in quantifying total ALP and ALP electrophoretic isoenzymes. ALP isoenzymes calculated activity values can be obtained by multiplying the corresponding electrophoretic percentage by the total ALP activity. Two buffers both at pH 10.4 (carbonate/bicarbonate, 50 mM final concentration, and 2-amino-2-methyl-1-propanol/HCl, 250 mM final concentration) and two substrates (α- and β-naphthyl phosphates, 4 mM final concentration) were used. Absorbance readings of the corresponding naphthols were done at 530 and 540 nm, respectively.

Study of ALP total activity against incubation time (10, 15, 30 and 45 min for a-naphthyl phosphate and 5, 10 and 20 min for β-naphthyl phosphate) and sample volume (0.02, 0.04 and 0.08 ml of serum for α-naphthyl phosphate and 0.005, 0.01, 0.02 and 0.04 ml of serum for β-naphthyl phosphate) was performed for both buffers: linearity of results was always achieved. Electrophoresis was performed on cellulose acetate allowing the separation of two ALP fractions. ALP fractions electrophoretic percentage against incubation time (10, 15 and 30 min for both buffers and substrates) was studied. A slightly linear variation of results was obtained only with β-naphthyl phosphate. The methods described are sufficiently sensitive and ensure reproducibility of experimental results.

From the same electrophoretic separation of serum ALP fractions/isoenzymes, different values for its percentage and calculated activities can be obtained by changing the assay conditions used for ALP visualization. The aim was to present the same set of experimental conditions for both procedures and not to optimize experimental conditions neither propose methods to be used routinely in a clinical laboratory. As a conclusion of this work, attention should be drawn to the fact that the same assay conditions for both procedures should be used, enabling a correct quantitation of isoenzyme activity in the biochemical problem being studied in each case.

AM Seligman, HH Chauncey, MM Nachlas, LH Manheimer, HA Ravin (1951). The colorimetric determination of phosphatases in human serum. J Biol Chem 190: 7-15.

AL Babson, PA Read (1959). A new assay for prostatic acid phosphatase in serum. Amer J Clin Path 32: 88-91.

MJ Martins, PO Dias, C Hipólito-Reis (1998). Rat serum alkaline phosphatase electrophoretic fractions: variations with feeding, starvation and cellulose fibre ingestion. Clinical Nutrition 17: 279-285.

C Hipólito-Reis, PO Dias, MJ Martins (1999). The importance of assay conditions in visualization and quantitation of serum alkaline phosphatase isoenzymes separated by electrophoresis. Scandinavian Journal of Clinical and Laboratory Investigation. In press.

Artigo II

Rat serum alkaline phosphatase electrophoretic fractions: variations with feeding, starvation and cellulose fibre ingestion.

Maria J. Martins, Paulo O. Dias, Cândido Hipólito-Reis.

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Clinical Nutrition (1998) 17(6): 279–285

Rat serum alkaline phosphatase electrophoretic fractions: variations with feeding, starvation and cellulose fibre ingestion

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Abstract—The effect of feeding, starvation and fibre ingestion on alkaline phosphatase (ALP) activity (E.C. 3.1.3.1) was studied in Wistar rat serum. Using identical assay conditions for total ALP activity determination and for electrophoretic ALP isoenzymes/fractions activity calculation, α- and β-naphthyl phosphates and p-nitrophenyl phosphate were used as substrates and 2-amino-2-methyl-1-propanol/HCl was used as buffer, respectively. Total activity with β-naphthyl phosphate was significantly higher than with α-naphthyl phosphate and p-nitrophenyl phosphate; with α-naphthyl phosphate it was significantly higher than with p-nitrophenyl phosphate. With all substrates, fed animals had significantly higher total activity than starving ones. Electrophoresis allowed the separation of two fractions. The second fraction activity was significantly higher in the fed group than in the starving ones, irrespective of the substrate used. Starving animals with fibre showed higher values of this fraction than starving animals without fibre, the difference reaching statistical significance with α-naphthyl phosphate. The first fraction predominated in both starved groups and the second in the fed group. The second fraction was identified as intestinal ALP. We conclude that the mechanical stimulation of the digestive tract appears to influence the passage of intestinal ALP to serum. The experimental conditions used enable quantification of electrophoretic fractions based on total activity. Activity depends on the substrate used.

Key words: alkaline phosphatase electrophoretic fractions; alimentary status

Introduction

One of the most frequently used laboratory tests in medical diagnosis is the assay of total serum ALP (orthophosphoric-monoester phosphohydrolase, alkaline optimum, E.C. 3.1.3.1) activity. The electrophoretic separation of serum ALP isoenzymes/fractions, identification and quantification is also frequently used. They are performed in order to clarify pathological conditions, mainly in the context of liver and bone pathology (although it should be recognised that a placental or intestinal-like enzyme may be found in certain malignant conditions) or to characterise some physiological situations, such as bone growth and placental function (1–11).

The physiological role of this enzyme is not yet completely clear although several hypothesis have been proposed (3, 6, 11, 12). A modulatory role upon the function of P-glycoprotein in hepatocytes has been recently suggested (13, 14).

Up to now, the use of the same method for quantification of total serum ALP activity and for electrophoretic serum ALP isoenzymes/fractions identification, quantification and/or activity calculation has not been developed and systematically used (11, 15–31). This would have the advantage of an accurate comparison between activity results obtained by measurement of total ALP and by electrophoretic separation of ALP isoenzymes/fractions (11), enabling correct quantification of each isoenzyme/fraction activity.

In the present work the aim was not to optimize experimental assay conditions but to draw some attention to the fact that the same assay conditions for both these experimental procedures should be used. Therefore, under the same biological conditions, we applied the same experimental conditions in the assay of both total ALP activity and isoenzymes/fractions activities present in each separated electrophoretic band, of the same serum samples, using α- and β-naphthyl phosphates as substrates and 2-amino-2-methyl-1-propanol/HCl, pH 10.4, as buffer. p-Nitrophenyl phosphate was also used in the assay of total ALP but not in electrophoretic identification of ALP isoenzymes/fractions. Our results show the interest of the technical improvement developed by us and the importance of the experimental conditions used and alimentary status of the animals.

This study was carried out using rats under conditions of starvation with no access to cellulose fibre, rats under conditions of starvation with access to fibre and rats under ad libitum conditions of feeding. The second group was included after the observation that some starved rats that had ingested cellulose fibre showed total serum ALP levels higher than those of starved rats with no access to fibre. Our results suggest that the mechanical stimulation of the digestive tract influences the passage of intestinal ALP (IntALP) to serum.

Materials and methods

Reagents and animals

α- and β-naphthyl phosphates (sodium salts) as well as orthodianisidine (tetrazotized), L-phenylalanine, L-homoarginine and (-)-p-bromotetramisole were purchased from Sigma Chemical Co, St. Louis, Mo, USA; p-nitrophenyl phosphate (sodium salt) from Kock-Light Laboratories, Ltd, Colnbrook Berks, England; 2-amino-2-methyl-1-propanol from BDH, England; fast violet B from Difco Laboratory, West Molesey, Surrey, UK and the Sepraphore III cellulose acetate electrophoretic strips from Gelman Sciences, Ann Arbor, MI, USA. All other reagents were of the highest quality and purity available. In this work, male adult Wistar rats (free from liver and bone diseases), supplied by the Gulbenkian Institute of Sciences, Lisboa, Portugal, were used. The animals were kept in our laboratory under controlled environmental conditions (12 h light/dark cycle and room temperature 24°C) and fed with a suitable commercial diet, supplied by Harlan Interfauna Iberica, S. A., Barcelona, Spain (protein 15.00%, ashes 5.70%, cellulose 5.20%, fat 2.20%, also including the appropriate vitamins and minerals) and water ad libitum. The animals were divided into three groups of eight animals 24 h before the experimental work: starvation in the absence of cellulose fibre (routinely used for bedding inside the animal cages and non-metabolised by the enzymes of the digestive tract), starvation with access to cellulose fibre and feeding ad libitum. They were weighed at the beginning of the experimental work. The weight range was 487–548 g, 500–593 g and 501–556 g for the first, second and third group of animals, respectively. There were no significant differences among the mean weight values, according to Student's t-test. An extra group of four animals, within the same weight range of the fed animals group, was used for the identification of ALP electrophoretic fractions.

Enzymatic assays

Total serum ALP activity. ALP activity was assayed according to methods improved in our laboratory and based on the original work of Babson and Read (32), Seligman et al. (33) and Bessey et al. (34). 2-Amino-2-methyl-1-propanol/HCl buffer 250 mmol/L (final concentration), pH 10.4, was used in all quantifications. $MgCl_2$ 5 mmol/L (final concentration) was added in all assays. The final concentrations of the substrates were: 4 mmol/L for α- and β-naphthyl phosphates and 10 mmol/L for p-nitrophenyl phosphate. The activity determinations were done at 30°C for 30, 15 and 5 min with α- and β-naphthyl phosphates and p-nitrophenyl phosphate, respectively. Enzymatic reactions were stopped by addition of sodium citrate 75 mmol/L (final concentration), pH 5.2, trichloroacetic acid 5% (final concentration) or perchloric acid 0.6 mmol/L (final concentration) when the substrates used were α- and β-naphthyl phosphates or p-nitrophenyl phosphate, respectively. The diazoic reaction was performed for 3 min with orthodianisidine, tetrazotized, differently with both naphthols: 30 and 21.5 mmol/L (final concentration), immediately after and before stopping the reactions as referred to previously, with α- and β-naphthols, respectively. Trichloroacetic acid 3.6% (final concentration) was added after the reaction between α-naphthol and orthodianisidine. The water insoluble coloured products of the diazoic reactions were extracted with ethyl acetate and their absorbance determined at 530 and 540 nm, respectively. When p-nitrophenyl phosphate was used, the enzymatic reaction was stopped with perchloric acid, which was removed by centrifugation after being neutralized with KOH, 1.3 mmol/L (2.1 ml for 1.5 ml of supernatant). Absorbance was determined at 410 nm (Spectronic Genesys 5, Milton Roy, Rochester, NY, USA).

Serum ACP isoenzymes/fraction electrophoretic separation. Electrophoresis was carried out on cellulose acetate strips for 45 minutes at 300 V using the diethylbarbiturate/HCl buffer 60 mmol/L, pH 8.6. Strips were incubated with α- or β-naphthyl phosphates and 2-amino-2-methyl-1-propanol/HCl buffer for 15 minutes in a humidified chamber. ALP isoenzymes/fractions were stained with fast violet *B* 2 g/L and evaluated by densitometry at 530 nm (Beckman Instruments INC, California). Using the same buffer and substrate for total ALP activity determination and ALP electrophoretic isoenzymes/fractions identification, the activity values for each fraction were obtained by multiplying the corresponding percentage by total ALP activity. In densitometric analysis, we have considered the two most evident and constant fractions (Fig. 1), whilst accepting that they are heterogeneous (1, 5, 6, 11).

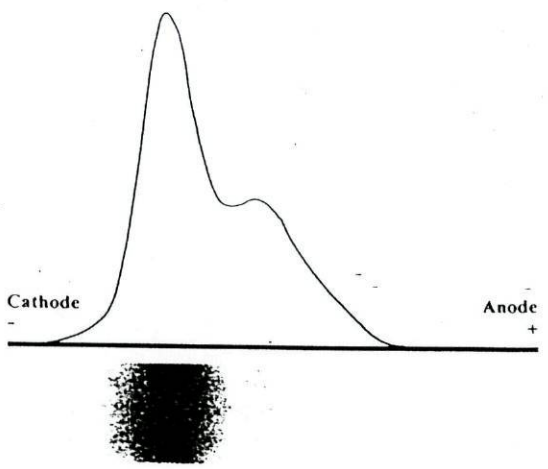

Fig. 1 Electrophoretic separation of serum alkaline phosphatase, from a fed rat, on cellulose acetate strips. Densitometric analysis of the two fractions at 530 nm after identification with β-naphthyl phosphate in 2-amino-2-methyl-1-propanol/HCl buffer and staining with fast violet *B*. The application point was near the cathode. The fast migrating fraction (anodal band) was named first fraction and the one presenting slower migration (cathodal band) was named the second fraction. First fraction is mainly tissue non-specific alkaline phosphatase and second fraction is mainly intestinal alkaline phosphatase.

Inhibition of serum ALP activity by various agents – identification of ALP electrophoretic fractions

L-Phenylalanine (5 mmol/L, final concentration), L-homoarginine (6 mmol/L, final concentration) and (-)-p-bromotetramisole (0.01 mmol/L, final concentration) (11) were added to the incubation medium used in total ALP and in ALP isoenzymes/fractions activities quantification, using β-naphthyl phosphate as the substrate.

Statistical analysis

All results are expressed as arithmetic mean ± standard deviation ($\bar{x} \pm SD$). The significance of differences between means was assessed by paired or unpaired Student's t-test.

Results

Total serum ALP activity

Table 1 shows the total mean ALP activity values obtained with different substrates in the serum of starved rats, with and without access to cellulose fibre, and fed ad libitum.

Total ALP activity obtained with β-naphthyl phosphate was always significantly higher than when α-naphthyl phosphate or p-nitrophenyl phosphate were used. The values obtained with α-naphthyl phosphate were always significantly higher than those obtained with p-nitrophenyl phosphate.

For each of the three substrates used, both groups of starved animals had statistically significant lower values of total ALP activity than the group of fed animals. When α- and β-naphthyl phosphates were used as substrates, the group without access to fibre showed a lower total ALP activity than the group with access to fibre, though the differences were not statistically significant. This fact was not observed with p-nitrophenyl phosphate. Many rats from this group had cellulose fibre in the gut.

Electrophoretic separation, percentage and calculated activity of the serum ALP fractions

Figure 1 shows the pattern of electrophoretic separation of the serum ALP from a fed rat. It also shows the corresponding electrophoretic strip. The application point was near the cathode. We label the fast migrating fraction (anodal band) the first fraction and the one presenting slower migration (cathodal band) the second fraction.

Figure 2 shows the percentage values of the two electrophoretic fractions above referred, using α- and β-naphthyl phosphates as substrates in the three experimental groups.

For both substrates, the second fraction showed the highest values in the fed group of animals and the first fraction was quantitatively the most important one under conditions of starvation.

In animals fed ad libitum or starving without access to cellulose fibre, the percentage values of the first fraction were higher when the substrate was α-naphthyl phosphate, whereas the percentage values of the cathodal band were higher when the substrate was β-naphthyl phosphate. For both animal groups, the difference between percentage values within each fraction obtained with both substrates was statistically significant.

For both substrates, the second fraction had significantly lower percentage values when the animals were starved than when the animals were fed. The second fraction percentage value was higher when the starving animals had access to fibre than when the animals did not have access to fibre. The difference was statistically significant when α-naphthyl phosphate was used.

In order to try to characterise the two fractions, differential inhibition studies of isoenzymes/fractions activity values, with serum from fed animals, were performed using L-phenylalanine, preferential inhibitor of IntALP, (-)-p-bromotetramisole and L-homoarginine, preferential inhibitors of tissue non-specific ALP (TNALP) (11).

Table 1 Total alkaline phosphatase activity values determined with different substrates in the serum of starved rats, with and without access to cellulose fibre, and fed ad libitum. Activity values found in starved animals without access to fibre were taken as 100%

Substrate	Starvation		Feeding	
	without fibre	with fibre	ad libitum	*P*
α-Naphthyl phosphate	0.039 ± 0.011 (a_1) 100%	0.050 ± 0.017 (a_2) 128%	0.081 ± 0.023 (a_3) 208%	($a_3 - a_2$) $P < 0.05$ ($a_3 - a_1$) $P < 0.005$ ($a_2 - a_1$) ns
β-Naphthyl phosphate	0.089 ± 0.038 (b_1) 100%	0.105 ± 0.035 (b_2) 118%	0.176 ± 0.075 (b_3) 197%	($b_3 - b_2$) $P < 0.05$ ($b_3 - b_1$) $P < 0.05$ ($b_2 - b_1$) ns
p-Nitro-phenyl phosphate	0.030 ± 0.007 (c_1) 100%	0.026 ± 0.005 (c_2) 87%	0.051 ± 0.007 (c_3) 170%	($c_3 - c_2$) $P < 0.005$ ($c_3 - c_1$) $P < 0.005$ ($c_2 - c_1$) ns
P	($a_1 - b_1$) $P < 0.05$ ($a_1 - c_1$) $P < 0.05$ ($b_1 - c_1$) $P < 0.005$	($a_2 - b_2$) $P < 0.005$ ($a_2 - c_2$) $P < 0.005$ ($b_2 - c_2$) $P < 0.005$	($a_3 - b_3$) $P < 0.005$ ($a_3 - c_3$) $P < 0.005$ ($b_3 - c_3$) $P < 0.005$	

Total activity values are expressed in μmoles of substrate metabolised per ml of serum per minute in the conditions described in Methods ($\bar{X} \pm SD$). The groups are composed of 8 animals with water ad libitum. Activity values were obtained form single determinations in the serum from each rat. ns: no significant difference.

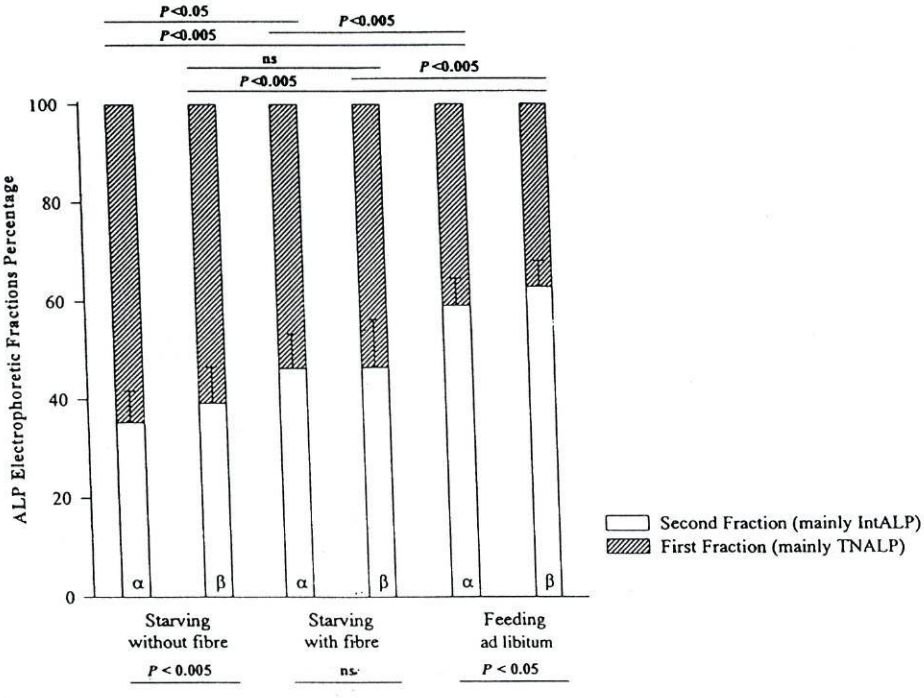

Fig. 2 Percentage values of alkaline phosphatase electrophoretic fractions determined with α- or β-naphthyl phosphates and 2-amino-2-methyl-1-propanol/HCl buffer in the serum of starved rats, with and without access to cellulose fibre, and fed ad libitum. First fraction (the fast migrating fraction) is mainly tissue non-specific alkaline phosphatase (TNALP) and second fraction (the slowest fraction) is mainly intestinal alkaline phosphatase (IntALP). ns: no significant difference.

Table 2 shows the results obtained when these inhibitors were added to the ALP incubation medium.

Owing to the poor resolution between liver and bone isoenzymes, found in cellulose acetate, and based on studies on the mechanism of serum ALP elevation in bile duct obstruction we suggest that our first fraction represents TNALP, including both bone and hepatic ALP (1, 5, 6, 11, 35, 36). Observing not only the isoenzyme patterns obtained (data not shown) for the three experimental groups, with both substrates, but also Figure 2, it can be seen that the second fraction increases with feeding and cellulose fibre ingestion. This information and the results of amino-acid

Table 2 Total alkaline phosphatase activity and alkaline phosphatase electrophoretic fractions calculated activity determined in the presence of alkaline phosphatase inhibitors values

Inhibitor	Total Activity	Calculated Activity	
		First Fraction (TNALP)	Second Fraction (IntALP)
Control	0.176 ± 0.042 (100%)	0.078 ± 0.012 (100%)	0.098 ± 0.030 (100%)
(-)-p-Bromotetramisole	0.118 ± 0.036 (67%)	0.055 ± 0.017 (71%)	0.063 ± 0.019 (64%)
L-Homoarginine	0.108 ± 0.034 (61%)	0.031 ± 0.014 (40%)	0.077 ± 0.030 (79%)
L-Phenylalanine	0.136 ± 0.021 (77%)	0.069 ± 0.005 (88%)	0.067 ± 0.016 (68%)

All values are expressed in μmoles of substrate metabolised per ml of serum per minute ($\bar{X}$ ± SD). Sera from 4 fed animals with water ad libitum were used. Values were obtained from single determinations in the serum from each rat. The activity of each fraction was calculated by multiplying the corresponding percentage by total alkaline phosphatase activity. First fraction is mainly tissue non-specific alkaline phosphatase (TNALP) and second fraction is mainly intestinal alkaline phosphatase (IntALP). Values obtained without alkaline phosphatase inhibitors were taken as 100%. L-Phenylalanine (5 mmol/L, final concentration). L-homoarginine (6 mmol/L, final concentration) and (-)-p-bromotetramisole (0.01 mmol/L, final concentration) were added to the incubation medium used in total alkaline phosphatase and in alkaline phosphatase isoenzymes/fractions activities quantification, using β-naphthyl phosphate as the substrate.

and (-)-p-bromotetramisole ALP inhibition indicate that the separation of the two fractions, under the electrophoretic conditions used, is not complete and that the first fraction consists primarily of TNALP while the second represents mainly intestinal ALP.

Table 3 shows the mean values of the calculated ALP activity of the two electrophoretic fractions considered.

For each fraction, and in all experimental groups, statistically significant differences were found between the ALP activity evaluated with α- and β-naphthyl phosphates: higher values were always obtained with the latter substrate. For α- and β-naphthyl phosphates, both starved without access to fibre and fed groups showed statistically significant differences between the calculated activity of the two fractions: the first fraction was higher in the first group and the second fraction in the second group.

The activity of the second fraction was significantly higher in the fed group than in the starving ones, irrespective of the substrate used.

When the starving animals had access to cellulose fibre, the second fraction activity was higher than when the animals did not have access to cellulose fibre. The difference was statistically significant when α-naphthyl phosphate was used.

Contrary to what was observed with the second fraction, the first fraction activity varied very little with the metabolic status of the animals. Only when α-naphthyl phosphate was used was a significant difference observed between the fed and the starving without fibre groups. Taking the ALP fractions calculated activity values found in starved animals without access to fibre as 100%, it can been seen that, with both substrates, second fraction calculated activity values increased more with fibre ingestion and feeding than first fraction calculated activity values.

Discussion

Since 1946 (34) p-nitrophenyl phosphate has been used as an ALP substrate in a vast number of experiments, mainly due to its relatively simple use. Several study groups use and/or recommend p-nitrophenyl phosphate as the substrate for ALP assays (37–40). p-Nitrophenyl phosphate is, however, not commonly used in electrophoretic isoenzymes/fractions identification although its use as a substrate for quantification of serum ALP isoenzyme activity after electrophoresis in polyacrylamide gels has been described (30). With this compound it is difficult to obtain insoluble, non diffusible and easily identifiable products on the electrophoretic separation medium.

Total serum ALP activity determination and/or ALP isoenzymes K_m calculation has been performed sometimes with α- and β-naphthyl phosphates using fluorimetric methods (41, 42) or specific light absorbance (43). Total serum ALP activity determination, using β-naphthyl phosphate as substrate, has also been performed by quantification of released β-naphthol, after its coupling with orthodianisidine, tetrazotized (33). Both substrates have already been used in ALP isoenzymes/fractions identification after its electrophoretic separation (15, 20, 23–25, 44–47).

The spectrophotometric method used in this work for quantification of total ALP activity was improved in our laboratory. It is sufficiently sensitive and ensures reproducibility of experimental results. Increasing the incubation time or the sample volume increased ALP activity (data not shown). Based on this work, it was possible to use the same method (the same experimental conditions, including incubation medium) for quantification of total ALP activity and for electrophoretic ALP isoenzymes/fractions identi-

Table 3 Alkaline phosphatase calculated activity values determined with different substrates. Serum electrophoretic fractions of starved rats, with and without access to cellulose fibre, and fed ad libitum. Calculated activity values found in starved animals without access to fibre were taken as 100%

Fraction/Substrate		Starvation without fibre	Starvation with fibre	Feeding ad libitum	*P*
1st Fraction (TNALP)	α-Naphthyl phosphate	0.025 ± 0.006 (a_1) 100%	0.027 ± 0.009 (a_2) 108%	0.033 ± 0.007 (a_3) 132%	($a_3 - a_2$) ns ($a_3 - a_1$) $P < 0.05$ ($a_2 - a_1$) ns
	β-Naphthyl phosphate	0.055 ± 0.024 (b_1) 100%	0.056 ± 0.018 (b_2) 102%	0.065 ±0.023 (b_3) 118%	($b_3 - b_2$) ns ($b_3 - b_1$) ns ($b_2 - b_1$) ns
2nd Fraction (IntALP)	α-Naphthyl phosphate	0.014 ± 0.005 (c_1) 100%	0.023 ± 0.008 (c_2) 164%	0.048 ± 0.016 (c_3) 342%	($c_3 - c_2$) $P < 0.005$ ($c_3 - c_1$) $P < 0.005$ ($c_2 - c_1$) $P < 0.05$
	β- Naphthyl phosphate	0.034 ± 0.014 (d_1) 100%	0.049 ± 0.017 (d_2) 144%	0.111 ± 0.052 (d_3) 326%	($d_3 - d_2$) $P < 0.05$ ($d_3 - d_1$) $P < 0.005$ ($d_2 - d_1$) ns
		($a_1 - b_1$) $P < 0.05$ ($c_1 - d_1$) $P = 0.005$ ($a_1 - c_1$) $P < 0.005$ ($b_1 - d_1$) $P = 0.005$	($a_2 - b_2$) $P < 0.005$ ($c_2 - d_2$) $P < 0.005$ ($a_2 - c_2$) ns ($b_2 - d_2$) ns	($a_3 - b_3$) $P < 0.005$ ($c_3 - d_3$) $P = 0.005$ ($a_3 - c_3$) $P < 0.05$ ($b_3 - d_3$) $P < 0.05$	

Calculated activity values are expressed in μmoles of substrate metabolised per ml of serum per min in the conditions described in Methods ($\bar{X} \pm SD$) and were obtained from single determinations in the serum from each rat. The groups are composed of 8 animals with water ad libitum. The activity of each fraction was calculated by multiplying the corresponding percentage by total alkaline phosphatase activity. First fraction is mainly tissue non-specific alkaline phosphatase (TNALP) and second fraction is mainly intestinal alkaline phosphatase (IntALP). ns: no significant difference.

fication, quantification and activity calculation, what has not been taken extensively into account (15–31, 44–46).

A given set of experimental conditions separately and independently affects each isoenzyme (11, 38). So, total serum ALP activity as well as serum ALP electrophoretic isoenzymes/fractions revelation, their percentage and activity calculation will be dependent on or influenced by initial serum isoenzymes/fractions proportions and experimental conditions. Only if both experimental procedures are equally influenced by assay conditions can the activity values for each isoenzyme/fraction be calculated based on total ALP activity (11, 37). Thus, a process was developed which enables quantification of each electrophoretic isoenzyme/fraction: multiplying the corresponding percentage by total ALP activity. For this purpose either α- or β-naphthyl phosphates could be used.

The observed total activity with α- and β-naphthyl phosphates was different and higher than that obtained with p-nitrophenyl phosphate (Table 1), enabling therefore a better understanding of the differences of activity when these naphthols monoesters were used as substrates. Total ALP activity was higher in the starving group with access to cellulose fibre than in the starving group without access to fibre. These differences, although without statistical significance, were observed with α- and β-naphthyl phosphates but not with p-nitrophenyl phosphate.

Independently of the substrate used, total ALP activity was always higher when the animals were fed ad libitum than when starved (Table 1). This confirms our previous results with p-nitrophenyl phosphate (2).

ALP isoenzymes/fractions separation with measurement of activity in each fraction has permitted a clearer characterisation of metabolic patterns. The most important fraction in the ad libitum feeding situation was the second (mainly IntALP) while under conditions of starvation the first (mainly TNALP) predominated. This was true for both percentage (Fig. 2) and calculated activity fraction values (Table 3). As to the effect of access to cellulose fibre by starving animals, activities present in the two electrophoretic fractions showed that these animals had significantly higher ALP activity in the second fraction than starved animals without fibre, when α-naphthyl phosphate was used.

These results suggest that we must take into due consideration the maintenance conditions of the animals (for example with and without cellulose fibre used in bedding). This, generally, is not taken into account under conditions of starvation.

It is known that after fat feeding ALP activity increases in serum (48–58). There are several hypothesis to explain/identify the pathways whereby IntALP could reach the serum (54, 55). In one of them bile salts, released into the intestinal lumen after a fatty meal, would be responsible for the shedding of membrane-bound IntALP rich vesicles and also for the release of anchor-bound IntALP (with the specific glycan phosphatidylinositol, GPI, anchor covalently attached to the COOH terminal of the enzyme), both from the shedded vesicles and from the enterocyte membrane. Soluble IntALP (IntALP isoform free from its GPI anchor) could be formed through the action of phospholipase D. Some of those IntALP isoforms might be implicated in the transport of lipids, either through enterocyte or via the intercellular space to the chyle and to the blood accompanying the absorption products (55).

Access to cellulose fibre by starving animals, who frequently ingested the fibre, suggests that the mechanical stimulation of the gut may be the primary cause to induce the passage of IntALP into the serum. Maybe the ingestion of fibre and the consequent mechanical stimulation of the intestine either increases desquamation of intestinal cells or the process of renewal of the brush border membrane or membrane shedding increasing the release of IntALP into the intestinal lumen and consequently its increase in serum (54).

Our results, despite enabling a consistent interpretation related to the different metabolic situations, by themselves do not enable a definitive determination of the origin of the electrophoretic fractions obtained from serum ALP. The shape of the curve that both fractions present in their electrophoretic pattern suggests their complexity. For example, in humans, TNALP consists of soluble bone ALP, eventually anchor-bound, and soluble liver ALP, eventually anchor-bound and/or membrane-bound; IntALP consists of one or more anchor-bound fractions and soluble IntALP (which is not the predominant form from IntALP unlike what is observed for TNALP) (6). Pursuing the study of the two fractions obtained (either by changing the electrophoretic media/suport or using specific ALP isoenzyme antibodies or using neuraminidase or performing extraction with organic solvents) will allow us to further clarify this complex pattern (6).

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References

1. Moss D W. Alkaline phosphatase isoenzymes. Clin Chem 1982; 28: 2007–2016
2. Hipólito-Reis C. Fosfátases alcalinas – Variações das actividades séricas e hepáticas com o jejum no rato. Arquivos de Medicina (Fac. Med. Porto) 1989; 3: 19–24
3. Epstein E, Kiechle F L, Artiss J D, Zak B. The clinical use of alkaline phosphatase enzymes. Clin Lab Med 1986; 6: 491–505
4. Moss D W. Perspectives in alkaline phosphatase research. Clin Chem 1992; 38: 2486–2492
5. Price C P. Multiple forms of human serum alkaline phosphatase: detection and quantification. Ann Clin Biochem 1993; 30: 355–372
6. Van Hoof V O, De Broe M E. Interpretation and clinical significance of alkaline phosphatase isoenzyme patterns. Crit Rev Clin Lab Sci 1994; 31: 197–293
7. Moss D W, Henderson A R. Methods for the determination of alkaline phosphatase activity. In: Burtis C A, Ashwood E R, eds. Tietz Textbook of Clinical Chemistry, 2nd Edition. Philadelphia: W B Saunders, 1994: 832–843
8. Balistreri W F, Rej R. Liver function. In: Burtis C A, Ashwood E R, eds. Tietz Textbook of Clinical Chemistry, 2nd Edition. Philadelphia: W B Saunders, 1994: 1449–1512

9. Endres D B, Rude R K. Mineral and bone metabolism. In: Burtis C A, Ashwood E R, eds. Tietz Textbook of Clinical Chemistry, 2nd Edition. Philadelphia: W B Saunders, 1994: 1887–1973
10. Moss D W. Electrophoresis of human alkaline and acid phosphatases. Clin Lab Med 1986; 6: 507–523
11. Crofton Ψ M. Biochemistry of alkaline phosphatase isoenzymes. Crit Rev Clin Lab Sci 1982; 16: 160–194
12. Hipólito-Reis C. Estudo bioquímico da intoxicação alcoólica – Reversão pelo etanol em dose elevada dos efeitos do jejum sobre a glicemia e actividades fosfatásicas alcalinas séricas. Arquivos de Medicina (Fac. Med. Porto) 1990; 4: 303–308
13. Martel F, Martins M J, Hipólito-Reis C, Azevedo I. Inward transport of [H^3]-1-methyl-4-phenylpyridinium in rat isolated hepatocytes: putative involvement of a P-glycoprotein transporter. Br J Pharmacol 1996; 119: 1519–1524
14. Martel F, Martins M J, Calhau C, Hipólito-Reis C, Azevedo I. Postnatal development of organic cation transport in the rat liver. Pharmacol Res 1998; 37: 131–136
15. Hodson A W, Latner A L, Raine L. Iso-enzymes of alkaline phosphatase. Clin Chim Acta 1962; 7: 255–261
16. Van Hoof V O, Lepoutre L G, Hoylaerts M F, Chevigne R, De Broe M E. Improved agarose electrophonetic method for separating alkaline phosphatase isoenzymes in serum. Clin Chem 1988; 34: 1857–1862
17. Rosalki S B, Ying Foo A. Two new methods for separating and quantifying bone and liver alkaline phosphatase isoenzymes in plasma. Clin Chem 1984; 30: 1182–1186
18. Demetriou J A, Beattie J M. Electrophoretic separation on agarose thin film of isoenzymes of alkaline phosphatase from human serum and tissue. Clin Chem 1971; 17: 290–295
19. Haije W G, De Jong M. Iso-enzyme patterns of serum alkaline phosphatase in agar-gel electrophoresis and their clinical significance. Clin Chim Acta 1963; 8: 620–623
20. Kaplan M M, Rogers L. Separation of human serum-alkaline-phosphatase isoenzymes by polyacrylamide gel electrophoresis. Lancet 1969; 2: 1029–1031
21. Fritsche H A, Adams-Park H R. High molecular weight isoenzymes of alkaline phosphatase in human serum. Demonstration by cellulose acetate electrophoresis and physico-chemical characterization. Clin Chim Acta 1974; 52: 81–89
22. Hamilton B A, McPhee J L , Hawrylak K, Stinson R A. Alkaline phosphatase releasing activity in human tissues. Clin Chim Acta 1989; 186: 249–254
23. Rhone D P, Mizuno F M. Separation of isoenzymes of alkaline phosphatase by substrate-gel imprint after electrophoresis on cellulose acetate. Clin Chem 1972; 18: 662–666
24. Kalimanovska V, Whitaker K B, Moss D W. Effect of bromelain on alkaline phosphatases of intestinal and non-intestinal tissues and serum. Clin Chim Acta 1987; 170: 219–226
25. Jennings R C, Brocklehurst D, Hirst M. Using starch-gel electrophoresis and sephadex gel-filtration with special reference to high molecular weight enzymes. Clin Chim Acta 1970; 30: 509–517
26. Rosalki S B, Ying Foo A. Incubation with neuraminidase and affinity electrophoresis with wheat-germ lectin compared for separating and quantifying alkaline phosphatase isoenzymes in plasma. Clin Chem 1985; 31: 1198–1200
27. Van Hoof V O, Hoylaerts M F, Geryl H, Van Mullem M, Lepoutre L G, De Broe M E. Age and sex distribution of alkaline phosphatase isoenzymes by agarose electrophoresis. Clin Chem 1990; 36: 875–878
28. Bentouati L, Baboli M S, Hachem H, Hamza M, Canal P, Soula G. Hyperphosphatasemia related to three intestinal alkaline phosphatase isoforms: biochemical study. Clin Chim Acta 1990; 189: 145–152
29. Johnson R B. A new fluorimetric method for the estimation or detection of total and fractionated alkaline phosphatase. Clin Chem 1969; 15: 108–123
30. Healy P J. Quantitative analysis of serum alkaline phosphatase isoenzyme activity. Clin Chim Acta 1974; 55: 407–409
31. Romel W C, LaMancusa S J, Dufrene J K. Detection of serum alkaline phosphatase isoenzymes with phenolphthalein monophosphate following cellulose acetate electrophoresis. Clin Chem 1968; 14: 47–57
32. Babson A L, Read P A. A new assay for prostatic acid phosphatase in serum. Amer J Clin Path 1959; 32: 88–91
33. Seligman A M, Chauncey H H, Nachlas M M, Manheimer L H, Ravin H A. The colorimetric determination of phosphatases in human serum. J Biol Chem 1951; 190: 7–15
34. Bessey O A, Lowry O H, Brock M J. A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. J Biol Chem 1946; 164: 321–329
35. Kaplan M M, Righetti A. Induction of rat liver alkaline phosphatase: the mechanism of the serum elevation in bile duct obstruction. J Clin Invest 1970; 49: 508–516
36. Hipólito-Reis C. Alkaline phosphatases – effects of bile duct obstruction on hepatic, renal, intestinal and serum activities in the rat. IV Portuguese – Spanish Biochemistry Congress. Póvoa de Varzim 1991; 7 P87-Mo
37. Bretaudière J-P, Vassault A, Amsellem L, Pourci M-L, Thie-Phung H, Bailly M. Criteria for establishing a standardized method for determining alkaline phosphatase activity in human serum. Clin Chem 1977; 23: 2263–2274
38. McComb R B, Bowers G N, Posen S, Eds. Alkaline phosphatase. New York: Plenum Press, 1979: 305–318
39. Committee on enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology. Recommended methods for the determination of four enzymes in blood. Scand J Clin Lab Invest 1974; 33: 291–306
40. International Federation of Clinical Chemistry, scientific committee, analytical section, expert panel on enzymes. IFCC methods for the mesurement of catalytic concentration of enzymes. Part 5. IFCC method for alkaline phosphatase (orthophosphoric-monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1). Clin Chim Acta 1983; 135: 339F–367F; and J Clin Chem Clin Biochem 1983; 21: 731–748
41. Moss D W. Kinetics of phosphatase action on naphthyl phosphates, determined by a highly sensitive spectrofluorimetric technique. Biochem J 1960; 76: 32P
42. Moss D W, Campbell D M, Anagnostou-Kakaras E, King E J. Characterization of tissue alkaline phosphatases and their partial purification by starch-gel electrophoresis. Biochem J 1961; 81: 441–447
43. Stevens J, Thomas F. Alkaline phosphatase: reaction rate analysis at 340 nm. Clin Chim Acta 1972; 37: 541–543
44. Moss D W, Edwards R K. Improved electrophoretic resolution of bone and liver alkaline phosphatases resulting from partial digestion with neuraminidase. Clin Chim Acta 1984; 143: 177–182
45. Lee L M Y, Kenny M A. Electrophoretic method for assessing the normal and pathological distribution of alkaline phosphatase isoenzymes in serum. Clin Chem 1975; 21: 1128–1135
46. Taswell H F, Jeffers D M. Isoenzymes of serum alkaline phosphatase in hepatobiliary and skeletal diseases. Am J Clin Pathol 1963; 40: 349
47. Smith I, Lighstone P J, Perry J D. Separation of human tissue alkaline phosphatases by electrophoresis on acrylamide disc gels. Clin Chim Acta 1968; 19: 499–505
48. Keiding N R. The alkaline phosphatase fractions of human lymph. Clin Sci 1964; 26: 291–297
49. Keiding N R. Intestinal alkaline phosphatase in human lymph and serum. Scand J Clin & Lab Invest 1966; 18: 134–140
50. Malagelada J R, Linscheer W C, Rufo M, Fishman W H. Two directional release of brush border alkaline phosphatase for fat absorption. Gastroenterology 1971; 60: 693
51. Madsen N B, Tuha J. On the source of the alkaline phosphatase in rat serum. J Biol Chem 1952; 195: 741–750
52. Young G P, Friedman S, Yedin S T, et al. Effect of fat feeding on intestinal alkaline phosphatase activity in tissue and serum. Am J Physiol 1981; 241: G461–G468
53. Day A P, Feher M D, Chopra R, et al. Triglyceride fatty acid chain length influences the post prandial rise in serum intestinal alkaline phosphatase activity. Ann Clin Biochem 1992; 29: 287–291
54. Alpers D H, Eliakim R, DeSchryver-Kecskemeti K. Secretion of hepatic and intestinal alkaline phosphatase: similarities and differences. Clin Chim Acta 1989; 186: 211–224
55. Van Hoof V O, Denj J T, De Broe M E. How do plasma membranes reach the circulation? Clin Chim Acta 1997; 266: 23–31
56. Moss D W. Release of membrane-bound enzymes from cells and the generation of isoforms. Clin Chim Acta 1994; 226: 131–142
57. Glickman R M, Alpers D H, Drummey G D, Isselbacher K J. Increased lymph alkaline phosphatase after fat feeding: effects of medium chain triglycerides and inhibition of protein synthesis. Biochim Biophys Acta 1970; 201: 226–235
58. Walker B A, Eze L C, Tweedie M C K, Price Evans D A. The influence of ABO blood groups, secretor status and fat ingestion on serum alkaline phosphatase. Clin Chim Acta 1971; 35: 433–444

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Artigo III

Importance of assay conditions in visualization and quantitation of serum alkaline phosphatase isoenzymes separated by electrophoresis.

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Importance of assay conditions in visualization and quantitation of serum alkaline phosphatase isoenzymes separated by electrophoresis

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Hipólito-Reis C, Dias PO, Martins MJ. Importance of assay conditions in visualization and quantitation of serum alkaline phosphatase isoenzymes separated by electrophoresis. Scand J Clin Lab Invest 1999; 59: 593–606.

The importance of separation and identification of serum alkaline phosphatase (ALP; E.C. 3.1.3.1) fractions/isoenzymes has been frequently reported. Each serum ALP fraction/isoenzyme quantitation has both practical and theoretical importance. In the present work, serum was collected from Wistar rats and, in identical experimental conditions, total serum ALP activity and serum ALP electrophoretic fractions/isoenzymes activities were quantified. Different results for both kinds of ALP activity were obtained when different buffers or mixture of these buffers (carbonate/bicarbonate; 2-amino-2-methyl-1-propanol/HCl; Veronal, sodium diethylbarbiturate/HCl), pH conditions (9.4 and 10.4) and substrates (α- and β-naphthyl phosphates) were used. Higher total serum ALP activity was always observed with β-naphthyl phosphate, independently of the buffer (or mixture of buffers) and pH used. Electrophoresis allowed the separation of two serum ALP fractions. Activity of both serum ALP electrophoretic fractions was always higher with β-naphthyl phosphate, except with carbonate/bicarbonate pH 10.4. The effect of a change in pH was buffer- (or mixture of buffers) and substrate-dependent; the addition of a second buffer (to that previously used) was not always accompanied by an increase or decrease (of the same magnitude) in our results. The results obtained with different buffers (or mixture of buffers) were not identical with substrates and pH values. It is concluded that (i) from the same electrophoretic separation of serum ALP fractions/isoenzymes, different values for its activity can be obtained by changing the assay conditions used for ALP visualization (revelation, staining); (ii) the same assay conditions for quantitation of total serum ALP and serum ALP electrophoretic fractions/isoenzymes should be used; (iii) the choice of assay conditions should take into account the biochemical problem being studied in each case.

Key words: Cellulose acetate; methods comparison

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INTRODUCTION

Human serum contains different alkaline phosphatase (ALP) fractions/isoenzymes (orthophosphoric-monoester phosphohydrolase, alkaline optimum, E.C. 3.1.3.1) in variable proportions, depending on physiological and pathological conditions. Thus, total serum ALP activity is the sum of serum fraction/isoenzyme activities. It is, therefore, important not only to quantitate total serum ALP, but also to identify and quantitate each serum ALP fraction/isoenzyme in order to allow clarification of those conditions. Placental ALP increases in maternal serum as pregnancy term approaches; ALP from liver and bone increases in serum with occurrence of obstructive liver disease and bone growth/rebuilding or osteomalacia, respectively. Although some situations are characterized by an elevated total serum ALP activity value, secondary to alterations in serum ALP fraction(s)/isoenzyme(s), the occurrence of a normal value for total serum ALP activity associated with an abnormal serum ALP electrophoretic isoenzyme pattern is possible [1–8].

It is important to bear in mind that ALP activity determination itself is peculiar in some ways; it can be modified by multiple factors in assay conditions. The enzyme reaction depends markedly not only on the type of buffer, buffer concentration and pH of the incubation medium in which it takes place, but also on the substrate used (the substrate specificity of the enzyme permits use of a wide variety of orthophosphoric-monoesters). All these factors are interdependent. A given set of experimental conditions separately and independently affects the activity of each isoenzyme, because each one possesses its own particular kinetic characteristics. Total serum ALP activity quantitation, as well as serum ALP electrophoretic fraction/isoenzyme visualization, their percentage and activity calculation is dependent on initial serum fraction/isoenzyme proportions and serum fraction/isoenzyme activity values modulation by assay conditions. If all experimental procedures are equally influenced by assay conditions, then close and accurate comparison between activities obtained by measurement of total serum ALP and after electrophoretic separation of serum ALP fractions/isoenzymes is possible [2, 9–24].

Separation, identification and/or quantitation of ALP fractions/isoenzymes are clinically very useful. For these purposes several methods have been described in the literature [1, 2, 5–7, 24–27]. Although these methods produce relatively good and reproducible results, some of them may be too laborious and time-consuming for routine work in a clinical laboratory. Others may lack sensitivity, may not allow good resolution or visualization of ALP fraction(s)/isoenzyme(s), or may even make it difficult to obtain quantitative results [1, 2, 5–7, 24–27].

Results on electrophoretic separation or characterization of ALP isoenzymes, their visualization and activity calculation were obtained varying the type of support medium and/or the strength of the electric field and/or using distinct substrates, buffers and pH values. Electrophoretic separation and identification of ALP fractions/isoenzymes has also been accomplished using neuraminidase or lectin, specific antibodies against ALP isoenzymes and ALP extraction with organic solvents [1, 2, 5–7, 13, 23–48]. In recent decades studies have been conducted for the purpose of improving electrophoretic separation and quantitation of ALP fractions/isoenzymes [6, 7, 31, 32, 34, 38–41, 43]. Currently, several commercial kits are available for electrophoretic separation of ALP fractions/isoenzymes and, when adequately used, these systems enable good and reproducible results [6]. However, until recently, the importance of using the same method for both quantitation of total serum ALP activity and serum ALP electrophoretic fraction/isoenzyme visualization and activity calculation has not been taken into account [8, 39–48].

Here, the results of a study of the influence of substrate (α- and β-naphthyl phosphates, α-NP and β-NP respectively), buffer (carbonate/bicarbonate; 2-amino-2-methyl-1-propanol/HCl, AMP; Veronal, sodium diethylbarbiturate/HCl) and pH value (9.4 and 10.4) on rat total serum ALP activity and rat serum ALP electrophoretic fraction/isoenzyme visualization (revelation, staining) and activity calculation are presented and discussed. The aim of this work was not to optimize experimental conditions, neither to propose routine methods to be used in a clinical laboratory, nor to present or study human or rodent ALP problems. Rather, we were particularly interested in drawing attention to the fact that the same assay conditions for both experimental procedures should be used,

enabling correct quantitation of each fraction/isoenzyme activity [23, 49].

MATERIALS AND METHODS

α-NP and β-NP (sodium salts), tetrazotized orthodianisidine (zinc chloride complex) and 2-amino-2-methyl-1-propanol were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and *fast violet B* from Difco Laboratories (West Molesey, Surrey, UK). All other reagents were of the highest quality and purity available.

Animals

In this study, eight male adult Wistar rats (free from liver and bone diseases), supplied by the Gulbenkian Institute of Sciences, Lisboa, Portugal, were used. The animals were kept in our laboratory under controlled environmental conditions (12 h light/dark cycle and room temperature 24°C) and fed with a suitable commercial diet, supplied by Harlan Interfauna Iberica S.A. (Barcelona, Spain) (protein 15.00%, ashes 5.70%, cellulose 5.20%, fat 2.20%, in addition to the appropriate vitamins and minerals) and water *ad libitum* until the time of the experiment. Animals' weight ranged from 492 g to 570 g. Animals were killed by decapitation and their blood was collected. Sera were used immediately in enzymatic assays.

Enzymatic assays

Total serum alkaline phosphatase activity. ALP activity was determined according to methods developed in our laboratory [49] and based on the original work of Babson and Read [18] and Seligman *et al.* [19], the substrates being α-NP and β-NP.

ALP activity was assayed in the serum and on the electrophoretic strips, after serum ALP fraction/isoenzyme separation, using different substrates, buffers (or mixture of buffers) and pH values, as listed in Table I.

The final concentration of both substrates was 4 mmol/L. $MgCl_2$ 5 mmol/L (final concentration) was added in all assays. Activity determinations were done at 30°C for 30 min and 15 min with α-NP and β-NP, respectively. Enzymatic reactions were stopped by the addition of sodium citrate, pH 5.2, 75 mmol/L (final concentration) and trichloroacetic acid 5% (final concentration) with substrates α-NP and β-NP, respectively. For α-naphthol, the diazotization reaction with orthodianisidine, 30.0 mmol/L (final concentration), was performed for 3 min immediately after stopping the reaction, as recommended for this naphthol. Trichloroacetic acid 3.6% (final concentration) was added after completion of the diazotization reaction. For β-naphthol, diazotization was also performed for 3 min, but using orthodianisidine 21.5 mmol/L (final concentration) immediately before stopping the reaction, as recommended for this naphthol.

The water insoluble coloured products of the diazoic reactions were extracted with ethyl acetate and their absorbances determined at 530 and 540 nm (Spectronic Genesys 5, Milton Roy, Rochester, NY, USA) for α- and β-naphthols, respectively. Enzyme activity was expressed as α- or β-naphthol released (nmol ml^{-1} min^{-1}).

TABLE I. Experimental conditions used for total serum alkaline phosphatase activity determination and serum alkaline phosphatase fraction/isoenzyme electrophoretic visualization, percentage quantitation and activity calculation.

	pH	
Buffer	9.4	10.4
Veronal*, 25 mmol/L	A	–
Carbonate/bicarbonate, 25 mmol/L	B	B
AMP, 1 mol/L	A	A
	B	B
Carbonate/bicarbonate, 25 mmol/L + Veronal*, 25 mmol/L	A	–
	B	–
AMP, 1 mol/L + Veronal*, 25 mmol/L	A	–
AMP, 1 mol/L + Carbonate/bicarbonate, 25 mmol/L	B	B

A and B represent different experimental groups of animals, both fed *ad libitum* and each with 4 rats. For total serum alkaline phosphatase activity determination, both groups of animals were used. For serum alkaline phosphatase electrophoretic fraction/isoenzyme visualization, percentage quantitation and activity calculation, animal groups were used as described (see text). α- and β-naphthyl phosphates were always used as substrates. Buffer concentrations shown in this table are final concentrations.

* Veronal pKa does not allow its utilization at pH 10.4.

AMP = 2-amino-2-methyl-1-propanol/HCl.

Serum ALP fraction/isoenzyme electrophoretic separation and visualization. Electrophoresis was carried out on cellulose acetate strips for 45 min at 300 V using diethylbarbiturate/HCl buffer, pH 8.6, 60 mmol/L. Strips were incubated for 15 min in a humidified chamber with different buffers (or mixture of buffers), substrates and pH values, as listed in Table I. After this incubation period, serum ALP fractions/isoenzymes bands were stained by coupling α- and β-naphthols, produced by ALP isoenzymes, with *fast violet B*, 2 g/L. Electrophoretic patterns were scanned with a densitometer at 530 nm (Beckman Instruments Inc., Fullerton, CA, USA).

Using the same buffer, substrate and pH value for total serum ALP activity and serum ALP electrophoretic fraction/isoenzyme visualization, calculated activity values for the various fractions were obtained by multiplying the corresponding percentage by the total serum ALP activity. Values were expressed as α- or β-naphthol released (nmol ml^{-1} min^{-1}). In densitometric analysis we considered the most evident and constant fractions, whilst accepting that they may sometimes be heterogeneous [5–7, 23, 44].

Statistical analysis

All results are expressed as arithmetic means ±standard deviation. The significance of differences between means was assessed by paired Student's *t*-test.

RESULTS AND DISCUSSION

Results published in the literature show that assay conditions are very important not only in quantifying total serum ALP and ALP from tissue extracts, but also in ALP isoenzyme inhibition studies [9, 11–16, 22, 50, 51]. For example, it is known that the choice of AMP buffer versus diethanolamine buffer is important for total serum ALP determination and for the selective inhibition of bone, liver and intestinal isoenzymes [13, 50]. Method parameters, such as reagent formulation, can also influence ALP activity measurement [52].

In addition to the different effects of buffer systems on ALP isoenzymes, some isoenzymes have different affinities for a given substrate. A given substrate and buffer system activating or inhibiting a particular isoenzyme can induce a more or less incorrect estimation of its own activity and, depending on the amount present in the sample, of total sample ALP activity, resulting in an incorrect evaluation of other isoenzymes present in the sample under study (if this calculation is based on the previous activity values) [13, 16, 22–24, 51].

The optimal pH for intestinal isoenzyme is 9.4 [53]. In serum in which this isoenzyme is the most abundant, total serum ALP activity will be greatly influenced (modified) if the pH chosen for ALP assay is quite different from the optimal one. Total ALP activity for this same sample may also be buffer-dependent. AMP, when compared to glycine, enhances more serum ALP from bone and liver than from intestine [16]; placental and intestinal isoenzymes are much more easily activated by N-ethylaminoethanol than by diethanolamine [11]; the same is true for serum containing these ALP isoenzymes [12].

Despite the fact that this information is widely known, researchers do not always ensure that the same method, i.e. the same set of assay conditions for quantitation of total serum ALP activity and for serum ALP electrophoretic fraction/isoenzyme visualization, percentage quantitation and activity calculation (when serum ALP fraction/isoenzyme calculated activity values are obtained by multiplying the corresponding percentage by the total serum ALP activity), is consistently used.

In some studies, the same substrate and buffer were used in total serum and tissue ALP activity determination and in electrophoretic identification of serum and tissue ALP fractions/isoenzymes, but the consequences of this usage were not sufficiently taken into consideration [45–48]. p-Nitrophenylphosphate is frequently used and recommended as substrate in the determination of total serum and tissue ALP activity [1, 2, 6, 8, 9, 11–13, 16, 22, 25, 26, 30, 32–37, 39–41, 43, 44, 48–52, 54, 55]. Although p-nitrophenylphosphate has already been used in the electrophoretic identification of serum ALP isoenzymes by direct scanning of the reaction product [48], to our knowledge, it is not usually used in electrophoresis [13, 16, 23, 25, 28–44] owing to some diffusion that can occur on the electrophoretic support medium. AS-MX phosphate is a

fluorogenic ALP substrate that enables good scanning of ALP fractions/isoenzymes, which can improve their electrophoretic quantitation. Its use in the measurement of total serum and tissue ALP activity and serum and of tissue ALP fraction(s)/isoenzyme(s) electrophoretic identification has been described but, as far as we know, its electrophoretic application has been more common [23, 32, 35, 46, 47]. The use of β-glycerophosphate and phenolphthalein monophosphate for both procedures has been described, but methods using these ALP substrates are now used relatively seldom [2, 16, 22, 29, 45].

Figure 1 shows the pattern of electrophoretic separation of serum ALP from a fed rat; the application point was near the cathode. We labelled the more rapid fraction the first fraction and the slowest one the second. Tables II to VI show the variation in results obtained for total serum ALP activity and serum ALP electrophoretic fractions percentage values and activity according to the different assay conditions used.

The effect of a change in pH was buffer- (or mixture of buffers) and substrate-dependent, modifying total serum ALP, and sometimes differently affecting each fraction. The addition of a second buffer to one previously used was not always accompanied by the same pattern of variation in our results, and the results obtained with different buffers (or mixture of buffers) were not identical with either substrate or pH values.

The phosphate group obtained from ALP activity is either transferred to water (hydrolytic ALP activity) or to phosphate acceptors when they are present in the reaction mixture (transphosphorylasic ALP activity). Although carbonate/bicarbonate and Veronal are not now routinely used in ALP quantitation, they were chosen because of their proven lack of ability to function as phospho-acceptors. AMP is a widely used and recommended buffer that can support transphosphorylation [8, 13–16, 22, 26, 32, 37, 45–47, 49–52, 55]. Using these three buffers (alone or in a mixture) it was possible to show differing results as a consequence of the modulation of hydrolytic and transphosphorylasic ALP activity according to pH value and substrate used. Buffer choice was also based on our knowledge that AMP, Veronal and carbonate/bicarbonate (alone or in a mixture) allow,

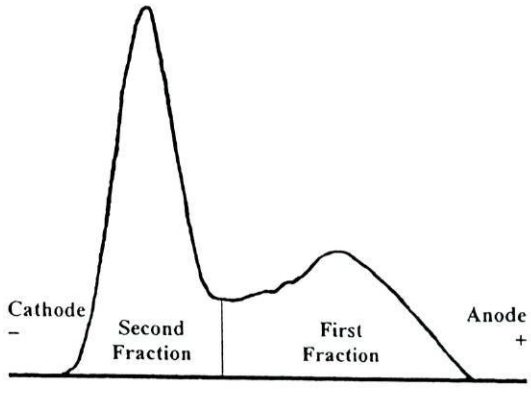

FIG. 1. Electrophoretic separation of serum alkaline phosphatase from a fed rat on cellulose acetate strips. Densitometric analysis at 530 nm, after 15 min incubation of both fractions with β-naphthyl phosphate (4 mmol/L) in 2-amino-2-methyl-1-propanol/HCl buffer (pH 10.4, 1 mol/L) and staining by coupling β-naphthol, produced by alkaline phosphatase isoenzymes, with *fast violet B* (2 g/L). The application point was near the cathode. We labelled the more rapid fraction the first fraction and the slowest one the second.

without interference, the appearance of colour after the coupling of tetrazotized orthodianisidine with α- and β-naphthols. α-NP and β-NP were chosen as substrates because with their reaction products insoluble, non-diffusible and easily identifiable compounds can be obtained on electrophoretic strips, enabling their scanning. This experimental work was performed using cellulose acetate as the electrophoretic support medium because of its ease of handling and speed of separation. The separation level, although incomplete for the two serum ALP fractions obtained, is sufficient to show that fraction/isoenzyme visualization (staining, revelation) was dependent on the experimental conditions used. The likely identification of the two ALP fractions is published elsewhere [49].

This study shows that differences in assay conditions may have different effects upon total serum ALP and serum ALP electrophoretic fractions activity, sometimes completely opposite effects owing to the different ways in which the measured activity of serum ALP fractions/isoenzymes can be affected by the buffer, pH and substrate system used. This has obvious implications for comparison and interpretation of both types of activity results. Nevertheless, comparison between isoenzyme patterns is feasible if one bears in mind that fraction(s)/

TABLE II. Serum alkaline phosphatase activity. Total activity determined with different substrates, buffers and pH values.

Buffer	Substrate: α-naphthyl phosphate (4 mmol/L) (nmol ml^{-1} min^{-1})	Substrate: β-naphthyl phosphate (4 mmol/L) (nmol ml^{-1} min^{-1})	p
Veronal, 25 mmol/L, pH 9.4	67.19 ± 8.36 (a_1)	120.10 ± 37.76 (a_2)	$p < 0.005$
Carbonate/bicarbonate, 25 mmol/L, pH 9.4	43.20 ± 8.18 (b_1)	112.45 ± 26.49 (b_2)	$p < 0.0005$
Carbonate/bicarbonate, 25 mmol/L, pH 10.4	117.26 ± 15.56 (c_1)	127.63 ± 37.91 (c_2)	ns
AMP, 1 mol/L, pH 9.4	108.74 ± 20.28 (d_1)	193.34 ± 42.21 (d_2)	$p < 0.005$
AMP, 1 mol/L, pH 10.4	54.79 ± 24.66 (e_1)	188.97 ± 39.27 (e_2)	$p < 0.0001$
Carbonate/bicarbonate, 25 mmol/L + Veronal, 25 mmol/L, pH 9.4	72.86 ± 16.97 (f_1)	133.82 ± 32.66 (f_2)	$p < 0.001$
AMP, 1 mol/L + Veronal, 25 mmol/L, pH 9.4	57.09 ± 16.56 (g_1)	200.81 ± 47.40 (g_2)	$p < 0.0001$
AMP, 1 mol/L + carbonate/bicarbonate, 25 mmol/L, pH 9.4	63.97 ± 15.44 (h_1)	189.31 ± 70.56 (h_2)	$p < 0.001$
AMP, 1 mol/L + carbonate/bicarbonate, 25 mmol/L, pH 10.4	50.77 ± 9.27 (i_1)	134.41 ± 25.78 (i_2)	$p < 0.0001$

p							
pH		AMP		Carbonate/bicarbonate			
$(b_1 - c_1)$ $p < 0.001$	$(b_2 - c_2)$ $p < 0.02$	$(b_1 - h_1)$ $p < 0.005$	$(b_2 - h_2)$ $p < 0.02$	$(a_1 - f_1)$ ns	$(a_2 - f_2)$ $p < 0.01$	$(d_1 - g_1)$ $p = 0.00001$	$(d_2 - g_2)$ ns
$(d_1 - e_1)$ $p < 0.001$	$(d_2 - e_2)$ ns	$(c_1 - i_1)$ $p < 0.001$	$(c_2 - i_2)$ ns	$(e_1 - i_1)$ ns	$(e_2 - i_2)$ $p < 0.01$	$(f_1 - g_1)$ $p < 0.001$	$(f_2 - g_2)$ $p = 0.0005$
$(h_1 - i_1)$ $p < 0.05$	$(h_2 - i_2)$ $p < 0.05$	$(a_1 - g_1)$ ns	$(a_2 - g_2)$ $p < 0.05$	$(d_1 - h_1)$ $p < 0.001$	$(d_2 - h_2)$ ns	$(b_1 - f_1)$ $p < 0.0005$	$(b_2 - f_2)$ $p < 0.0002$

Results are expressed as means ± standard deviation, in nmol ml^{-1} min^{-1} (n = 8 with feeding *ad libitum*). AMP = 2-amino-2-methyl-1-propanol/HCl. ns = no significant difference. Total activity values were obtained from single determinations in serum from each rat.

TABLE III. Serum alkaline phosphatase activity. First fraction percentage values. Electrophoretic fraction visualization performed with different substrates, buffers and pH values (and with *fast violet B*).

Buffer	Substrate: α-naphthyl phosphate (4 mmol/L) %	Substrate: β-naphthyl phosphate (4 mmol/L) %	p
Veronal, 25 mmol/L, pH 9.4	14.38 ± 2.74 (a_1)	12.93 ± 2.80 (a_2)	ns
Carbonate/bicarbonate, 25 mmol/L, pH 9.4	13.90 ± 3.23 (b_1)	13.63 ± 5.05 (b_2)	ns
Carbonate/bicarbonate, 25 mmol/L, pH 10.4	15.73 ± 4.63 (c_1)	14.28 ± 4.19 (c_2)	ns
AMP, 1 mol/L, pH 9.4	24.02 ± 2.51 (d_1) (n=8)	24.51 ± 2.76 (d_2) (n=8)	ns
AMP, 1 mol/L, pH 10.4	35.61 ± 3.99 (e_1) (n=8)	35.11 ± 5.86 (e_2) (n=8)	ns
Carbonate/bicarbonate, 25 mmol/L + Veronal, 25 mmol/L, pH 9.4	16.55 ± 6.71 (f_1) (n=8)	13.95 ± 5.21 (f_2) (n=8)	ns
AMP, 1 mol/L + Veronal, 25 mmol/L, pH 9.4	25.55 ± 4.07 (g_1)	29.05 ± 4.88 (g_2)	ns
AMP, 1 mol/L + carbonate/bicarbonate, 25 mmol/L, pH 9.4	28.67 ± 3.48 (h_1)	30.87 ± 6.45 (h_2)	ns
AMP, 1 mol/L + carbonate/bicarbonate, 25 mmol/L, pH 10.4	48.72 ± 7.73 (i_1)	42.53 ± 6.80 (i_2)	ns

p

pH

$(b_1 - c_1)$ ns	$(b_2 - c_2)$ ns
$(d_1 - e_1)$ $p < 0.001$	$(d_2 - e_2)$ $p < 0.001$
$(h_1 - i_1)$ $p < 0.005$	$(h_2 - i_2)$ $p < 0.05$

AMP

$(b_1 - h_1)$ $p < 0.002$	$(b_2 - h_2)$ $p < 0.005$
$(c_1 - i_1)$ $p = 0.002$	$(c_2 - i_2)$ $p < 0.001$
$(a_1 - g_1)$ $p < 0.05$	$(a_2 - g_2)$ $p < 0.005$

Carbonate/bicarbonate

$(a_1 - f_1)$ ns	$(a_2 - f_2)$ ns	$(d_1 - g_1)$ ns	$(d_2 - g_2)$ ns
$(d_1 - h_1)$ $p = 0.005$	$(d_2 - h_2)$ $p < 0.05$	$(f_1 - g_1)$ $p = 0.05$	$(f_2 - g_2)$ $p < 0.001$
$(e_1 - i_1)$ $p < 0.05$	$(e_2 - i_2)$ $p = 0.05$	$(b_1 - f_1)$ ns	$(b_2 - f_2)$ ns

Results are expressed as means ± standard deviation (n=4, when not indicated, with feeding *ad libitum*). AMP = 2-amino-2-methyl-1-propanol/HCl. ns = no significant difference. Percentage values were obtained from single determinations in serum from each rat.

TABLE IV. Serum alkaline phosphatase activity. Second fraction percentage values. Electrophoretic fraction visualization performed with different substrates, buffers and pH values (and with *fast violet B*).

	Substrate		
	α-naphthyl phosphate (4 mmol/L)	β-naphthyl phosphate (4 mmol/L)	
Buffer	%	%	p
Veronal, 25 mmol/L, pH 9.4	85.63 ± 2.74 (a_1)	87.08 ± 2.80 (a_2)	ns
Carbonate/bicarbonate, 25 mmol/L, pH 9.4	86.10 ± 3.23 (b_1)	86.38 ± 5.05 (b_2)	ns
Carbonate/bicarbonate, 25 mmol/L, pH 10.4	84.28 ± 4.63 (c_1)	85.72 ± 4.19 (c_2)	ns
AMP, 1 mol/L, pH 9.4	75.98 ± 2.51 (d_1) (n = 8)	75.49 ± 2.76 (d_2) (n = 8)	ns
AMP, 1 mol/L, pH 10.4	64.39 ± 3.99 (e_1) (n = 8)	64.89 ± 5.86 (e_2) (n = 8)	ns
Carbonate/bicarbonate, 25 mmol/L + Veronal, 25 mmol/L, pH 9.4	83.45 ± 6.71 (f_1) (n = 8)	86.05 ± 5.21 (f_2) (n = 8)	ns
AMP, 1 mol/L + Veronal, 25 mmol/L, pH 9.4	74.45 ± 4.07 (g_1)	70.95 ± 4.88 (g_2)	ns
AMP, 1 mol/L + carbonate/bicarbonate, 25 mmol/L, pH 9.4	71.33 ± 3.48 (h_1)	69.13 ± 6.45 (h_2)	ns
AMP, 1 mol/L + carbonate/bicarbonate, 25 mmol/L, pH 10.4	51.28 ± 7.73 (i_1)	57.48 ± 6.80 (i_2)	ns

p

pH

($b_1 - c_1$) ns; ($b_2 - c_2$) ns

($d_1 - e_1$) $p < 0.001$; ($d_2 - e_2$) $p < 0.001$

($h_1 - i_1$) $p < 0.005$; ($h_2 - i_2$) $p < 0.05$

AMP

($b_1 - h_1$) $p < 0.002$; ($b_2 - h_2$) $p < 0.005$

($c_1 - i_1$) $p = 0.002$; ($c_2 - i_2$) $p < 0.001$

($a_1 - g_1$) $p < 0.05$; ($a_2 - g_2$) $p < 0.005$

Carbonate/bicarbonate

($a_1 - f_1$) ns; ($a_2 - f_2$) ns; ($d_1 - g_1$) ns; ($d_2 - g_2$) ns

($d_1 - h_1$) $p = 0.005$; ($d_2 - h_2$) $p < 0.05$; ($f_1 - g_1$) $p = 0.05$; ($f_2 - g_2$) $p < 0.001$

($e_1 - i_1$) $p < 0.05$; ($e_2 - i_2$) $p = 0.05$; ($b_1 - f_1$) ns; ($b_2 - f_2$) ns

Results are expressed as means ± standard deviation (n = 4, when not indicated, with feeding *ad libitum*). AMP = 2-amino-2-methyl-1-propanol/HCl. ns = no significant difference. Percentage values were obtained from single determinations in serum from each rat.

TABLE V. Serum alkaline phosphatase activity. First fraction calculated activity values. Electrophoretic fraction visualization performed with different substrates, buffers and pH values (and with *fast violet B*).

	Substrate	
	α-naphthyl phosphate (4 mmol/L)	β-naphthyl phosphate (4 mmol/L)
Buffer	(nmol ml^{-1} min^{-1})	(nmol ml^{-1} min^{-1})	p
Veronal, 25 mmol/L, pH 9.4	9.53 ± 2.20 (a_1)	17.09 ± 7.01 (a_2)	ns
Carbonate/bicarbonate, 25 mmol/L, pH 9.4	6.86 ± 1.56 (b_1)	15.52 ± 8.71 (b_2)	ns
Carbonate/bicarbonate, 25 mmol/L, pH 10.4	19.77 ± 4.59 (c_1)	17.93 ± 8.06 (c_2)	ns
AMP, 1 mol/L, pH 9.4	26.35 ± 6.81 (d_1) (n=8)	47.99 ± 14.16 (d_2) (n=8)	p<0.01
AMP, 1 M, pH 10.4	20.00 ± 10.90 (e_1) (n=8)	66.46 ± 19.36 (e_2) (n=8)	p<0.0001
Carbonate/bicarbonate, 25 mmol/L + Veronal, 25 mmol/L, pH 9.4	12.25 ± 7.16 (f_1) (n=8)	18.91 ± 9.52 (f_2) (n=8)	p<0.001
AMP, 1 mol/L + Veronal, 25 mmol/L, pH 9.4	12.16 ± 4.06 (g_1)	57.70 ± 16.53 (g_2)	p=0.02
AMP, 1 mol/L + carbonate/bicarbonate, 25 mmol/L, pH 9.4	21.45 ± 6.90 (h_1)	60.92 ± 38.50 (h_2)	ns
AMP, 1 mol/L + carbonate/bicarbonate, 25 mmol/L, pH 10.4	25.91 ± 10.92 (i_1)	57.49 ± 23.95 (i_2)	p<0.05

p							
pH		AMP		Carbonate/bicarbonate			
($b_1 - c_1$) p<0.01	($b_2 - c_2$) ns	($b_1 - h_1$) p<0.02	($b_2 - h_2$) ns	($a_1 - f_1$) ns	($a_2 - f_2$) ns	($d_1 - g_1$) p<0.01	($d_2 - g_2$) ns
($d_1 - e_1$) p = 0.05	($d_2 - e_2$) ns	($c_1 - i_1$) ns	($c_2 - i_2$) p<0.02	($e_1 - i_1$) ns	($e_2 - i_2$) p<0.01	($f_1 - g_1$) ns	($f_2 - g_2$) p<0.01
($h_1 - i_1$) ns	($h_2 - i_2$) ns	($a_1 - g_1$) ns	($a_2 - g_2$) p<0.01	($d_1 - h_1$) p<0.01	($d_2 - h_2$) ns	($b_1 - f_1$) ns	($b_2 - f_2$) ns

Results are expressed as means ± standard deviation, in nmol ml^{-1} min^{-1} (n=4, when not indicated, with feeding *ad libitum*). AMP = 2-amino-2-methyl-1-propanol/HCl. ns = no significant difference. Calculated activity values were obtained from single determinations in serum from each rat and by multiplying the percentage by the total ALP activity. The sum of the first and second (Table VI) fractions calculated activity values matches with total ALP activity values (Table II) only when n=8.

TABLE VI. Serum alkaline phosphatase activity. Second fraction calculated activity values. Electrophoretic fraction visualization performed with different substrates, buffers and pH values (and with *fast violet B*).

	Substrate	
	α-naphthyl phosphate (4 mmol/L)	β-naphthyl phosphate (4 mmol/L)
Buffer	(nmol ml^{-1} min^{-1})	(nmol ml^{-1} min^{-1})
Veronal, 25 mmol/L, pH 9.4	56.45 ± 2.21 (a_1)	113.69 ± 40.23 (a_2)
Carbonate/bicarbonate, 25 mmol/L, pH 9.4	42.68 ± 4.59 (b_1)	92.42 ± 17.98 (b_2)
Carbonate/bicarbonate, 25 mmol/L, pH 10.4	108.17 ± 15.40 (c_1)	102.89 ± 19.87 (c_2)
AMP, 1 mol/L, pH 9.4	82.39 ± 14.28 (d_1) (n = 8)	145.34 ± 28.73 (d_2) (n = 8)
AMP, 1 mol/L, pH 10.4	34.79 ± 14.21 (e_1) (n = 8)	122.50 ± 27.74 (e_2) (n = 8)
Carbonate/bicarbonate, 25 mmol/L + Veronal, 25 mmol/L, pH 9.4	60.60 ± 13.85 (f_1) (n = 8)	114.89 ± 28.73 (f_2) (n = 8)
AMP, 1 mol/L + Veronal, 25 mmol/L, pH 9.4	35.24 ± 10.27 (g_1)	140.63 ± 29.21 (g_2)
AMP, 1 mol/L + carbonate/bicarbonate, 25 mmol/L, pH 9.4	52.19 ± 9.05 (h_1)	127.73 ± 65.60 (h_2)
AMP, 1 mol/L + carbonate/bicarbonate, 25 mmol/L, pH 10.4	25.71 ± 3.19 (i_1)	74.01 ± 12.89 (i_2)

p							
pH		AMP		Carbonate/bicarbonate			
($b_1 - c_1$) $p < 0.01$	($b_2 - c_2$) ns	($b_1 - h_1$) ns	($b_2 - h_2$) ns	($a_1 - f_1$) ns	($a_2 - f_2$) $p < 0.05$	($d_1 - g_1$) $p < 0.01$	($d_2 - g_2$) ns
($d_1 - e_1$) $p < 0.0001$	($d_2 - e_2$) ns	($c_1 - i_1$) $p = 0.002$	($c_2 - i_2$) $p = 0.05$	($e_1 - i_1$) ns	($e_2 - i_2$) $p < 0.05$	($f_1 - g_1$) $p < 0.01$	($f_2 - g_2$) ns
($h_1 - i_1$) $p < 0.01$	($h_2 - i_2$) ns	($a_1 - g_1$) $p < 0.02$	($a_2 - g_2$) $p = 0.05$	($d_1 - h_1$) $p < 0.005$	($d_2 - h_2$) ns	($b_1 - f_1$) $p = 0.001$	($b_2 - f_2$) $p = 0.05$

Results are expressed as means ± standard deviation, in nmol ml^{-1} min^{-1} (n = 4, when not indicated, with feeding *ad libitum*). AMP = 2-amino-2-methyl-1-propanol/HCl. ns = no significant difference. Calculated activity values were obtained from single determinations in the serum from each rat and by multiplying the percentage by the total ALP activity. The sum of the first (Table V) and second fractions calculated activity values matches with total ALP activity values (Table II) only when n = 8.

isoenzyme(s) migration can be influenced in a familiar way, for example, by sample treatment before/during ALP extraction, by using a certain electrophoretic system or by addition of a certain enzyme to an ALP sample that will modify ALP fraction(s)/isoenzyme(s) electrophoretic characteristics [6, 23, 24, 32, 40, 56].

Special attention is needed when determining pathophysiological conditions because method sensibility or resolution, influenced by assay conditions, may induce variations in results independent of the metabolic alterations under investigation. These problems are especially interesting for isoenzyme characterization in terms of origin, identification and determination. Detailed attention to the influence of assay conditions upon tissue and type of ALP isoenzyme activity will allow us to draw conclusions, under different pathophysiological conditions, as to the origin of serum isoenzymes. The importance of isoenzyme characterization in biochemical research and its application in clinical medicine is obvious.

In conclusion, in the electrophoretic characterization and quantitation of serum ALP isoenzymes, the biochemical visualization of the fractions is crucial; the same assay conditions for quantitation of total serum ALP and serum ALP electrophoretic fractions should be used.

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REFERENCES

1 Epstein E, Kiechle FL, Artiss JD, Zak B. The clinical use of alkaline phosphatase enzymes. Clin Lab Med 1986; 6: 491–505.

2 Moss DW, Henderson AR. Enzymes In: Burtis CA, Ashwood ER, editors. Tietz textbook of clinical chemistry. 2nd ed. Philadelphia: WB Saunders, 1994: 830–43.

3 Balistreri WF, Rej R. Liver function. In: Burtis CA, Ashwood ER, editors. Tietz textbook of clinical chemistry. 2nd ed. Philadelphia: WB Saunders, 1994: 1449–512.

4 Endres DB, Rude RK. Mineral and bone metabolism. In: Burtis CA, Ashwood ER, editors. Tietz textbook of clinical chemistry. 2nd ed. Philadelphia: WB Saunders, 1994: 1887–973.

5 Moss DW. Alkaline phosphatase isoenzymes. Clin Chem 1982; 28: 2007–16.

6 Van Hoof VO, De Broe ME. Interpretation and clinical significance of alkaline phosphatase isoenzyme patterns. Crit Rev Clin Lab Sci 1994; 31: 197–293.

7 Price CP. Multiple forms of human serum alkaline phosphatase: detection and quantitation. Ann Clin Biochem 1993; 30: 355–72.

8 Panteghini M. Benign inherited hyperphosphatasemia of intestinal origin: report of two cases and a brief review of the literature. Clin Chem 1991; 37: 1449–52.

9 McComb RB, Bowers Jr GN. Study of optimum buffer conditions for measuring alkaline phosphatase activity in human serum. Clin Chem 1972; 18: 97–104.

10 Morton RK. Transphosphorylation by phosphatases. Methods Enzymol 1955; II: 556–61.

11 Van Belle H. Alkaline phosphatase. I. Kinetics and inhibition by levamisole of purified isoenzymes from humans. Clin Chem 1976; 22: 972–6.

12 Van Belle H. Alkaline phosphatase. II. Conditions affecting determination of total activity in serum. Clin Chem 1976; 22: 977–81.

13 Bretaudière J-P, Vassault A, Amsellem L, Pourci M-L, Thieu-Phung H, Bailly M. Criteria for establishing a standardized method for determining alkaline phosphatase activity in human serum. Clin Chem 1977; 23: 2263–74.

14 Bowers GN, Kelley M-L, McComb RB. Continuous spectrophotometric measurement of serum alkaline phosphatase activity. Relative molecular activity and sensitivity of nine self-indicating phenolic substrates in 2-amino-2-methyl-1-propanol buffer. Clin Chem 1968; 14: 608–10.

15 Babson AL, Greeley SJ, Coleman CM, Phillips GE. Phenolphthalein monophosphate as a substrate for serum alkaline phosphatase. Clin Chem 1966; 12: 482–90.

16 Haije WG. Influence of buffer conditions on the activities of some iso-enzymes of alkaline phosphatase in the serum. Clin Chim Acta 1973; 48: 23–6.

17 Moss DW. Kinetics of phosphatase action on naphthyl phosphates, determined by a highly sensitive spectrofluorimetric technique. Biochem J 1960; 76: 32P.

18 Babson AL, Read PA. A new assay for prostatic acid phosphatase in serum. Am J Clin Pathol 1959; 32: 88–91.

19 Seligman AM, Chauncey HH, Nachlas MM, Manheimer LH, Ravin HA. The colorimetric determination of phosphatases in human serum. J Biol Chem 1951; 190: 7–15.

20 Stevens J, Thomas F. Alkaline phosphatase:

reaction rate analysis at 340 nm. Clin Chim Acta 1972; 37: 541–3.
21 Kind PRN, King EJ. Estimation of plasma phosphatase by determination of hydrolysed phenol with amino-antipyrine. J Clin Pathol 1954; 7: 322–6.
22 McComb RB, Bowers Jr GN, Posen S. Alkaline phosphatase. New York: Plenum Press, 1979.
23 Crofton PM. Biochemistry of alkaline phosphatase isoenzymes. Crit Rev Clin Lab Sci 1982; 16: 160–94.
24 Phosphatase isoenzymes. In: Wilkinson JH, editor. Isoenzymes. 2nd ed. London: Chapman & Hall, 1970: 239–79.
25 Withold W, Schulte U, Reinauer H. Method for determination of bone alkaline phosphatase activity: analytical performance and clinical usefulness in patients with metabolic and malignant diseases. Clin Chem 1996; 42: 210–7.
26 Price CP, Milligan TP, Darte C. Direct comparison of performance characteristics of two immunoassays for bone isoform of alkaline phosphatase in serum. Clin Chem 1997; 43: 2052–7.
27 Otto VI, Schär BK, Sulser T, Hänseler E. Specific determination of germ cell alkaline phosphatase for early diagnosis and monitoring of seminoma: performance and limitations of different analytical techniques. Clin Chim Acta 1998; 273: 131–47.
28 Hodson AW, Latner AL, Raine L. Iso-enzymes of alkaline phosphatase. Clin Chim Acta 1962; 7: 255–61.
29 Haije WG, De Jong M. Iso-enzyme patterns of serum alkaline phosphatase in agar-gel electrophoresis and their clinical significance. Clin Chim Acta 1963; 8: 620–3.
30 Kaplan MM, Rogers L. Separation of human serum-alkaline-phosphatase isoenzymes by polyacrylamide gel electrophoresis. Lancet 1969; 2: 1029–31.
31 Rhone DP, Mizuno FM. Separation of isoenzymes of alkaline phosphatase by substrate-gel imprint after electrophoresis on cellulose acetate. Clin Chem 1972; 18: 662–6.
32 Fritsche HA, Adams-Park HR. High molecular weight isoenzymes of alkaline phosphatase in human serum. Demonstration by cellulose acetate electrophoresis and physico-chemical characterization. Clin Chim Acta 1974; 52: 81–9.
33 Kalimanovska V, Whitaker KB, Moss DW. Effect of bromelain on alkaline phosphatases of intestinal and non-intestinal tissues and serum. Clin Chim Acta 1987; 170: 219–26.
34 Van Hoof VO, Lepoutre LG, Hoylaerts MF, Chevigné R, De Broe ME. Improved agarose electrophoretic method for separating alkaline phosphatase isoenzymes in serum. Clin Chem 1988; 34: 1857–62.
35 Hamilton BA, McPhee JL, Hawrylak K, Stinson RA. Alkaline phosphatase releasing activity in human tissues. Clin Chim Acta 1989; 186: 249–54.
36 Bentouati L, Baboli MS, Hachem H, Hamza M, Canal P, Soula G. Hyperphosphatasemia related to three intestinal alkaline phosphatase isoforms: biochemical study. Clin Chim Acta 1990; 193: 93–102.
37 Van Hoof VO, Hoylaerts MF, Geryl H, Van Mullem M, Lepoutre LG, De Broe ME. Age and sex distribution of alkaline phosphatase isoenzymes by agarose electrophoresis. Clin Chem 1990; 36: 875–8.
38 Murthy VV, Malikin G, Karmen A. Agarose gel electrophoresis of alkaline phosphatase isoenzymes with wheat germ lectin and fluorogenic substrate. Clin Chem 1991; 37: 1309–10.
39 Rosalki SB, Ying Foo A. Two new methods for separating and quantifying bone and liver alkaline phosphatase isoenzymes in plasma. Clin Chem 1984; 30: 1182–6.
40 Moss DW, Edwards RK. Improved electrophoretic resolution of bone and liver alkaline phosphatases resulting from partial digestion with neuraminidase. Clin Chim Acta 1984; 143: 177–82.
41 Rosalki SB, Ying Foo A. Incubation with neuraminidase and affinity electrophoresis with wheat-germ lectin compared for separating and quantifying alkaline phosphatase isoenzymes in plasma. Clin Chem 1985; 31: 1198–200.
42 Amacher DE, Higgins CV, Schomaker SJ, Clay RJ. Further charaterization of serum alkaline phosphatase from male and female beagle dogs. Enzyme 1989; 42: 1–7.
43 Wei JS, Shih CL, Chung NC, Wang J-Y. Quantitative determination of high molecular weight alkaline phosphatase in patients with colorectal cancer by polyacrylamide gel electrophoresis. Enzyme 1990; 43: 188–91.
44 Griffiths WC, Camara PD, Rosner M, Lev R, Brooks EM. Prevalence and properties of the intestinal alkaline phosphatase identified in serum by cellulose acetate electrophoresis. Clin Chem 1992; 38: 507–11.
45 Romel WC, LaMancusa SJ, Dufrene JK. Detection of serum alkaline phosphatase isoenzymes with phenolphthalein monophosphate following cellulose acetate electrophoresis. Clin Chem 1968; 14: 47–57.
46 Johnson Jr RB. A new fluorometric method for the estimation or detection of total and fractionated alkaline phosphatase. Clin Chem 1969; 15: 108–23.
47 Demetriou JA, Beattie JM. Electrophoretic separation on agarose thin film of isoenzymes of alkaline phosphatase from human serum and tissue. Clin Chem 1971; 17: 290–5.
48 Healy PJ. Quantitative analysis of serum alkaline phosphatase isoenzyme activity. Clin Chim Acta 1974; 55: 407–9.
49 Martins MJ, Dias PO, Hipólito-Reis C. Rat serum alkaline phosphatase electrophoretic fractions: variations with feeding, starvation and cellulose fibre ingestion. Clin Nutr 1998; 17: 279–85.
50 Steele BW, Nord M, Statland BE. Comparative effects of DEA versus AMP buffer on alkaline phosphatase isoenzymes. Clin Chem 1976; 22: 1221.

51 Van Hoof VO, Van Campenhout CM, De Broe ME, Lepoutre LG. Variations in measured alkaline phosphatase activity: influence of isoenzymes and buffer systems. Clin Chem 1990; 36: 2012–3.
52 Lamb EJ, Browne M, John WG, Price CP. Alkaline phosphatase activity measurement in the UK by AMP-buffered methods: an appraisal of current practice. Ann Clin Biochem 1998; 35: 120–7.
53 Fernley HN. Mammalian alkaline phosphatases. In: Boyer PD, editor. The enzymes. Vol IV. New York: Academic Press, 1971: 417–47.
54 Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology. Recommended methods for the determination of four enzymes in blood. Scand J Clin Lab Invest 1974; 33: 291–306.
55 International Federation of Clinical Chemistry. Scientific Committee, Analytical Section, Expert Panel on Enzymes. IFCC methods for the measurement of catalytic concentration of enzymes. Part 5. IFCC method for alkaline phosphatase (orthophosphoric-monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1). Clin Chim Acta 1983; 135: 339F–67F. J Clin Chem Clin Biochem 1983; 21: 731–48.
56 Van Hoof VO, Van Mullem M. Comparison of two commercially available systems for the electrophoretic separation of alkaline phosphatase isoenzymes. J Chromatogr 1993; 646: 235–43.

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2.2 - Modulação de actividades fosfatásicas alcalinas

2.2.a - Inibição da fosfátase alcalina hepática do Rato por sais biliares em concentrações fisiológicas sem modificar a isozima intestinal (Artigo IV).

2.2.b - Diferente modulação da fosfátase alcalina do fígado e do rim do Rato por moduladores clássicos da enzima e por moduladores de diferentes sistemas de transporte (Artigo V).

Artigo IV

Physiologic concentrations of bile salts inhibit rat hepatic alkaline phosphatase but not the intestinal isoenzyme.

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Physiologic Concentrations of Bile Salts Inhibit Rat Hepatic Alkaline Phosphatase but Not the Intestinal Isoenzyme

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Objective: The effect of bile salts on alkaline phosphatase (EC 3.1.3.1) activity from Wistar rat liver, duodenum, jejunum, and serum was investigated.

Design and results: For concentrations higher than 1 mM conjugated bile salts (glycocholate, glycochenodeoxycholate, taurocholate, taurodeoxycholate, and taurochenodeoxycholate) inhibited hepatic ALP but, up to concentrations of 10 mM, had no effect on intestinal ALP. Also cholate, deoxycholate, and chenodeoxycholate, within the same concentration range, did not have any effect on intestinal ALP. ALP inhibition induced by conjugated bile salts was significantly higher in serum of starved rats than in serum of fed animals, what is in good agreement with the known higher proportion of hepatic ALP and lower proportion of intestinal ALP in serum of starved rats.

Conclusions: Bile salts can, thus, be used to help discriminating between tissue-nonspecific and intestinal ALP isoenzymes and identifying pathologic conditions where the relative quantities of these isoenzymes are altered in serum. Inhibition of hepatic ALP by physiologic concentrations of bile salts may bear some relation to the bile salts effects on their own enterohepatic circulation and/or biosynthesis.

KEY WORDS: alkaline phosphatase inhibition; ALP isoforms; feeding; starvation; bile salts.

Introduction

Alkaline phosphatase (ALP; EC 3.1.3.1, orthophosphoric-monoester phosphohydrolase, alkaline optimum) is a family of phosphomonoesterases, which are encoded by at least four different gene loci: tissue-nonspecific, intestinal, placental, and germ-cell ALP (1). ALP binds to the cellular surface *via* a glycosyl-phosphatidylinositol anchor linkage and can be released into intestinal lumen, plasma, or bile in various isoforms (2–8). Depending on the physiologic and on the pathologic conditions ALP can be found in those biologic compartments with or without its anchor and in the former case associated or not with membrane fragments (2–8). For example, in cholestasis serum ALP activity increases owing to an increase in serum hepatic ALP and membrane-bound liver ALP will become more or less prominent (1,8,9). Anchor-bound ALP can undergo aggregation and complexation to give rise to high-molecular-weight isoforms (2–8).

Although the specific function of ALP is not yet known, the fact that it is ubiquitous—from bacteria to humans—and that, in mammals, it is located mainly in the plasma membranes of exchange surfaces (in liver, kidney, bone, intestine, and placenta), where extensive transport takes place, suggests a fundamental biologic role probably related to transport processes (1,9,10). ALP might be involved in intestinal lipid transport, in bone mineralization process, in regulation of a phosphorylated low-conductance chloride channel in human pancreatic duct cells and of P-glycoprotein (Pgp) in rat hepatocytes (1,11,12).

Bile salts are water-soluble amphipathic products of cholesterol catabolism: hydroxyl groups are inserted in positions 3, 6, 7, and/or 12 of the cholesterol molecule and its side chain is oxidized to form an acid group which is largely ionized at the bile pH value (13–15). Bile salts ability to alter cellular functions is thought to be related to their relative hydrophobicity-hydrophilicity character and detergent effect. The number, location, and orientation of the hydroxyl groups, among other factors, determine the hydrophobic-hydrophilic balance of bile salts (generally the more hydrophobic bile salts have fewer hydroxyl groups). Bile salts conjugation in the liver to either taurine or glycine (primary bile salts) decreases hydrophobicity, whereas deconjugation and dehydroxylation (secondary bile salts) by intestinal bacteria increases relative hydrophobicity (13–23). Bile salts have a solubilizing capacity for phospholipids and cholesterol (13,14,16,17), regulate

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turnover of intestinal hydrolases (22) and release proteins from cell membranes (14,16–18); in the particular case of ALP bile salts release it from enterocytes in an anchor-bound form into the intestinal lumen (1,2,8). Bile salts modify anchor-bound ALP electrophoretic mobility (4), can produce cell lysis (14,16,18,24,25), have antibacterial activity (21), inhibit mitochondrial oxidative phosphorylation (26), activate protein kinase C (27–29), and probably can induce colonic cancer (27). Bile salts transcriptionally regulate their biosynthesis and enterohepatic transport (30).

In our previous work we have shown that not only the reversal of transport through Pgp may be related to the loss of efficacy of ALP in isolated hepatocytes but also that mechanical stimulation of the digestive tract (owing to cellulose fiber ingestion, sawdust) is followed by the passage of intestinal ALP to serum (11,12,31).

As feeding increases intestinal ALP activity in serum (31–33) and bile salts can release ALP from cellular membranes (1,2,8,22), we investigated whether bile salts can affect hepatic and intestinal ALP activity. A preliminary account of some of this work has already appeared (34,35).

Materials and methods

Materials

p-Nitrophenylphosphate, an ALP substrate, was purchased from Sigma (St. Louis, MO, USA; Sigma 104D). All bile salts (sodium salts, minimum purity: 97–99%) were also purchased from Sigma. All other reagents were of the highest quality and purity available.

Methods

In this work, 19 male adult Wistar rats (free from liver and bone diseases), 382 to 509 g of body weight, supplied by the Gulbenkian Institute of Sciences (Lisboa, Portugal) were used. The animals were kept under controlled environmental conditions (12 h light/dark cycle and room temperature 24 °C) and fed with a suitable commercial diet, supplied by Harlan Interfauna Iberica (Barcelona, Spain; protein 15.0%, ashes 5.7%, cellulose 5.2%, fat 2.2%, also including the appropriate vitamins and minerals) and water *ad lib* until the moment of the experiment. Some of these animals (n = 7) were starved for 24 h with water *ad lib* (31,33).

The tissue enzyme samples were prepared as previously described (10). Immediately after decapitation, blood from both groups of animals was collected in plastic tubes and allowed to clot at room temperature. Samples were then centrifuged, also at room temperature, and sera collected. Sera were kept at −20 °C and used within 1 week. The entire small bowel, only from fed animals, was removed, rinsed with ice-cold isotonic NaCl, and divided into duodenum and jejunum. All duodenum (from pylorus to ligament of Treitz) and jejunum, from 3 to 33 cm, were used in the experiments. Mucosa was collected from each intestinal segment by scraping the opened gut. Scrapings were placed in 20× (mL/g) solution A (phosphate-buffered saline, pH 7.4, 0.5% Triton X-100; 4:1). Liver, only from fed animals, was removed, rinsed with ice-cold isotonic NaCl, and placed in 2× (mL/g) solution A. All tissue samples were kept at −80 °C for 1 month at the most.

On the day of the experiment, all tissue samples were homogenized by using a Thomas Teflon homogenizer and kept on ice. The assays were carried out in duplicate as previously described (10,33). The reaction mixtures contained in a final volume of 500 μL: 80 mM Tris-HCl (pH 10.2), 0.4 mM $MgCl_2$, 0.376 mg of *p*-nitrophenylphosphate, 0.008% Triton X-100, 0 to 10 mM bile salt, and ALP sample. The reactions were started with the addition of the tissue enzyme samples [serum 100 μL (without dilution), liver 60 μL (3× diluted in solution A), duodenum and jejunum 15 μL (546 and 294× diluted in solution A, respectively)]. Incubations took place at 37 °C for 6 min and were stopped by the addition of 2 mL of 20 mM NaOH. ALP activity was determined by reading *p*-nitrophenol absorbance at 400 nm (Spectronic Genesys 5, Milton Roy, Rochester, NY, USA). The pH optimum for hepatic and intestinal ALP activity assays is not the same (33,36,37) but we used pH 10.2 for all samples used in this work to avoid one more different assay condition when comparing the bile salts effect on ALP activity.

Statistical analysis

All results are expressed as arithmetic mean ± standard deviation ($\bar{x} \pm SD$). The significance of differences between means was assessed by unpaired Student's t-test.

IC 50% calculation: IC 50% represents the bile salts concentration that inhibits ALP activity by 50%, in the assay conditions above referred.

Results

The effect of five conjugated bile salts on the activity of rat liver, duodenum and jejunum ALP was studied. We observed that, at concentrations higher than 1 mM, bile salts inhibited hepatic ALP activity (Figure 1): glycochenodeoxycholate (GCDC; 3α-OH, 7α-OH; glycine conjugated), glycocholate (GC; 3α-OH, 7α-OH, 12α-OH; glycine conjugated), and taurochenodeoxycholate (TCDC; 3α-OH, 7α-OH; taurine conjugated) were the most potent hepatic ALP inhibitors, followed by taurodeoxycholate (TDC; 3α-OH, 12α-OH; taurine conjugated) and taurocholate (TC; 3α-OH, 7α-OH, 12α-OH; taurine conjugated) (Table 1). On the opposite, the same bile salts, even at the concentration of 10 mM, had no effect on rat duodenal and jejunal ALP (results not shown). We also studied the effect of these bile salts (by using the concentration values that gave higher hepatic ALP activity inhibition) on total

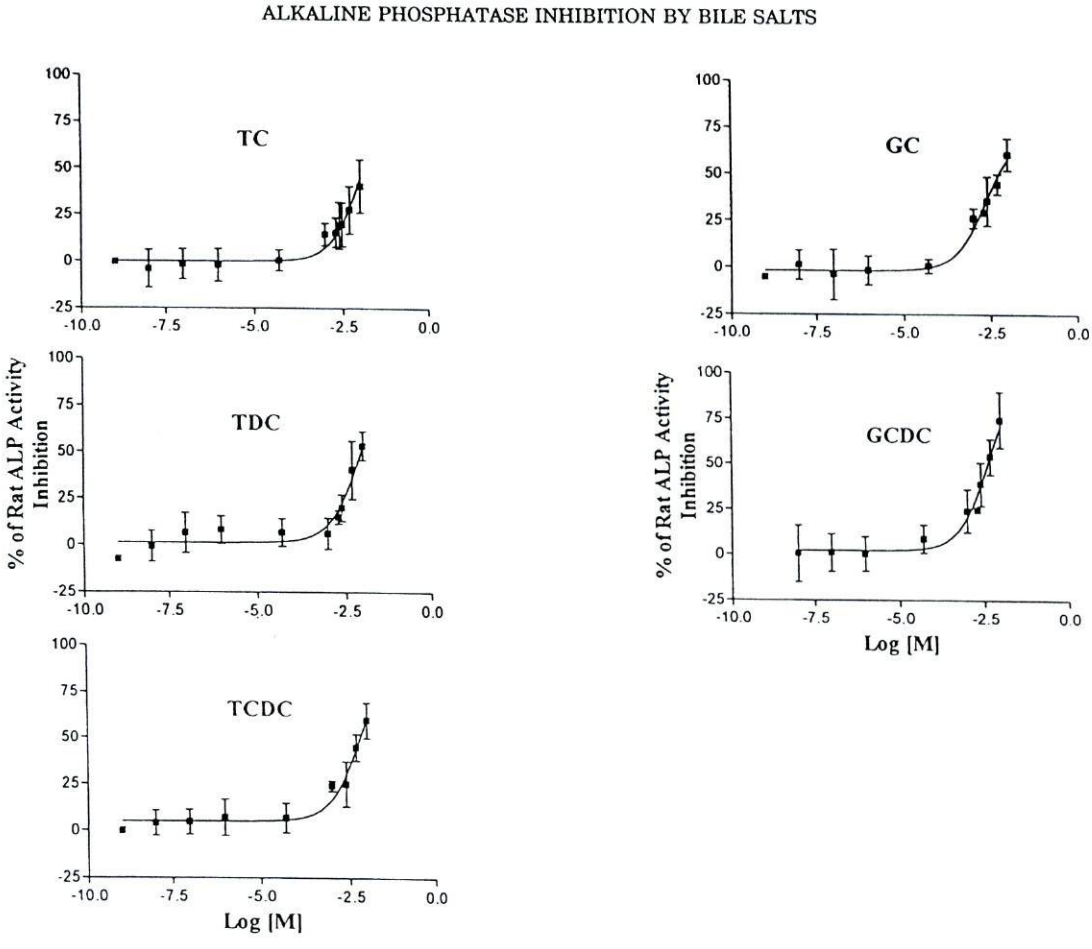

Figure 1 — Effect of conjugated bile salts on hepatic ALP activity from fed *ad lib* Wistar rats. All results (%) are expressed as arithmetic mean ± SD ($\bar{x} \pm SD$); $1 \leq n \leq 9$. All the assays were carried out in duplicate and the conditions in which they were performed are described in Materials and Methods. TCDC: taurochenodeoxycholate; TDC: taurodeoxycholate; TC: taurocholate; GCDC: glycochenodeoxycholate; GC: glycocholate.

TABLE 1
Inhibition of Rat Liver ALP Activity by Conjugated Bile Salts

Bile Salt	IC 50% (mM)	95% Confidence Interval (mM)
GCDC[a]	4.40	[3.40, 5.71]
GC	5.07	[3.80, 6.75]
TCDC	7.21	[5.51, 9.46]
TDC	9.34	[6.47,13.47]
TC	12.50	[9.95,15.72]

IC 50% with the corresponding 95% confidence intervals are shown ($1 \leq n \leq 9$). IC 50% represents the bile salt concentration that inhibits rat hepatic ALP activity by 50%. Liver homogenates from fed *ad libitum* Wistar rats were incubated at 37°C for 6 min with the indicated bile salts (in a concentration range 0.001 μM to 10 mM, final concentrations). See assay conditions in Materials and Methods.

[a]TCDC: taurochenodeoxycholate; TDC: taurodeoxycholate; TC: taurocholate; GCDC; glycochenodeoxycholate; GC: glycocholate.

serum ALP activity from both fasted and fed rats. Serum ALP activity inhibition was higher in the former group of animals (Figure 2).

The lack of effect on the intestinal isoenzyme was also observed when we tested (up to 10 mM) three deconjugated bile salts: cholate (C; 3α-OH, 7α-OH, 12α-OH; deconjugated), deoxycholate (DC; 3α-OH, 12α-OH; deconjugated), and chenodeoxycholate (CDC; 3α-OH, 7α-OH; deconjugated) (results not shown).

The reverse-phase high-performance liquid chromatography retention factor of bile salts observed by other authors (44) is negatively correlated (Figure 3, $r^2 = 0.86$) with the IC 50% values found by us for the effect of the conjugated bile salts on hepatic ALP activity (Table 1).

Discussion

Bile salts, particularly DC, are frequently used at concentrations of up to 24 mM to solubilize various components from isolated membrane preparations (19). Their total concentration in hepatic bile from rat and man is in this range: ~20 mM for rat and 14

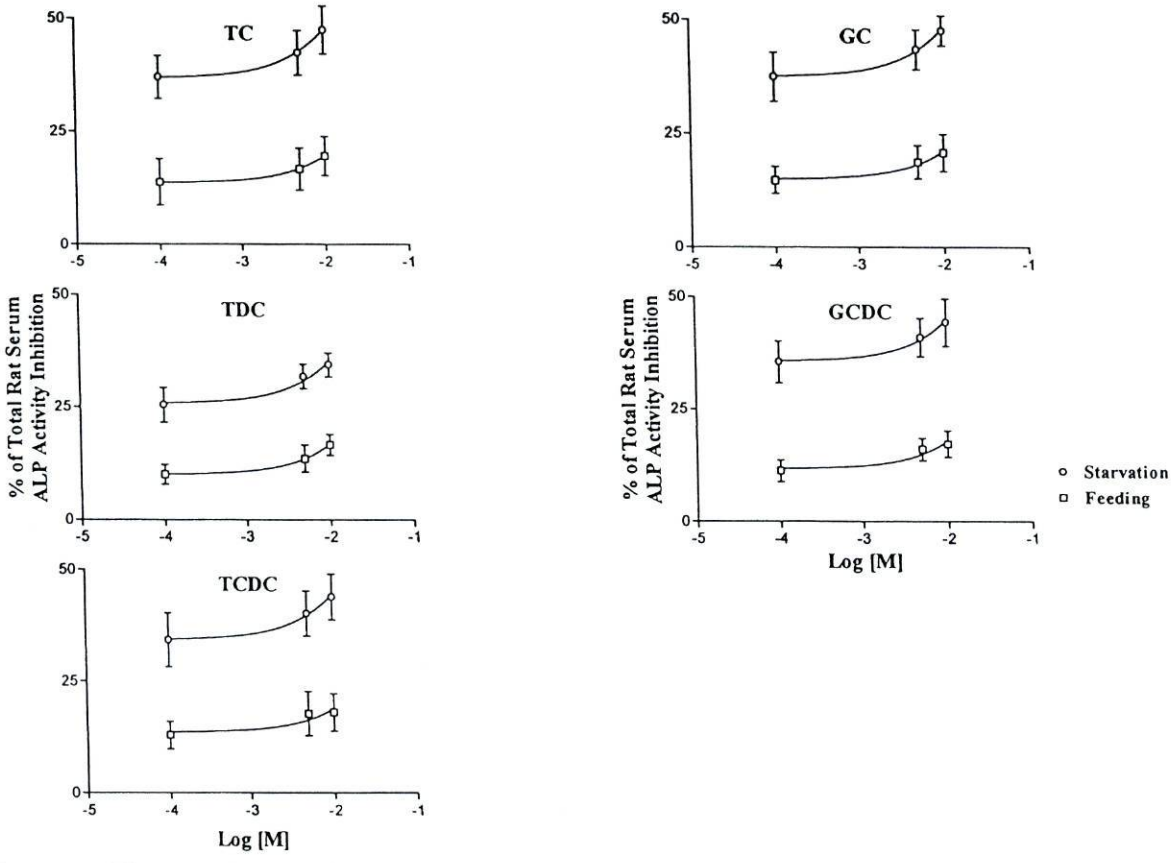

Figure 2 — Effect of conjugated bile salts on serum ALP activity of starved and fed *ad lib* Wistar rats. All results (%) are expressed as arithmetic mean ± standard deviation ($\bar{x} \pm SD$). All assays were carried out in duplicate and the conditions in which they were performed are described in Materials and Methods. Starvation (24 h): $5 \leq n \leq 6$; feeding *ad lib*: $4 \leq n \leq 6$. TCDC: taurochenodeoxycholate; TDC: taurodeoxycholate; TC: taurocholate; GCDC: glycochenodeoxycholate; GC: glycocholate.

mM for man (19). At maximum secretory rate, rat liver can deliver hepatic bile containing ~80 mM bile salts (14). Trihydroxy bile salts make up an important proportion of rat and human bile (19). In rat liver, there are powerful rehydroxylation enzymes and, thus, the bile contains only small amounts of the secondary bile salts produced in the intestine (14). Mono and dihydroxylated deconjugated bile salts are those most likely to be found in the intestine of both man and rat (14,27). These facts partially support our choice of bile salts and concentration values used in this work.

The effect of some of the bile salts used here (C, DC, CDC, TC, and TDC; alone or in a mixture) on rat renal ALP activity have been referred on two published reports (26,38). The observation that TC (1 mM, 24% of inhibition) was a more potent inhibitor of renal ALP activity than TDC (1 mM, 7% of inhibition) (38) are in line with our own results for hepatic ALP activity inhibition but, as we used a wider range of concentrations, we could observe that at concentrations higher than 3 mM TC became a weaker inhibitor of hepatic ALP than TDC (Figure 1).

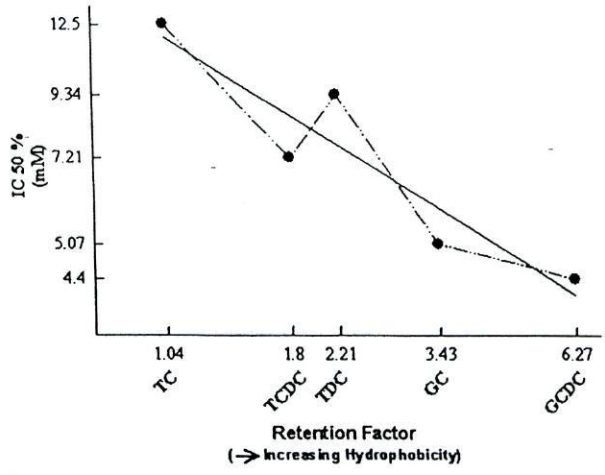

Figure 3 — Plot IC 50% of bile salts effect on liver ALP activity from fed *ad lib* Wistar rats vs. reverse-phase high-performance liquid chromatography retention factor (hydrophobicity) of bile salts ($r^2 = 0.86$). TCDC: taurochenodeoxycholate; TDC: taurodeoxycholate; TC: taurocholate; GCDC: glycochenodeoxycholate; GC: glycocholate.

The lack of effect of C, DC and CDC (5 mM), and TC (10 and 20 mM) on intestinal ALP had already been published (22,39).

In starvation and in feeding *ad lib* conditions rat serum possesses different ratios of hepatic ALP to intestinal ALP, intestinal ALP being higher in a well-fed state (31–33). Therefore, we decided to test the effect of the above-referred conjugated bile salts on the ALP activity from serum samples of rats under those two different metabolic conditions.

In good agreement with the ratio of hepatic ALP to intestinal ALP in these two groups of animals, ALP inhibition by conjugated bile salts in serum from starved rats was always significantly higher ($p < 0.005$) than that in serum from fed *ad lib* animals (Figure 2). This differential effect should allow discrimination between pathologic conditions where variations in the hepatic ALP/intestinal ALP proportion in the serum occur.

To our knowledge, this is the first report about the effect of the same conjugated bile salts (within the same concentration range) on the hepatic and intestinal ALP activities and showing differential inhibition of these ALP isoenzymes by conjugated bile salts. So when studying ALP activity, particularly in serum from patients suffering of biliary obstruction or in other conditions with the possibility of contamination with bile salts, attention should be directed to the possibility of the above-referred interference.

ALP found in body fluids such as serum or bile, tissue homogenates or present in supernatants after extraction from membrane fractions prepared from tissues or cells, occurs in various different isoforms. The structure and composition of these isoforms as well as their relative proportions depend on tissue or cell source, the biologic mechanisms of release or the specific conditions of physicochemical extraction. An example of the latter is the different isoforms obtained after butanol extraction at high and low pH that has been attributed to activation of inositol-specific phospholipase in acid conditions (2–8,40). In mammalian cells, ALP is a membrane-bound protein that cannot be easily extracted from the membranes with Triton X-100 (7,40) but, in normal conditions, blood ALP is not predominantly membrane bound (1,8).

To avoid any possible interference of Triton X-100 on ALP activity (41) we equalised the final concentration of this detergent in all the reaction mixtures (see Materials and Methods). It has been reported that solubilization of different ALP samples in Hepes buffer, pH 7.4, at 4 °C overnight in nonionic detergent Triton X-100 did not affect ALP activity (41).

Changes in protein concentration and in incubation time of the ALP samples in the presence of bile salts could produce variations in the relative quantities of the enzyme isoforms and on the bile salts effect on ALP activity. We tested different dilution factors and incubation times (which increased proportionately to the increase in the dilution factor and up to 60 min) from the ones referred in Material and Methods for all ALP samples being used from fed animals and no modification on the pattern of the TC effect on ALP activity was observed (results not shown). Also, increasing the incubation time (up to 60 min) but keeping constant the dilution factor did not modify TC inhibition of serum ALP activity from fasted rats (results not shown).

Results published in the literature, including from our lab, show that assay conditions are very important not only in quantifying total serum ALP and tissue ALP, but also in ALP isoenzyme inhibition studies (36). So, we tested the effect of TDC on jejunal and liver ALP activity by using Tris-HCl with pH values from 8.2 to 10.7: there was no modification both in the inhibition of the hepatic isoenzyme and in the lack of effect on the intestinal isoenzyme (results not shown).

Despite (i) the different ALP isoforms present in the samples used for ALP quantification and their possible distinct modification by bile salts, (ii) the variability in the hepatic/intestinal ALP proportions in each one of the serum samples from the two groups of animals under study, and (iii) the different total protein concentration present in the liver, intestine, and serum samples, these variations did not seem to influence the serum demonstration of the bile salts differential ALP isoenzymes inhibition. Our results suggest that the tecidular origin of the ALP enzyme is the major determinant in the bile salts inhibitory effect.

As several amino acids have been reported to be inhibitors of ALP (1,9,42,43) we asked weather these effects could somehow be related to the ones observed by us.

The first amino acid to be discovered as an ALP inhibitor was L-phenylalanine, which inhibits more intestinal and placental ALP than the liver and bone isoenzymes. L-tryptophan has a similar specificity. In contrast, L-homoarginine inhibits liver and bone ALP to a much greater extent than the intestinal and placental isoenzymes. For all these amino acids hydrophobic regions in the molecule are necessary for inhibition to take place. The inference seems to be that they bind to a hydrophobic site on the enzyme molecule. The susceptibility of ALP to inhibition by specific amino acids may imply that its catalytic function may be affected *in vivo* by specific microenvironments, such as local amino acid concentration (1,9,42,43).

Bile salts are relatively mild in their effects on membrane proteins, although, in some cases, conformational change in the protein may be produced as the shell of detergent molecules replaces the lipid environment. This is evidenced by a change or loss in enzymatic activities, or the loss of interactions with other specific proteins, as solubilization proceeds. In general, when it occurs, the conformational effect is greater with increasing hydrophobicity of the bile salt nucleus (14).

Hydrophobicity of common bile salts have been shown to be directly correlated with their reverse-phase high-performance liquid chromatography re-

tention factor which increase in the order: C < CDC < DC (44). Reverse-phase high-performance liquid chromatography retention factors of the taurine conjugates are lower than those of the glycine conjugates, which in turn are lower than those of the corresponding free bile salts. C and its conjugates are rendered less hydrophobic by the presence of a third hydroxyl group (44). In our results there was a change in the expected order of the IC 50% value of TDC and the IC 50% value of TCDC but, in general, the hydrophobic character of the bile salts correlated well with their inhibitory potency (Figure 3). As in the case of the amino acids, the hydrophobic character seems to play an important role on the bile salts inhibitory potency of hepatic ALP activity.

Levamisole and bromotetramisole have been described as strongly inhibiting liver, bone, kidney, and spleen ALP but having very little effect on intestinal and placental ALP (1,9). As in the case of levamisole and L-phenylalanine (1,9,42,43), bile salts would be useful helping to discriminate between ALP isoenzymes separated by electrophoresis and in the identification of pathologic conditions where their relative quantities are altered in the serum.

Recent data suggest a possible role for Pgp in cholesterol (sterols) trafficking from the plasma membrane to the endoplasmic reticulum (45–47). Progesterone inhibits cholesterol esterification, possibly by blocking the transport of cholesterol to the endoplasmic reticulum (48). There is a strong correlation between a steroid hormone's hydrophobicity and its ability to inhibit both cholesterol esterification and Pgp-catalyzed drug efflux (48). An interaction between ALP and Pgp activities has already been proposed by us (11,12) and, in line with that proposition, it is possible that the hepatic ALP inhibition by bile salts (or other inhibitors) affects cholesterol transport and esterification.

Bile salts are synthesized in the liver and secreted into bile. In the small intestine, they assist in the lipolysis of dietary triacylglycerols and then, in the jejunum and ileum, present the resulting fatty acids and monoacylglycerols for absorption. In the terminal ileum bile salts are absorbed by transport systems, enter the portal blood and are then returned to the liver to be secreted once more into bile (14). This enterohepatic circulation has been recently reported to be regulated by bile salts (30). When bile salts (specially CDC) are bound to farnesoid X receptor (bile salts are physiologic ligands for the farnesoid X receptor, an orphan nuclear receptor) there is an activation of the gene encoding an intestinal bile salt binding-protein, which is a cytosolic protein that serves as a component of the bile salt transport system in the ileal enterocytes (30). Moreover, when bound to bile salts, farnesoid X receptor represses transcription of the gene encoding the rate-limiting enzyme in bile salts synthesis, cholesterol 7α-hydroxylase (30).

At present, the possible existence of a link between the bile salts differential inhibition of intestinal ALP and hepatic ALP and their enterohepatic circulation and/or biosynthesis is speculative. However, the evidence of that differential effect at physiologic concentrations of bile salts strongly supports the investigation of that hypothesis.

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References

1. Van Hoof VO, De Broe ME. Interpretation and clinical significance of alkaline phosphatase isoenzyme patterns. *Crit Rev Clin Lab Sci* 1994; **31**: 197–293.
2. Alpers DH, Eliakim R, DeSchryver–Kecskemeti K. Secretion of hepatic and intestinal alkaline phosphatases: similarities and differences. *Clin Chim Acta* 1989; **186**: 211–24.
3. Hamilton BA, McPhee JL, Hawrylak K, Stinson RA. Alkaline phosphatase releasing activity in human tissues. *Clin Chim Acta* 1989; **186**: 249–54.
4. Deng JT, Hoylaerts MF, Van Hoof VO, De Broe ME. Differential release of human intestinal alkaline phosphatase in duodenal fluid and serum. *Clin Chem* 1992; **38**: 2532–8.
5. Moss DW. Release of membrane-bound enzymes from cells and the generation of isoforms. *Clin Chim Acta* 1994; **226**: 131–42.
6. Raymond FD, Moss DW, Fisher D. Separation of alkaline phosphatase isoforms with and without intact glycan-phosphatidylinositol anchors in aqueous polymer phase systems. *Clin Chim Acta* 1994; **227**: 111–20.
7. Deng JT, Hoylaerts MF, Nouwen EJ, De Broe ME, Van Hoof VO. Purification of circulating liver plasma membrane fragments using a monoclonal antileucine aminopeptidase antibody. *Hepatology* 1996; **23**: 445–54.
8. Van Hoof VO, Deng JT, De Broe ME. How do plasma membranes reach the circulation? *Clin Chim Acta* 1997; **266**: 23–31.
9. Crofton PM. Biochemistry of alkaline phosphatase isoenzymes. *Crit Rev Clin Lab Sci* 1982; **16**: 160–94.
10. Calhau C, Hipólito–Reis C, Azevedo I. Alkaline phosphatase and exchange surfaces. *Clin Biochem* 1999; **32**: 153–4.
11. Martel F, Martins MJ, Hipólito–Reis C, Azevedo I. Inward transport of [^{3}H]-1-methyl-4-phenylpyridinium in rat isolated hepatocytes: putative involvement of a P-glycoprotein transporter. *Br J Pharmacol* 1996; **119**: 1519–24.
12. Martel F, Martins MJ, Calhau C, Hipólito–Reis C, Azevedo I. Postnatal development of organic cation transport in the rat liver. *Pharmacol Res* 1998; **37**: 131–6.
13. Hofmann AF, Small DM. Detergent properties of bile salts: correlation with physiological function. *Annu Rev Med* 1967; **18**: 333–76.
14. Coleman R. Bile salts and biliary lipids. *Biochem Soc Trans* 1987; 15: 68–80S.
15. Brunton LL. Agents affecting gastrointestinal water flux and motibility, digestants, and bile acids. In:

Gilman AG, Rall TW, Nies AS, Taylor P, Eds. *The pharmacological basis of therapeutics.* Pp. 914–32, 8th ed. New York: Pergamon Press, 1990.
16. Coleman R, Holdsworth G. The release of membrane components prior to haemolysis during extraction of intact erythrocytes with bile salts. *Biochim Biophys Acta* 1976; **426**: 776–80.
17. Vyvoda OS, Coleman R, Holdsworth G. Effects of different bile salts upon the composition and morphology of a liver plasma membrane preparation. Deoxycholate is more membrane damaging than cholate and its conjugates. *Biochim Biophys Acta* 1977; **465**: 68–76.
18. Billington D, Evans CE, Godfrey PP, Coleman R. Effects of bile salts on the plasma membranes of isolated rat hepatocytes. *Biochem J* 1980; **188**: 321–7.
19. Coleman R, Iqbal S, Godfrey PP, Billington D. Membranes and bile formation. Composition of several mammalian biles and their membrane-damaging properties. *Biochem J* 1979; **178**: 201–8.
20. Attili AF, Angelico M, Cantafora A, Alvaro D, Capoccaccia L. Bile acid-induced liver toxicity: relation to the hydrophobic-hydrophilic balance of bile acids. *Med Hypotheses* 1986; 19: 57–69.
21. Sung JY, Shaffer EA, Costerton JW. Antibacterial activity of bile salts against common biliary pathogens. Effects of hydrophobicity of the molecule and in the presence of phospholipids. *Dig Dis Sci* 1993; **38**: 2104–12.
22. Shiozaki H, Yoshioka M, Miura S, *et al.* Conjugated bile salts regulate turnover of rat intestinal brush border membrane hydrolases. *Dig Dis Sci* 1995; **40**: 1193–8.
23. Heuman DM, Pandak WM, Hylemon PB, Vlahceciv ZR. Conjugates of ursodeoxycholate protect against cytotoxicity of more hydrophobic bile salts: in vitro studies in rat hepatocytes and human erythrocytes. *Hepatology* 1991; **14**: 920–6.
24. Coleman R, Lowe P. Membrane damage caused by bile salts. *Biochem Soc Trans* 1980; **8**: 126–7.
25. Ilani A, Granoth R. The pH dependence of the hemolytic potency of bile salts. *Biochim Biophys Acta* 1990; **1027**: 199–204.
26. Morgan WA, Kaler B, Bach PH. The effects of bile salts on the kinetic properties of enzymes used in the diagnosis of liver and kidney damage. *Biochem Soc Trans* 1997; **25**: 76S.
27. Morgan WA, Sharma P, Kaler B, Bach PH. The modulation of protein kinase C by bile salts. *Biochem Soc Trans* 1997; **25**: 75S.
28. Beuers U. Effects of bile acids on hepatocellular signaling and secretion. *Yale J Biol Med* 1997; **70**: 341–6.
29. Beuers U, Probst I, Soroka C, Boyer JL, Kullak–Ublick G-A, Paumgartner G. Modulation of protein kinase C by taurolithocholic acid in isolated rat hepatocytes. *Hepatology* 1999; **29**: 477–82.
30. Makishima M, Okamoto AY, Repa JJ, *et al.* Identification of a nuclear receptor for bile acids. *Science* 1999; **284**: 1362–8.
31. Martins MJ, Dias PO, Hipólito–Reis C. Rat serum alkaline phosphatase electrophoretic fractions: variations with feeding, starvation and cellulose fibre ingestion. *Clin Nutr* 1998; **17**: 279–85.
32. Glickman RM, Alpers DH, Drummey GD, Isselbacher KJ. Increased lymph alkaline phosphatase after fat feeding: effects of medium chain triglycerides and inhibition of protein synthesis. *Biochim Biophys Acta* 1970; **201**: 226–35.
33. Young GP, Friedman S, Yedlin ST, Alpers DH. Effect of fat feeding on intestinal alkaline phosphatase activity in tissue and serum. *Am J Physiol* 1981; **241**: G461–8.
34. Martins MJ, Negrão MR, Hipólito–Reis C. Effect of bile salts on serum alkaline phosphatase activity from starved and fed rats. *Ital Biochem Soc Trans* 1999; **13**: 256.
35. Martins MJ, Negrão MR, Hipólito–Reis C. Effect of bile salts on rat hepatic and intestinal alkaline phosphatase. *Ital Biochem Soc Trans* 1999; **13**: 257.
36. Hipólito–Reis C, Dias PO, Martins MJ. Importance of assay conditions in visualization and quantitation of serum alkaline phosphatase isoenzymes separated by electrophoresis. *Scand J Clin Lab Invest* 1999; **59**: 593–606.
37. Fernley HN. Mammalian alkaline phosphatases. In: Boyer PD, Ed. *The enzymes,* Vol. IV. Pp. 417–47. New York: Academic Press, 1971.
38. Israeli B-A, Bogin E. Biochemical changes in liver, kidney and blood associated with common bile duct ligation. *Clin Chim Acta* 1986; **160**: 211–21.
39. Hepner GW, Hofmann AF. Different effects of free and conjugated bile acids and their keto derivatives on (Na^+,K^+)-stimulated and Mg^{2+}-ATPase of rat intestinal mucosa. *Biochim Biophys Acta* 1973; **291**: 237–45.
40. Miki A, Tanaka Y, Ogata S, Ikehara Y. Selective preparation and characterization of membranous and soluble forms of alkaline phosphatase from rat tissues. A comparison with the serum enzyme. *Eur J Biochem* 1986; **160**: 41–8.
41. Miura S, Yoshioka M, Hamada Y, Morita A, Asakura H, Tsuchiya M. Alkaline phosphatase of rat intestinal lymph: its characterization and the effect of fat administration. *Clin Chim Acta* 1989; **186**: 239–48.
42. McComb RB, Bowers GN, Posen S. *Alkaline phosphatase.* Pp. 289–372. New York: Plenum Press, 1979.
43. Fishman WH, Sie H-G. Organ-specific inhibition of human alkaline phosphatase isoenzymes of liver, bone, intestine and placenta; L-phenylalanine, L-tryptophan and L-homoarginine. *Enzymologia* 1971; **41**: 141–67.
44. Armstrong MJ, Carey MC. The hydrophobic-hydrophilic balance of bile salts. Inverse correlation between reverse-phase high performance liquid chromatographic mobilities and micellar cholesterol-solubilizing capacities. *J Lipid Res* 1982; **23**: 70–80.
45. Luker GD, Nilsson KR, Covey DF, Piwnica–Worms D. Multidrug resistance (MDR1) P-glycoprotein enhances esterification of plasma membrane cholesterol. *J Biol Chem* 1999; **274**: 6979–91.
46. Metherall JE, Li H, Waugh K. Role of multidrug resistance P-glycoproteins in cholesterol biosynthesis. *J Biol Chem* 1996; **271**: 2634–40.
47. Field FJ, Born E, Murthy S, Mathur SN. Transport of cholesterol from the endoplasmic reticulum to the plasma membrane is constitutive in CaCo-2 cells and differs from the transport of plasma membrane cholesterol to the endoplasmic reticulum. *J Lipid Res* 1998; **39**: 333–43.
48. Debry P, Nash EA, Neklason DW, Metherall JE. Role of multidrug resistance P-glycoproteins in cholesterol esterification. *J Biol Chem* 1997; 272: 1026–31.

Artigo V[3]

Alkaline phosphatase from rat liver and kidney is differentially modulated.

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Alkaline phosphatase from rat liver and kidney is differentially modulated

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Abstract

Objective: To investigate the effect of inhibitors of alkaline phosphatase (ALP) and modulators of P-glycoprotein (Pgp), multidrug resistance protein (MRP) and hepatic taurocholate uptake on the activity of tissue-nonspecific ALP (TNALP) in liver and kidney.
Design and Results: ALP activity was determined in rat liver and kidney homogenates. Levamisole had a stronger inhibitory effect on renal TNALP than on the hepatic isoform. 1,3-dimethylxanthine (theophylline) almost abolished renal TNALP activity whereas its effect on hepatic TNALP was less intense. 3-isobutyl-1-methylxanthine (IBMX) and lidocaine produced opposite effects, activating hepatic TNALP and inhibiting the kidney isoform. Quinidine significantly inhibited renal TNALP without affecting hepatic TNALP. Kaempferol activated both liver and kidney isoforms, the effect being more pronounced on hepatic TNALP.
Conclusions: a) renal TNALP seems to be more sensitive to inhibition than hepatic TNALP, b) TNALP activity studies should take into account the source of ALP isoform and c) ALP pharmacological manipulation *in vivo* may produce different and even opposite effects in different tissues/organs.

Keywords: Alkaline phosphatase; Modulation; Levamisole; Theophylline; IBMX; Lidocaine; Quinidine; Kaempferol

1. Introduction

ALP (EC 3.1.3.1; orthophosphoric-monoester phosphohydrolase, alkaline optimum) is a family of nonspecific ecto-phosphomonoesterases. ALP binds to the cellular membrane *via* a glycosyl-phosphatidylinositol anchor linkage and can be differently released into intestinal lumen, plasma or bile depending on both physiologic and pathologic conditions [1–4]. Human ALPs are encoded by four different gene *loci* which express the kidney/bone/liver-type (TNALP), intestinal (IntALP), placental (PALP) and germ-cell (GALP) isoenzymes. Since kidney, bone and liver express relatively high amounts of the same ALP protein (isoform), this is usually denominated TNALP. IntALP, PALP and GALP are expressed in high amounts in intestine, placenta and germ cells, respectively, and are denominated tissue-specific ALP [1].

Quantification of total ALP activity in serum and/or of serum ALP electrophoretic fractions/isoenzymes is frequently used in clinical medicine. In previous work, we have shown the importance of using the same assay conditions in both determinations [1,5,6]. PALP appears in maternal serum in increasing amounts as pregnancy term approaches; TNALP from liver and bone increases in serum when obstructive liver disease and bone growth/rebuilding or osteomalacia occur, respectively. ALP cancer related research is focused on the altered expression/activity and posttranslational modifications of the enzyme, such as changes in glycosylation, in several malignancies [1,7,8].

Differential processing of the TNALP and IntALP gene products, through differential glycosylation, gives rise to tissue-specific isoforms [1,7,9–12]. ALPs are glycoproteins that, regarding their glycan moities, differ not only in the

Abbreviations: ABC, ATP binding cassette; ALP, alkaline phosphatase; ATP-DPH, ATP-diphosphohydrolase; DMSO, dimethylsulphoxide; GALP, germ-cell alkaline phosphatase; IBMX, 3-isobutyl-1-methylxanthine; IntALP, intestinal alkaline phosphatase; MRP, multidrug resistance protein; PBS, phosphate buffered saline; PALP, placental alkaline phosphatase; Pgp, P-glycoprotein; p-NPL, p-nitrophenol; p-NPP, p-nitrophenylphosphate; theophylline, 1,3-dimethylxanthine; TNALP, tissue-nonspecific alkaline phosphatase.

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way oligosaccharide chains are linked to the protein but also on the type of sugar-chain present in the molecule [1,7,11–14]. It has, for example, been observed that TNALP isoforms from kidney, bone and liver are N-glycosylated but, contrary to isoforms from kidney and bone, the hepatic isoform does not seem to be O-glycosylated [12,14]. The study of ALP sugar-chain heterogeneity revealed a difference not only among the genetically different TNALP, IntALP and PALP isoenzymes but also among TNALP isoforms from kidney, bone and liver [7]. ALP glycosylation seems to be organ specific rather than being species-specific [7,14]. The influence of oligosaccharides on ALP has been studied by various authors [9,12,13,15–17]. As hepatic and renal TNALP have different glycosylation patterns [7,12] we decided to investigate whether these differences could determine distinct responses of the two isoforms when being challenged by various drugs.

ALP specific function remains unclear. ALP cellular location mainly in the plasma membranes of exchange surfaces (for example in liver, kidney, bone, intestine and placenta), where extensive transport takes place, is suggestive of a biologic role related to transport processes [1]. ALP might be involved in cellular migration, in intestinal lipid transport, in bone mineralization and in regulation of a low-conductance chloride channel [1]. Previous studies from our group further support a link between ALP and transmembrane transport activities [18,19]. Physical separation of ALP from Pgp and ALP inhibition seem to favor drug import through Pgp [transport system that usually exports drugs from cells causing multidrug resistance and that belongs to the superfamily of ATP binding cassette (ABC) transporter proteins] [18,19]. Unpublished results from our laboratory also show that modulators of ALP activity can modify taurocholate uptake by freshly isolated rat hepatocytes.

Following our overall aim of clarifying ALP physiologic role(s), we have tested the effect of known ALP inhibitors (levamisole, theophylline and IBMX) [1,20–23], modulators of Pgp or MRP (another ABC transporter protein also causing multidrug resistance) (verapamil, kaempferol, genistein, quinidine, progesterone and corticosterone) [18, 19,24–28] and modulators of hepatic taurocholate uptake (progesterone, verapamil, lidocaine, quinidine and bupivacaine) [29,30] on ALP activity in liver and kidney homogenates. Distinct and, in some cases, opposite effects were observed on TNALP isoforms for some drugs. Previous reports have shown that hepatic and renal TNALP activity can be differently modulated [20,21,31–33] but, in our opinion, the search for new differential modulators and the study of differences in hepatic and renal TNALP behavior have not yet been object of the attention that the theme deserves.

A preliminary account of a portion of this work has already been presented in abstract form [34].

2. Materials and methods

2.1. Materials

p-Nitrophenylphosphate (p-NPP; Sigma 104D), p-nitrophenol (Sigma 104–8), Brilliant Blue G (Coomassie Brilliant Blue G, B-0770), albumin (A-1653), levamisole (L-9756), theophylline (T-1633), IBMX (I-5879), ± -verapamil (V-4629), progesterone (P-0130), corticosterone (C-2505), kaempferol (K-0133), genistein (G-6776), bupivacaine (B-5274), lidocaine (L-5647) and quinidine (Q-0875) were purchased from Sigma (Sigma Alcobendas), Madrid, Spain. All other reagents were of the highest quality and purity available.

2.2. Methods

2.2.1. Animals and sample collection

18 male adult Wistar rats (free from liver and bone diseases) weighing from 320 to 610 g of body weight (CRIFFA, Barcelona, Spain) were used. The animals were kept under controlled environmental conditions (12 h light/dark cycle and room temperature 24°C), fed with a suitable commercial diet (Harlan Interfauna Iberica, SA, Barcelona, Spain) with water *ad lib.*

Immediately after decapitation liver and kidneys were simultaneously perfused *in situ* with ice-cold isotonic NaCl to wash out the blood inside the organs. Organs were then removed and rinsed in ice-cold isotonic NaCl, which was followed, in kidney samples only, by the discharge of the medulla. The liver and the kidney cortex were placed, respectively, in 2 or 5 times (mL/g) solution A [phosphate buffered saline (PBS), pH 7.4: 0.5% Triton X-100; 4:1] and kept at −80°C until the moment of the experiment.

2.2.2. ALP activity quantification

On the day of ALP quantification, tissue samples were homogenized and kept on ice. ALP activity assays were carried out in duplicate, as previously described [35]. The reaction mixtures contained in a final volume of 500 μL: 80 mM Tris-HCl (pH 10.4), 0.4 mM $MgCl_2$, 0.376 mg p-NPP, the compound to be tested (0–10 mM) and liver or kidney homogenate. We equalized the final concentration of Triton X-100 (0.01%) and PBS (8%) in all reaction mixtures. The reactions were started by the addition of the tissue enzyme samples [60 μL of liver homogenate or 10 μL of kidney homogenate (both homogenates in a final dilution of 1/6 in solution A)]. Minimum and maximum values of protein in incubation medium were 0.9/5.2 mg and 0.2/0.4 mg for liver and kidney assays, respectively. Levamisole (0.1–1 mM), verapamil (0.001–0.5 mM), bupivacaine (0.2×10^{-6}–10 mM), lidocaine (0.2×10^{-6}–10 mM) and quinidine (0.2×10^{-6}–2 mM) were dissolved in water. Progesterone (0.01–5 mM) and corticosterone (0.001–5 mM) were dissolved in ethanol. Genistein (1 and 5 mM), kaempferol (0.1 and 0.5 mM), theophylline (0.001–5 mM) and IBMX (0.001–10

mM) were dissolved in dimethylsulphoxide (DMSO). Controls for drugs were run in the presence of correspondent volume of solvent. Incubations took place at 37°C for 12 or 2 min for liver and kidney homogenates, respectively, and the reactions were stopped by adding 2 mL of 20 mM NaOH. ALP activity was determined by reading, at 400 nm, the absorbance of the p-nitrophenol (p-NPL) produced. Although kaempferol has a yellow color, intensified in the incubation medium, it did not interfere in p-NPL quantification. The effect of each drug tested against ALP activity was determined as a percentage of the correspondent ALP control activity.

2.2.3. *Protein determination*

Protein concentration was determined as described by Bradford, with human serum albumin as standard [36].

2.2.4. *Statistical analysis*

All results are expressed as arithmetic mean ± standard deviation and represent a percentage of the correspondent control activity (n = number of cases). The significance of differences between means was assessed by paired or unpaired Student's t-test.

3. Results

The effect of eleven different drugs, belonging to distinct chemical and/or pharmacological classes, was studied on the ALP activity from rat liver and kidney homogenates. The assay conditions used for the quantification of ALP activity (which were the same for both tissue homogenates) allowed linearity of the enzymatic reaction (data not shown).

Levamisole significantly inhibited ALP activity from both samples but the effect was significantly higher on kidney homogenate (Figure 1). 0.5 and 1.0 mM levamisole inhibited approximately 40% of hepatic TNALP activity while kidney TNALP activity was almost completely abolished (Figure 1).

Theophylline had a much stronger (significantly different) inhibitory effect on ALP activity from kidney than on that from liver (Figure 2a).

IBMX and lidocaine had opposing effects on the two TNALP samples (Figures 2b and 3a, respectively). For both compounds, at 10 mM, it was observed a significant activation of hepatic TNALP activity and a significant inhibition of kidney TNALP activity.

2 mM quinidine had a significant inhibitory effect on renal TNALP activity while the hepatic TNALP activity was not affected (Figure 3b). Inhibition of renal TNALP activity by bupivacaine was slightly higher than that of hepatic TNALP activity (Figure 3c).

0.5 mM verapamil (data not shown) had a significantly different effect on the two TNALP samples, slightly reducing kidney TNALP activity and having no effect on hepatic TNALP activity.

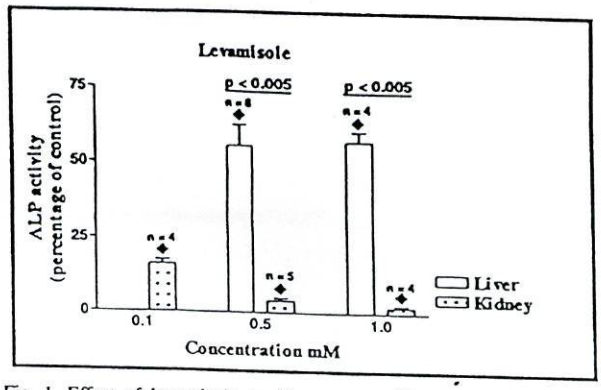

Fig. 1. Effect of levamisole on tissue-nonspecific alkaline phosphatase activity from rat liver and kidney homogenates (significantly different from control: ♦ $p \leq 0.005$). The ALP specific activity in the control was 1.90 ± 0.59 nmol p-nitrophenol mg^{-1} protein min^{-1} for liver homogenate and 272.52 ± 87.52 nmol p-nitrophenol mg^{-1} protein min^{-1} for kidney homogenate.

0.5 mM kaempferol had a slightly stronger activating effect on hepatic TNALP activity than on renal TNALP activity (Figure 4). 0.1 mM kaempferol (Figure 4) and 1 mM genistein (data not shown) had slightly different effects on hepatic and renal TNALP: significantly reducing kidney TNALP without affecting liver TNALP.

5 mM corticosterone significantly inhibited renal TNALP activity (data not shown). Progesterone had no effect on both ALP activities.

Hepatic and renal TNALP responded significantly differently when treated with levamisole, theophylline, IBMX, lidocaine, quinidine, bupivacaine, verapamil, kaempferol and genistein. Levamisole was the most potent inhibitor of renal TNALP, followed by theophylline and IBMX.

As hepatic TNALP showed a lower sensitivity to inhibition, we performed some additional sets of experiments to exclude possible interferences resulting from the use of crude tissue homogenates as ALP source and from the presence of different protein concentrations in the liver and kidney assay media.

We tested the effect of IBMX (using the same assay conditions) upon TNALP activity from both homogenates in separate and upon mixtures of the two homogenates. In one type of experiment liver and kidney homogenates were added to the assays and the incubation time was 2 min; in the other type of experiment liver and kidney homogenates were added to the assays (with kidney homogenate previously diluted 36 times in solution A) and the incubation time was 12 min. When testing the effect of IBMX on the two homogenates in separate (and for both incubation periods) TNALP hepatic activity was increased to 115% and TNALP renal activity was reduced to 17%. The results obtained with the two types of homogenates mixtures reflected these two opposite effects and the different proportions of hepatic and kidney TNALP in the two homogenates

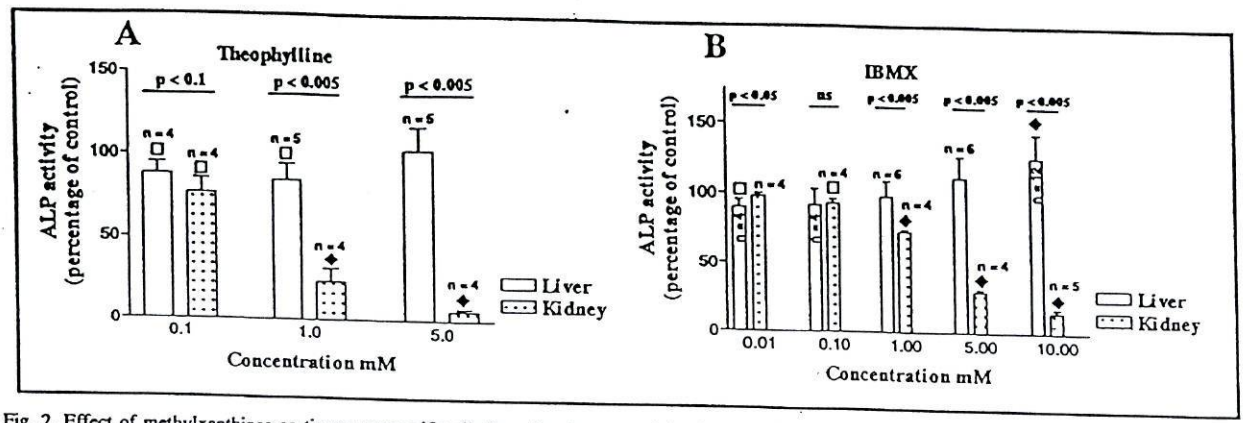

Fig. 2. Effect of methylxanthines on tissue-nonspecific alkaline phosphatase activity from rat liver and kidney homogenates (significantly different from control: □ $p \leq 0.05$ and ♦ $p \leq 0.005$). The ALP specific activity in the control was 1.72 ± 0.45 nmol p-nitrophenol mg^{-1} protein min^{-1} for liver homogenate and 156.08 ± 49.95 nmol p-nitrophenol mg^{-1} protein min^{-1} for kidney homogenate. A: Theophylline. B: IBMX.

mixtures. In the experiment where the kidney had not been diluted IBMX reduced TNALP activity of the mixture to 30%; the effect of IBMX was much less pronounced when kidney had been diluted since TNALP activity of the mixture was just reduced to 60%. No evidence of mutual interference of the homogenates on the effects of IBMX was observed.

In routine ALP liver homogenate assays there was a higher concentration of protein in the incubation media when compared to kidney homogenate. When the quantity of liver homogenate in the assay media was decreased the effects of levamisole and IBMX remained almost unchanged. When the liver homogenate was used to final dilutions, in solution A, of 1/12 and 1/30 (and in the latter situation increasing the incubation time from 12 to 60 min) the inhibitory effect of levamisole was 52% and 57%, respectively; the activating effect of IBMX was 41% and 32%, respectively.

These experiments allowed us to exclude a) the presence of any specific endogenous hepatic compound interfering with ALP sensitivity to drugs, b) drug metabolization by liver tissue and c) drug adsorption to liver protein.

4. Discussion

As already mentioned there are a few reports describing small differences in the behavior of hepatic and renal TNALP activity when challenged by some compounds. Those reports include the effects of p-chloromercuribenzoate, EDTA, levamisole, L-phenylalanine, urea, $CdCl_2$, $MgCl_2$ and $ZnCl_2$ [20,21,31–33].

Supporting the hypothesis that different glycosylation patterns in liver and renal TNALP determine differences in their functional behavior, here we present evidence of differential modulation of rat hepatic and renal TNALP by theophylline (Figure 2a), IBMX (Figure 2b), lidocaine (Figure 3a), quinidine (Figure 3b), bupivacaine (Figure 3c), verapamil (data not shown), kaempferol (Figure 4), genistein (data not shown), and confirm published results regarding levamisole (Figure 1). In our study, 1 mM levamisole inhibited approximately 40% of hepatic TNALP while the inhibition of renal TNALP was almost complete. Van Belle *et al.* had already reported that rat renal TNALP activity was totally inhibited by 1 mM levamisole whereas only 80% of the rat hepatic isoform was inhibited, albeit

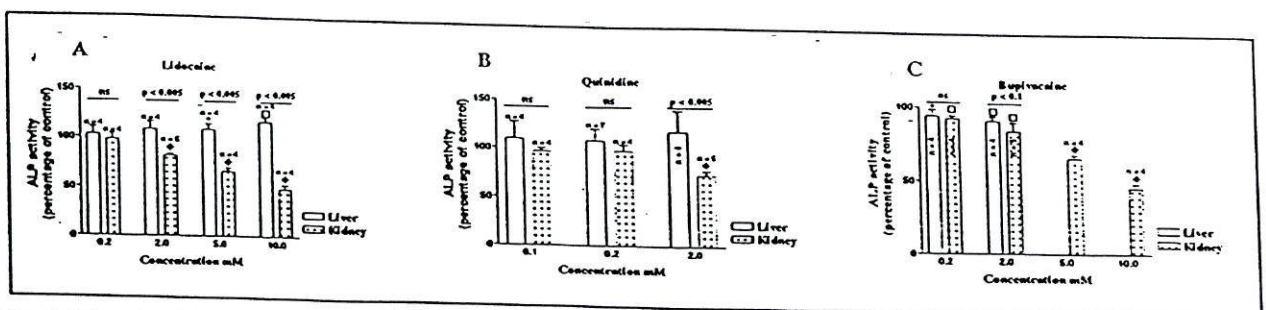

Fig. 3. Effect of modulators of taurocholate hepatic uptake on tissue-nonspecific alkaline phosphatase activity from rat liver and kidney homogenates (significantly different from control: • $p < 0.1$, □ $p \leq 0.05$ and ♦ $p \leq 0.005$). The ALP specific activity in the control was 1.90 ± 0.59 nmol p-nitrophenol mg^{-1} protein min^{-1} for liver homogenate and 272.52 ± 87.52 nmol p-nitrophenol mg^{-1} protein min^{-1} for kidney homogenate. A: Lidocaine. B: Quinidine. C: Bupivacaine.

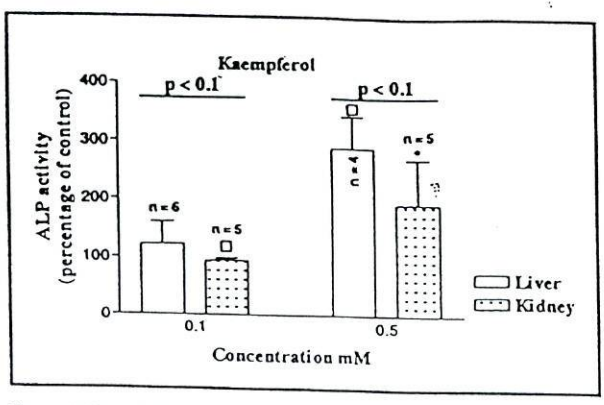

Fig. 4. Effect of kaempferol on tissue-nonspecific alkaline phosphatase activity from rat liver and kidney homogenates (significantly different from control: • $p < 0.1$ and □ $p \leq 0.05$). The ALP specific activity in the control was 1.72 ± 0.45 nmol p-nitrophenol mg^{-1} protein min^{-1} for liver homogenate and 156.08 ± 49.95 nmol p-nitrophenol mg^{-1} protein min^{-1} for kidney homogenate.

their tissue preparation and ALP assay conditions differed from the ones presented here [20].

It should be mentioned, however, that in the cases of progesterone and corticosterone our results were similar on both hepatic and renal TNALP.

Oligosaccharides may affect proteins in several ways and the effect of sugar chains on ALP has been studied by various authors [9,12,13,15–17]. Elimination of O-glycosylations affects hepatic and renal TNALP activities differently; N-glycosylation is absolutely essential for the activity of kidney, bone and liver TNALP, but not for that of IntALP, PALP or GALP [12,16,17]. The difference in heat stability between liver and bone TNALP is much reduced, but not abolished, by glycosidase treatment [9]. So, as part of our research, we have hypothesized that the distinct glycosylation of hepatic and renal TNALP could contribute to differences in their functional behavior toward several drugs.

Our results indicate that modulation studies of TNALP activity should consider the source of the TNALP isoform. Until now mainly "class-specific" inhibitions have been considered [1,5,15,16,22,23,31,33,35].

Lidocaine, at a concentration of 102 mM, has been reported to be a selective inhibitor of ATP-diphosphohydrolase (ATP-DPH) from human placental microvillar membrane and that it could be used when measuring PALP activity toward nucleotides [37]. However, we observed that 10 mM lidocaine activated rat hepatic TNALP and inhibited rat renal TNALP (Figure 3a), therefore raising concerns regarding the alleged selectivity of lidocaine as an ATP-DPH inhibitor.

Although *in vitro* modulation studies of ALP enzymes are recurrent in the literature [1,5,15,16,20,23,31–33,35], this study is, to our knowledge, the first report describing the effects of lidocaine, quinidine, bupivacaine, verapamil, kaempferol, genistein, corticosterone and progesterone on ALP activity from tissue homogenates (liver and kidney).

We have observed that modulators of Pgp or MRP (verapamil, kaempferol, genistein, quinidine and corticosterone) and also of hepatic taurocholate uptake (verapamil, lidocaine, quinidine and bupivacaine) affected hepatic and/or renal TNALP activity. These observations are in line with our previous results [18,19] and reinforce our suggestion that the physiologic role of ALP might be related with membrane transport modulation. It has been suggested by other authors that ALP activity might be involved in the control of plasma membrane functions early in the differentiation pathway [38]. ALP can hydrolyze phosphates bound to cell surface phosphoproteins and, in this way, it may modulate their biologic activities [38,39].

The data presented here encourages future investigation searching for a more precise and definite relation between ALP and ecto-phosphorylation/ecto-dephosphorylation processes in plasma membranes and for possible differential physiologic and pharmacological regulation of these processes by distinct ALP isoforms/isoenzymes. It has already been suggested by other authors that both protein kinases and protein phosphatases are required for reversible control of extracellular phosphorylation processes (see 38 for several examples). Ecto-protein kinases, involved in the regulation of several cellular functions, have been described as putative targets for pharmacological manipulation [40]. The differential modulation of hepatic and renal TNALP by drugs that have been shown to modulate the activity of membrane transport systems may be related to differences on the biologic roles of TNALP in the two tissues and opens the possibility of tissue specific ALP pharmacological manipulation *in vivo*.

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, POCTI and FEDER

References

[1] Van Hoof VO, De Broe ME. Interpretation and clinical significance of alkaline phosphatase isoenzyme patterns. Crit Rev Clin Lab Sci 1994;31:197–293.

[2] Alpers DH, Eliakim R, DeSchryver-Kecskemeti K. Secretion of hepatic and intestinal alkaline phosphatases: similarities and differences. Clin Chim Acta 1989;186:211–24.

[3] Deng JT, Hoylaerts MF, Van Hoof VO, De Broe ME. Differential release of human intestinal alkaline phosphatase in duodenal fluid and serum. Clin Chem 1992;38:2532–8.

[4] Van Hoof VO, Deng JT, De Broe ME. How do plasma membranes reach the circulation? Clin Chim Acta 1997;266:23–31.

[5] Martins MJ, Dias PO, Hipólito-Reis C. Rat serum alkaline phosphatase electrophoretic fractions: variations with feeding, starvation and cellulose fibre ingestion. Clin Nutr 1998;17:279–85.

[6] Hipólito-Reis C, Dias PO, Martins MJ. Importance of assay conditions in visualization and quantitation of serum alkaline phosphatase isoenzymes separated by electrophoresis. Scand J Clin Lab Invest 1999;59:593–606.

[7] Koyama I, Miura M, Matsuzaki H, Sakagishi Y, Komoda T. Sugar-chain heterogeneity of human alkaline phosphatases: differences between normal and tumour-associated isozymes. J Chromatogr 1987; 413:65–78.

[8] Millán JL, Fishman WH. Biology of human alkaline phosphatases with special references to cancer. Crit Rev Clin Lab Sci 1995;32:1–39.

[9] Moss DW, Whitaker KB. Modification of alkaline phosphatases by treatment with glycosidases. Enzyme 1985;34:212–6.

[10] Nair BC, Johnson DE, Majeska RJ, Rodkey JA, Bennett CD, Rodan GA. Rat alkaline phosphatase. II: Structural similarities between the osteosarcoma, bone, kidney, and placenta isoenzymes. Arch Biochem Biophys 1987;254:28–34.

[11] Fujimori-Arai Y, Koyama I, Hirano K, Komoda T. Intestinal alkaline phosphatase isoforms in rabbit tissues differ in glycosylation patterns. Clin Biochem 1997;30:545–51.

[12] Nosjean O, Koyama I, Goseki M, Roux B, Komoda T. Human tissue non-specific alkaline phosphatases: sugar-moiety-induced enzymic and antigenic modulations and genetic aspects. Biochem J 1997;321: 297–303.

[13] Miura M, Sakagishi Y, Hata K, Komoda T. Differences between the sugar moieties of liver- and bone-type alkaline phosphatases: a re-evaluation. Ann Clin Biochem 1994;31:25–30.

[14] Koyama I, Fujimori-Arai Y, Hirota N, Sakai T, Sakagishi Y, Komoda T. Liver-like alkaline phosphatase in the tissue-unspecific type enzyme found in rabbit organs. Biochim Biophys Acta 1991;1080:165–72.

[15] Mueller WH, Kleefeld D, Khattab B, Meissner JD, Scheibe RJ. Effects of retinoic acid on N-glycosylation and mRNA stability of the liver/bone/kidney alkaline phosphatase in neuronal cells. J Cell Physiol 2000;182:50–61.

[16] Pan C-J, Sartwell AD, Chou JY. Transcriptional regulation and the effects of sodium butyrate and glycosylation on catalytic activity of human germ cell alkaline phosphatase. Cancer Res 1991;51:2058–62.

[17] Watanabe T, Wada N, Chou JY. Structural and functional analysis of human germ cell alkaline phosphatase by site-specific mutagenesis. Biochemistry 1992;31:3051–8.

[18] Martel F, Martins MJ, Hipólito-Reis C, Azevedo I. Inward transport of [^{3}H]-1-methyl-4-phenylpyridinium in rat isolated hepatocytes: putative involvement of a P-glycoprotein transporter. Br J Pharmacol 1996;119:1519–24.

[19] Martel F, Martins MJ, Calhau C, Hipólito-Reis C, Azevedo I. Postnatal development of organic cation transport in the rat liver. Pharmacol Res 1998;37:131–6.

[20] Van Belle H. Kinetics and inhibition of rat and avian alkaline phosphatases. Gen Pharmacol 1976;7:53–8.

[21] Van Belle H. Kinetics and inhibition of alkaline phosphatases from canine tissues. Biochim Biophys Acta 1972;289:158–68.

[22] Farley JR, Ivey JL, Baylink DJ. Human skeletal alkaline phosphatase. Kinetic studies including pH dependence and inhibition by theophylline. J Biol Chem 1980;255:4680–6.

[23] Vinet B, Zizian L, Gauthier B. Characteristics of the inhibition of serum alkaline phosphatase by theophylline. Clin Biochem 1978;11: 57–61.

[24] Elbling L, Berger W, Rehberger A, Waldhör T, Micksche M. P-Glycoprotein regulates chemosensitivity in early developmental stages of the mouse. Faseb J 1993;7:1499–506.

[25] Ford JM, Hait WN. Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol Rev 1990;42:155–99.

[26] Sharom FJ. The p-glycoprotein efflux pump: how does it transport drugs? J Membrane Biol 1997;160:161–75.

[27] Chieli E, Romiti N, Cervelli F, Tongiani R. Effects of flavonols on p-glycoprotein activity in cultured rat hepatocytes. Life Sci 1995;57: 1741–51.

[28] Hooijberg JH, Broxterman HJ, Heijn M, Fles DLA, Lankelma J, Pinedo HM. Modulation by (iso)flavonoids of the ATPase activity of the multidrug resistance protein. FEBS Lett 1997;413:344–8.

[29] Kim RB, Leake B, Cvetkovic M, Roden MM, Nadeau J, Walubo A, Wilkinson GR. Modulation by drugs of human hepatic sodium-dependent bile acid transporter (sodium taurocholate cotransporting polypeptide) activity. J Pharmacol Exp Ther 1999;291:1204–9.

[30] Zimmerli B, Valantinas J, Meier PJ. Multispecificity of Na^+-dependent taurocholate uptake in basolateral (sinusoidal) rat liver plasma membrane vesicles. J Pharmacol Exp Ther 1989;250:301–8.

[31] Vanneuville FJ, Leroy JG. Properties of alkaline phosphatase in crude homogenates of human diploid fibroblasts. Arch Internat Physiol Biochim 1980;88:155–61.

[32] Fräki J, Ruuskanen O, Kouvalainen K. Biochemical characterization of alkaline phosphatase in guinea pig thymus. Biochim Biophys Acta 1977;482:370–8.

[33] Hodson AW. Separation of alkaline phosphatase isoenzymes from human kidney and comparison with alkaline phosphatases from other human tissues, urine, and *Escheria Coli*. Clin Chem 1983;29:100–6.

[34] Martins MJ, Negrão MR, Hipólito-Reis C, Azevedo L Tissue non-specific alkaline phosphatase activity inhibition seems to be dependent on the source of the enzyme. XII Congresso Nacional de Bioquímica 2000:Abstract 11:21.

[35] Martins MJ, Negrão MR, Hipólito-Reis C, Azevedo L Physiologic concentrations of bile salts inhibit rat hepatic alkaline phosphatase but not the intestinal isoenzyme. Clin Biochem 2000;33:611–7.

[36] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

[37] Chayet L, Collados L, Kettlun AM, Campos E, Traverso-Cori A, Garcia L, Valenzuela MA. Human placental ecto-enzymes: studies on the plasma membrane anchorage and effect of inhibitors of ATP-metabolizing enzymes. Res Commun Mol Pathol Pharmacol 1997; 96:14–24.

[38] Scheibe RJ, Kuehl H, Krautwald S, Meissner JD, Mueller WH. Ecto-alkaline phosphatase activity identified at physiological pH range on intact P19 and HL-60 cells is induced by retinoic acid. J Cell Biochem 2000;76:420–36.

[39] Burch WM, Hamner G, Wuthier RE. Phosphotyrosine and phosphoprotein phosphatase activity of alkaline phosphatase in mineralizing cartilage. Metabolism 1985;34:169–75.

[40] Redegeld FA, Caldwell CC, Sitkovsky MV. Ecto-protein kinases: ectodomain phosphorylation as a novel target for pharmacological manipulation? Trends Pharmacol Sci 1999;20:453–9.

2.3 - Participação da fosfátase alcalina em sistemas de transporte

2.3.a - Fosfátase alcalina e captação do taurocolato em hepatócitos isolados do Rato: possível relação (Artigo VI).

Artigo VI

Putative involvement of alkaline phosphatase in the modulation of taurocholate uptake by rat isolated hepatocytes.

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Enviado para publicação

Putative involvement of alkaline phosphatase in the modulation of taurocholate uptake by rat isolated hepatocytes

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Abstract: Alkaline phosphatase (ALP) was recently shown to modulate the active transport of different substances into various types of cells, including hepatocytes. As some drugs with ALP activating effects are known to induce the formation of gallstones, and the bile salts decrease ALP activity, we decided to investigate, in freshly isolated rat hepatocytes, the effect of ALP activity modulators on taurocholate uptake. We observed that modulation of hepatic ALP by octreotide or kaempferol (ALP activators) or by making the incubation medium more alkaline inhibited taurocholate transport. Inversely, acidifying the incubation medium resulted in activation of the mentioned uptake. Levamisole, Edta, vanadate or theophylline (ALP inhibitors) did not interfere on bile salt uptake, but the addition of theophylline to octeotride or kaempferol reduced the effect of these drugs on the uptake of taurocholate. Our results support the hypothesis of ALP being involved in the modulation of taurocholate uptake by isolated hepatocytes.

Introduction

Alkaline phosphatase (ALP) is a group of nonspecific phosphomonoesterases located primarily in cell plasma membranes, whose physiological role remains controversial. It was recently demonstrated that ALP concentration in different tissues is positively correlated with the extent of exchange surface per unit volume of the tissue, suggesting an association between ALP and transport systems (Calhau *et al*, 1999). Additionally, evidence was obtained for an involvement of ALP in the modulation of organic cation transport in hepatocytes (Martel *et al*, 1996b; 1998b,c), in Caco-2 cells (Calhau *et al*, 2001a) and in RBE4 cells (Calhau *et al*, 2001c). In RBE4 cells (rat brain microvessel endothelial cells) the transport of insulin was also shown to be modulated by ALP (Calhau *et al*, 2001b). Reciprocally, we have also recently shown that modulators of several transport systems modify ALP activity in rat liver and kidney homogenates (Martins et al, 2001).

Progesterone and octreotide (a stable somatostatin analogue) were recently described as activators of ALP (Calhau *et al*, 2000a, 2000b; Calhau *et al*, 2001b,c). As it is known that both of them lead to an increase in the incidence of gallstones (Carr and Wilson1987; Bigg-Wither *et al*, 1992), the large majority of gallstones is made of cholesterol, and bile acids are of utmost importance for the dissolution of cholesterol in bile (Guyton and Hall, 1996), we raised the hypothesis of an effect of ALP in the transport

of bile acids in hepatocytes. Under our hypothesis, activation of ALP would lead to a decrease in bile acid transport, and ALP inhibition would facilitate that transport. Bile salts inhibit ALP activity (Martins *et al*, 2000).

Here, we present results on the uptake of ^{3}H-taurocholate by freshly isolated rat hepatocytes, supporting an involvement of ALP in the modulation of that transport.

Material and Methods

Material

^{3}H-Taurocholate (specific activity 2.0Ci/mmol) (Net-322) was purchased from NEN™ Life Science Products, Boston, USA.

Taurocholate (T-4009), choline chloride (C-1879), lithium chloride (L-0505), levamisole (L-9756), Edta (E-1644), orthovanadate (S-6508), theophylline (T-1633), and kaempferol (K-0133) were purchased from Sigma (Sigma Alcobendas), Madrid, Spain. Theophylline and kaempferol were dissolved in dimethylsulphoxide.

Octreotide was generously provided by Novartis.

All other reagents were of the highest quality and purity available.

Membrane filters (Suport®-800, ∅ 25mm, 0.8 μm) were purchased from GelmanSciences, Michigan, USA.´

Methods

- Animals and isolation of hepatocytes.

10 male adult Wistar rats (free from liver and bone diseases) weighing from 300 to 600 g (CRIFFA, Barcelona, Spain) were used. The animals were kept under controlled environmental conditions (12 h light/dark cycle and room temperature 24 °C), fed with a suitable commercial diet (Harlan Interfauna Iberica, SA, Barcelona, Spain) with water *ad libitum*. Hepatocytes were isolated according to a procedure described previously (Martel *et al* 1996a,b; Martel *et al* 1998a). Preparations with over 75 % viable cells were used.

- Uptake of [^{3}H]-taurocholate into hepatocytes.

Cells were diluted in Krebs-Henseleit medium to a final concentration of 2-3 x 10^6 cells/mL. Cell suspension (1 mL) was incubated at 37°C under continuous shaking and gassing with 95% O_2 and 5% CO_2.

After a 5 min pre-incubation period, incubation with 25 μM [^{3}H]-taurocholate was started by adding 1 mL Krebs-Henseleit medium containing [^{3}H]-taurocholate to the cell suspension.

Incubation was stopped by rapid filtration through membrane filters, resulting in separation of the cells (retained on the filters) from the incubation medium. The filters were washed twice, with 4 mL ice-cold Krebs-Henseleit medium, and placed in 2 mL perchloric acid 0.2 M. Radioactivity retained on the filters was determined by liquid scintillation counting.

Controls for drugs were run in the presence of correspondent volume of solvent. Blanks were submitted to the same conditions, hepatocytes being added just before the filtration step.

The final concentration of viable hepatocytes and the protein content in one sample of the cell suspension carried throughout the experiment were used to express results.

- Effect of ionic composition of the incubation medium.

To study the effect of sodium on [^{3}H]-taurocholate uptake, cells were pre-incubated and incubated in NaCl-free Krebs-Henseleit medium. The composition of the NaCl-free Krebs-Henseleit medium was the same as the Krebs-Henseleit medium except that NaCl (corresponding to 137 mM) was isotonically replaced either with choline chloride or lithium chloride.

- Modulation of alkaline phosphatase activity.

Alkaline phosphatase activity was modulated by changing the pH value of the incubation medium (from 7.4 either to 6.2 or to 8.2) or by the presence of levamisole, Edta, vanadate, theophylline, octreotide or kaempferol.

When the effect of pH change was tested both the pH of pre-incubation and incubation media was modified. The viability of hepatocytes was not affected by pH changes. When the effect of drugs was tested these compounds were present during both the pre-incubation and incubation periods.

- Protein determination.

Protein concentration was determined as described by Bradford, with human serum albumin as standard (Bradford 1976).

- Statistical analysis.

All results are expressed as arithmetic mean ± s.e.mean (n = number of cases). The significance of differences between means was assessed by Student's t-test.

Results

Freshly isolated rat hepatocytes took up and accumulated ^{3}H-taurocholate in a saturable (Fig 1) and time-dependent (Fig 2) way.

Lowering the pH of incubation medium to 6.2 led to a marked increase in the accumulation of ^{3}H-taurocholate (to 175% of control), whereas increasing the pH to 8.2 had the opposite effect (reducing accumulation to 64% of control) (Fig 3).

Substitution of choline for sodium reduced ^{3}H-taurocholate accumulation to 49% of control, but when lithium was used to substitute sodium the observed decrease in ^{3}H-taurocholate accumulation was of a smaller magnitude, just to 67% of control (Fig 4).

Levamisole (500 μM), EDTA (1 mM), vanadate (100 μM) and theophylline (5 mM),

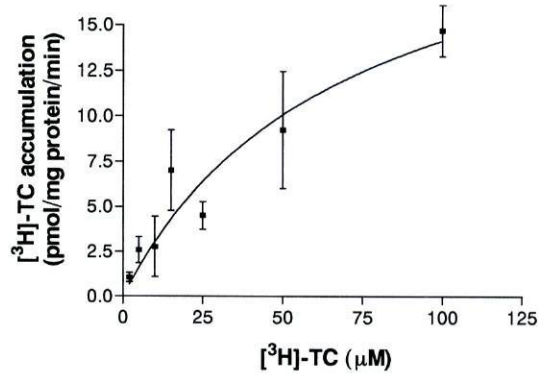

Figure 1. Accumulation of [^{3}H]-taurocholate ([^{3}H]-TC) in freshly isolated rat hepatocytes. The cells were incubated at 37°C for 15 min with various concentrations of [^{3}H]-TC. Shown are arithmetic means ± s.e.mean (n=3).

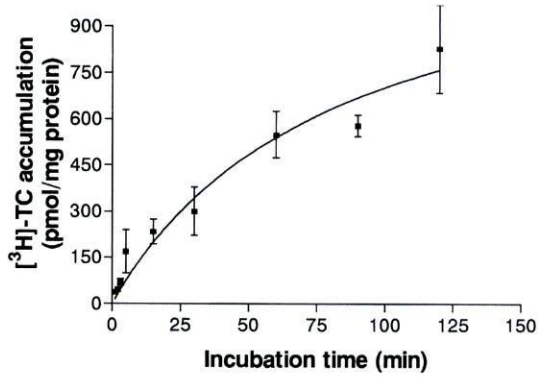

Figure 2. Time course of [^{3}H]-taurocholate ([^{3}H]-TC) accumulation in freshly isolated rat hepatocytes. The cells were incubated at 37°C with 25 mM of [^{3}H]-TC for various periods of time. Shown are arithmetic means ± s.e.mean (n=3).

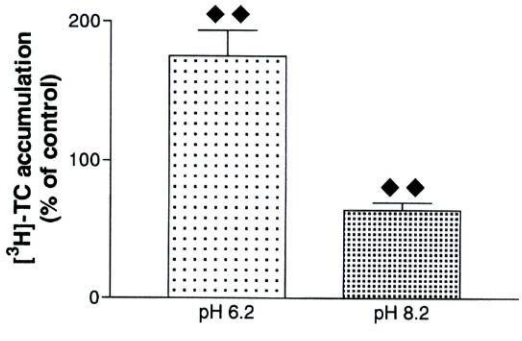

Figure 3. Effect of the pH of the Krebs-Henseleit medium on [^{3}H]-taurocholate ([^{3}H]-TC) accumulation in freshly isolated rat hepatocytes. Cells were incubated at 37°C with 25 μM [^{3}H]-TC for 15 min. Experiments were performed at pH 6.2 (n=5) or pH 8.2 (n=4). Results are shown as percentage of control (100%) value (arithmetic means ± s.e.mean).

◆◆ Significantly different from control $p < 0.05$.

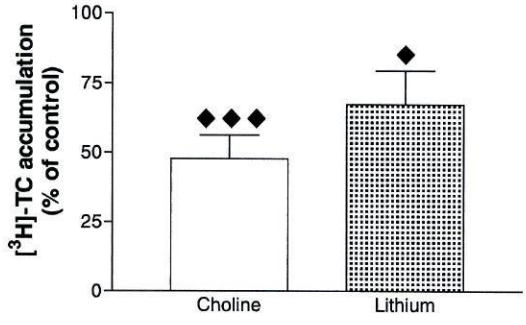

Figure 4. Effect of sodium on [^{3}H]-taurocholate ([^{3}H]-TC) accumulation in freshly isolated rat hepatocytes. Cells were incubated at 37°C with 25 μM [^{3}H]-TC for 15 min. Sodium chloride in the Krebs-Henseleit medium was replaced by either choline chloride (Choline; n=5) or lithium chloride (Lithium; n=4). Results are shown as percentage of control (100%) value (arithmetic means ± s.e.mean).
◆◆◆ Significantly different from control $p < 0.005$; ◆ significantly different from control $p < 0.1$.

drugs known as inhibitors of ALP activity, had no effect on ^{3}H-taurocholate accumulation (Fig 5).

On the contrary, octreotide (100 μg/9.8 mL) and kaempferol (100 μM), drugs recently described as activators of ALP activity, significantly reduced the accumulation of ^{3}H-taurocholate by hepatocytes (to 72% and to 32% of control, respectively) (Figs 6 and 7). Interestingly, in both cases, addition of theophylline (5 mM) led to a decrease of the effect of octreotide and kaempferol (to 98% and to 43% of control, respectively) (Figs 6 and 7).

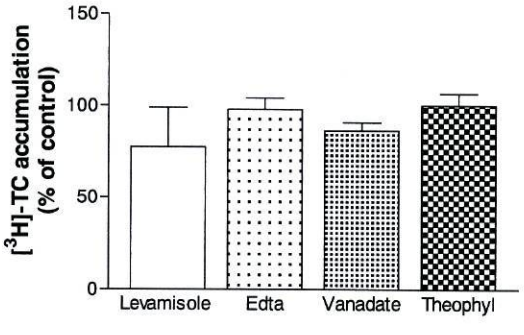

Figure 5. Effect of levamisole 500 μM (n=4), Edta 1 mM (n=5), vanadate 100 μM (n=3) and theophylline 5 mM (Theophyl; n=4) on [^{3}H]-taurocholate ([^{3}H]-TC) accumulation in freshly isolated rat hepatocytes. Cells were incubated at 37°C with 25 μM [^{3}H]-TC for 15 min. Results are shown as percentage of control (100%) value (arithmetic means ± s.e.mean).

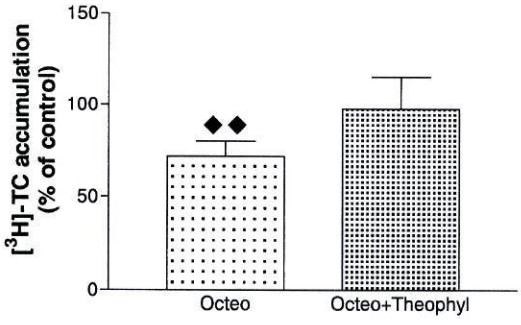

Figure 6. Effect of octeotride 100 μg/9.8 mL (Octeo; n=5) and octeotride 100 μg/9.8 mL plus theophylline 5 mM (Octeo+Theophyl; n=3) on [^{3}H]-taurocholate ([^{3}H]-TC) accumulation in freshly isolated rat hepatocytes. Cells were incubated at 37°C with 25 μM [^{3}H]-TC for 15 min. Results are shown as percentage of control (100%) value (arithmetic means ± s.e.mean).
◆◆ Significantly different from control $p < 0.05$.

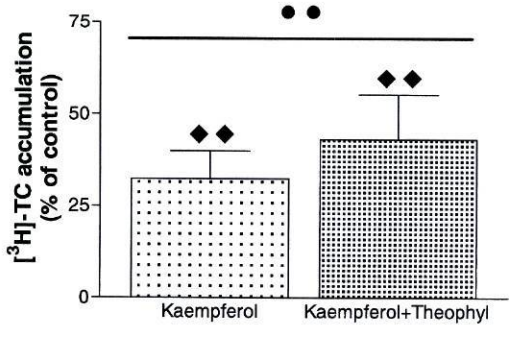

Figure 7. Effect of kaempferol 100 μM (n=5) and kaempferol 100 μM plus theophylline 5 mM (kaempferol+Theophyl; n=5) on [^{3}H]-taurocholate ([^{3}H]-TC) accumulation in freshly isolated rat hepatocytes. Cells were incubated at 37°C with 25 μM [^{3}H]-TC for 15 min. Results are shown as percentage of control (100%) value (arithmetic means ± s.e.mean).
◆◆ Significantly different from control $p < 0.05$.
● ●·Significantly different from each other $p < 0.05$.

Discussion

Hepatic extraction of bile acids from portal blood is a very efficient process, the concentration of bile acids in peripheral blood being considerably lower than that in portal blood (Meier *et al*, 1999; Agellon and Torchia, 2000). Uptake of monomeric bile acids by hepatocytes occurs mainly through two types of transport systems, the sodium-dependent sodium-taurocholate cotransporting polypeptide (Elferink *et al*, 1995; Meier *et al*, 1995; Meier *et al*, 1997; Kullak-Ublick 1999; Agellon and Torchia, 2000) and a transporter belonging to the organic anion cotransporting polypeptide family (Elferink *et al*, 1995; Meier *et al*, 1995; Meier *et al*, 1997; Kullak-Ublick 1999; Agellon and Torchia, 2000).

Omission of sodium from the pre-incubation and incubation media (when replaced by lithium) led to a 33% reduction in taurocholate accumulation, suggesting that in our experimental conditions sodium-independent mechanisms are preferentially responsible for the uptake of taurocholate.

The aim of our study was to investigate a putative involvement of ALP in the modulation of bile acid uptake, irrespective of the specific transport system involved. An investigation focused on the specific systems is justified by our present positive results, and will be better conducted with transfected cells.

The striking effect of medium pH modification on the uptake of taurocholate had not, to our knowledge, been described. An H^+ symport activity could explain our observation of an increase in taurocholate uptake through lowering of pH and a decrease in the uptake through an increase in pH. The most important uptake systems for bile salts in hepatocytes, the sodium taurocholate cotransport polipeptyde and the organic anion cotransport polipeptyde have no atributed qualities of H^+ symport or antiport (Dawson and Oelkers, 1995; Elferink *et al*, 1995; Meier, 1995; Love and Dawson, 1998; Eckardt *et al*, 1999; Kamisako *et al*, 1999; Kullak-Ublick, 1999), however.

ALP is an enzyme that, albeit able to function at the physiological pH (Swarup *et al*, 1981; Chan and Stinson, 1986; Fedde *et al*, 1988; Fedde *et al*, 1990; Calhau et al, 2001a; Scheibe *et al*, 2000), has been so named because of its alkaline pH optimum (McComb *et al*, 1979). Our results are thus compatible with the hypothesis of the pH effects on the taurocholate uptake being due to an activation and an inhibition of ALP by the alkaline and acid pH, respectively.

Drugs with ALP inhibitory effects, at the used concentrations, had no effect on taurocholate accumulation. On the other hand, octreotide and kaempferol, drugs that activate ALP (Calhau *et al*, 2000a,b; Martins *et al*, 2001), had an inhibitory effect on taurocholate uptake. Most interestingly, addition of theophylline, an ALP inhibitor (Vinet *et al*, 1978; Farley *et al*, 1980; Martins *et al* 2001), to either octreotide or kaempferol antagonized the effect of the ALP activators on the taurocholate transport. Octreotide has been described as a substrate for bile acid transporters (Fricker *et al*, 1994; Terasaki *et al*, 1995), and that characteristic may justify the decrease of taurocholate uptake induced by that drug. The antagonism, by theophylline, of the octreotide effect supports better the octreotide effect through activation of ALP. As to kaempferol, for which an ALP activating effect has also been verified (Calhau *et al*, 2000a; Martins *et al*, 2001), we have no other clue for its effect on the taurocholate transport than this one. Moreover, theophylline was also able to reduce kaempferol effect.

Overall, the results support the hypothesis of ALP being able to modify the transport of taurocholate in hepatocytes: an increase in ALP activity reduces the transport, whereas a decrease in ALP activity favors the transport.

The absence of effect of ALP inhibitory drugs here tested against the transport of taurocholate may be due to the fact that ALP is already under the inhibitory action of taurocholate itself (Martins *et al*, 2000).

We suggest that the effect of progesterone, cyclosporine, verapamil (Calhau *et al*, 2000a; Calhau *et al*, 2001c) and interleukin-6 (Gallo *et al*, 1997) on ALP may at least partly contribute to their effect on bile acids transport in the liver (Zimmerli *et al*, 1989; Elferink *et al*, 1995; Meier *et al*, 1995; Meier *et al*, 1999; Mizuta *et al*, 1999; Green *et al*, 1994).

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References

AGELLON LB, Torchia EC (2000) Intracellular transport of bile acids. Biochim Biophys Acta 1486: 198-209.

BRADFORD MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem; 72: 248-54.

BIGG-WITHER GW, Ho KK, Grunstein RR, Sullivan CE, Doust BD (1992) Effects of long-term octreotide on gall stone formation and gall bladder function. Br Med J 304: 1611-1612.

CALHAU C, Hipólito-Reis C, Azevedo I (1999) Alkaline phosphatase and exchange surfaces. Clin Biochem 32: 153-154.

CALHAU C, Martel F, Hipólito-Reis C, Azevedo I (2000a) Effect of P-glycoprotein modulators on alkaline phosphatase activity in cultured rat hepatocytes. Cell Physiol Biochem 10: 177-186.

CALHAU C, Martel F, Hipólito-Reis C, Azevedo I (2000b) Differences between duodenal and jejunal rat alkaline phosphatase. Clin Biochem 33: 571-577.

CALHAU C, Martel F, Hipólito-Reis C, Azevedo I (2001a) Regulation of ^{3}H-MPP^{+} uptake in Caco-2 cells by an ecto-phosphatase activity. Pharmacol & Toxicol P33.

CALHAU C, Martel F, Pinheiro-Silva S, Soares-da-Silva P, Hipólito-Reis C, Azevedo I (2001b) The putative involvement of an ecto-phosphatase on insulin internalization into RBE4 cells. Faseb J, 15:A130.

CALHAU C, Martel F, Soares-da-Silva P, Hipólito-Reis C, Azevedo I (2001c) Modulation of ^{3}H-MPP^{+} uptake in RBE4 cells by an ecto-phosphatase activity. Faseb J, 15: A130.

CARR BR, Wilson JD (1987) Disorders of the ovary and female reproductive tract. In: Principles of Internal Medicine 2 (Braunwald E, Isselbacher KJ, Petersdorf RG, Wilson JD, Martin JB, Fauci AS, Eds), 11th Ed, New York: McGraw-Hill Book Company.

CHAN JRA, Stinson RA (1986) Dephosphorylation of phosphoproteins of human liver plasma membranes by endogenous and purified liver alkaline phosphatases. J Biol Chem 261: 7635-7639.

ECKARDT U, Schroeder A, Stieger B, Hochli M, Landmann L, Tynes R, Meier PJ, Hagenbuch B (1999) Polyspecific substrate uptake by the hepatic organic anion transporter Oatp1 in stably transfected CHO cells. Am J Physiol 276 (Gastrointest Liver Physiol 39): G1037-G1042.

ELFERINK RPJO, Meijer DKF, Kuipers F, Jansen PLM, Groen AK, Groothuis GMM (1995) Hepatobiliary secretion of organic compounds; molecular mechanisms of membrane transport. Biochim Biophys Acta 1241: 215-268.

FARLEY JR, Ivey JL, Baylink DJ (1980) Human skeletal alkaline phosphatase. Kinetic studies including pH dependence and inhibition by theophylline. J Biol Chem 255: 4680-4686.

FEDDE KN, Lane CC, Whyte MP (1988) Alkaline phosphatase is an ectoenzyme that acts on micromolar concentrations of natural substrates at physiologic pH in human osteosarcoma (SAOS-2) cells. Arch Biochemy Biophys 264: 400-409.

FEDDE KN, Whyte MP (1990) Alkaline phosphatase (tissue-nonspecific isoenzyme) is a phosphoethanolamine and pyridoxal-5'-phosphate ectophosphatase: normal and hypophosphatasia fibroblast study. Am J Hum Genet 47: 767-775.

FRICKER G, Dubost V, Schwab D, Bruns C, Thiele C (1994) Heterogeneity in hepatic transport of somatostatin analog octapeptides. Hepatology 20: 191-200.

GALLO RL, Dorschner RA, Takashima S, Klagsbrun M, Eriksson E, Bernfield M (1997) Endothelial cell surface alkaline phosphatase activity is induced by IL-6 released during wound repair. J Invest Dermatol 109: 597-603.

GREEN RM, Whiting JF, Rosenbluth AB, Beier D, Gollan JL (1994) Interleukin-6 inhibits hepatocyte taurocholate uptake and sodium-potassium-adenosinetriphosphatase activity. Am J Physiol 267 (Gastrointest Liver Physiol 30): G1094-G1100.

GUYTON AC, Hall, JE (1996) Textbook of Medical Physiology, 9th Ed, W B Philadelphia: Saunders Company.

KAMISAKO T, Gabazza EC, Ishihara T, Adachi Y (1999) Molecular aspects of organic compound transport across the plasma membrane of hepatocytes. J Gastroent Hepatol 14: 405-412.

KULLAK-UBLICK GA (1999) Regulation of organic anion and drug transporters of the sinusoidal membrane. J Hepatol 31: 563-573.

LOVE MW, Dawson PA (1998) New insights into bile acid transport. Curr Opin Lipidol 9:225-229.

MARTEL F, Martins MJ, Azevedo I (1996a) Inward transport of ^{3}H-MPP^{+} in freshly isolated rat hepatocytes: evidence for interaction with catecholamines. Naunyn-Schmiedeberg's Arch Pharmacol 354: 305-311.

MARTEL F, Martins MJ, Hipólito-Reis C, Azevedo I (1996b) Inward transport of ^{3}H-1-phenylpyridinium in rat isolated hepatocytes: putative involvement of a P-glycoprotein transporter. Br J Pharmacol 119: 1519-1524.

MARTEL F, Calhau C, Martins MJ, Azevedo I (1998a) Uptake of ^{3}H-adrenaline by freshly isolated rat hepatocytes: putative involvement of P-glycoprotein. J Auton Pharmacol 18: 57-64.

MARTEL F, Calhau C, Hipólito-Reis C (1998b) Effect of bile duct obstruction on hepatic uptake of 1-methyl-4-phenylpyridinium in the rat. Pharmacol Res 37: 497-500.

MARTEL F, Martins MJ, Calhau C, Hipólito-Reis C, Azevedo I (1998c) Postnatal development of organic cation transport in the rat liver. Pharmacol Res 37: 131-136.

MARTINS MJ, Negrão MR, Hipólito-Reis C (2001) Alkaline phosphatase from rat liver and kidney is differentially modulated. Clin Biochem 34: 463-468.

MARTINS MJ, Negrão MR, Hipólito-Reis C, Azevedo I (2000) Physiologic concentrations of bile salts inhibit hepatic alkaline phosphatase but not the intestinal isoenzyme. Clin Biochem 33: 611-617.

MCCOMB RB, Bowers GN, Posen J, Posen S (1979) Alkaline phosphatase, New York: Plenum.

MEIER PJ (1995) Molecular mechanisms of hepatic bile salt transport from sinusoidal blood into bile. Am J

Physiol 269 (Gastrointest Liver Physiol 32): G801-G812.

MEIER PJ, Eckhardt U, Schroeder A, Hagenbuch B, Stieger B (1997) Substrate specificity of sinusoidal bile acid and organic anion uptake systems in rat and human liver. Hepatology 26: 1667-1677.

MIZUTA K, Kobayashi E, Uchida H, Ogino Y, Fujimura A, Kawarasaki H, Hashizume K (1999) Cyclosporine inhibits transport of bile acid in rats: comparison of bile acid composition between liver and bile. Transplant Proc 31: 2755-2756.

SEARGEANT LE, Stinson RA (1979) Inhibition of human alkaline phosphatase by vanadate. Biochem J 181: 247-250.

SCHEIBE RJ, Kuehl H, Krautwald S, Meissner JD, Mueller WH (2000) Ecto-alkaline phosphatase activity identified at physiological pH range on intact P19 and HL-60 cells is induced by retinoic acid. J Cell Biochem 76: 420-436.

SWARUP G, Cohen S, Garbers DL (1981) Selective dephosphorylation of proteins containing phosphotyrosine by alkaline phosphatase. J Biol Chem 256: 8197-8201.

TERASAKI T, Mizuguchi H, Itoho C, Tamai I, Lemaire M, Tsuji A (1995) Hepatic uptake of octreotide, a long-acting somatostatin analog via a bile acid transport system. Pharmacol Res 12: 12-17.

VINET B, Zizian L, Gauthier B (1978) Characteristics of the inhibition of serum alkaline phosphatase by theophylline. Clin Biochem 11: 57-61.

ZIMMERLI B, Valantinas J, Meier PJ (1989) Multispecificity of Na^+-dependent taurocholate uptake in basolateral (sinusoidal) rat liver plasma membrane vesicles. J Pharmacol Exp Ther 250: 301-308.

3 – RESUMO DA CONTRIBUIÇÃO EXPERIMENTAL

Quantificação de actividades fosfatásicas alcalinas

Nos Artigos I, II e III descrevem-se os protocolos experimentais elaborados para o doseamento de actividades fosfatásicas alcalinas, nomeadamente no que se refere a substratos, amortecedores de pH e valores de pH utilizados, em que as condições de ensaio são as mesmas na quantificação da actividade fosfatásica alcalina sérica total do Rato e na quantificação da actividade das fracções enzímicas da FA obtidas por separação electroforética em acetato de celulose. Nos Artigos II e III descrevem-se os resultados do estudo do efeito da mesma variação das condições experimentais nessas quantificações. No Artigo II mostra-se que o estudo de uma determinada situação susceptível de induzir modulação na actividade sérica total da FA, no Rato, em consequência da modulação da actividade de uma ou mais fracções electroforéticas da enzima, podia depender do conjunto das mesmas condições de ensaio escolhidas para a quantificação da actividade total e da das fracções. No Artigo III descrevem-se os resultados de um estudo mais alargado daquele efeito mostrando-se claramente que, sobre a mesma separação electroforética das espécies moleculares, o tipo de revelação das actividades enzímicas presentes nas fracções (condicionando a identificação e quantificação de cada uma delas) era crítico interessando, por isso, tanto como a sua própria separação. O valor obtido na quantificação da actividade total era igualmente muito influenciado pelas condições de ensaio associadas a uma dada revelação das fracções.

Modulação de actividades fosfatásicas alcalinas

Nos Artigos II, IV e V descrevem-se os resultados do estudo da modulação da actividade da FA em diferentes condições de nutrição [alimentação normal, jejum e ingestão única de fibras de celulose (serrim)] e por acção de compostos endógenos (sais biliares conjugados) e de fármacos [moduladores da enzima e de diferentes sistemas de transporte (da Pgp, da MRP e da captação hepática do taurocolato)]. No Artigo II observou-se que a ingestão única de fibras celulósicas grosseiras (serrim) tinha um efeito parecido ao da alimentação: nas duas situações, a actividade da FA sérica total no Rato aumentava em consequência do aumento da fracção electroforética que correspondia predominantemente à FAint. No Artigo IV observou-se que a FAtne hepática do Rato era inibida por sais biliares conjugados, em

concentrações fisiológicas, não se observando qualquer efeito destes compostos na isozima intestinal. No Artigo V observámos efeitos distintos e, até mesmo opostos, nas isoformas hepática e renal da FAtne do Rato por fármacos moduladores da FAtne e de diferentes sistemas de transporte. Com nenhum dos fármacos se observou activação da FAtne renal e, quando se observou inibição das duas isoformas, a inibição da FAtne renal foi sempre muito mais acentuada que a da isoforma hepática. No Artigo VI descreve-se uma possível relação entre a FA, na presença de moduladores da actividade da FAtne (pH e fármacos), e o transporte hepático, no Rato, de um sal biliar conjugado.

Participação da fosfátase alcalina em sistemas de transporte

No Artigo VI observou-se, no Rato, que um aumento da captação hepática do taurocolato estava associado à diminuição do valor de pH do meio de incubação ou à presença de inibidores da actividade fosfatásica alcalina e que, inversamente, uma diminuição dessa captação estava associada ao aumento do valor de pH do meio de incubação ou à presença de activadores da actividade fosfatásica alcalina.

4 – DISCUSSÃO GERAL E CONCLUSÕES FINAIS

Na revisão da literatura sobre a FA, feita na Introdução desta dissertação, pusemos em evidência a diversidade de condições de ensaio utilizadas na quantificação da FA.

Desde o seu início os trabalhos que realizámos tiveram a intenção não só de estudar condições fisiológicas e bioquímicas em que se pudesse observar a intervenção da FA mas também encontrar métodos adequados para o efeito e conhecer as relações entre uns e outros. Embora interessados no estudo do papel fisiológico da FA no Homem usámos sempre o Rato como animal de experiência.

Com base em métodos anteriormente descritos para o estudo da FA sérica total humana, em que se usava o α-naftilfosfato no doseamento da actividade da fosfátase ácida prostática (162) e o β-naftilfosfato no doseamento das fosfátases ácida e alcalina (159), conseguimos obter novos métodos que se encontram descritos nos Artigos I, II e III.

Nos métodos desenvolvidos, o carbonato/bicarbonato e o 2A2M1P (ambos a pH 10,4) foram usados como amortecedor de pH e o α- e o β-naftilfosfato como substrato. Os novos métodos foram testados, doseando-se a actividade fosfatásica alcalina sérica total, e mostraram-se adequados: a quantidade de produto formado aumentava de forma linear com o tempo de incubação e a quantidade de soro na gama de valores usados (Artigo I).

Esses métodos (os mesmos substratos, amortecedores de pH e valor de pH) foram também utilizados na revelação e quantificação de fracções da FA obtidas por separação electroforética em acetato de celulose (Artigo I). A electroforese permitiu a separação de duas fracções: a mais rápida (banda anódica) foi denominada primeira fracção e a mais lenta (banda catódica) segunda fracção. O estudo da influência do tempo de incubação na revelação dessas fracções mostrou que, independentemente do amortecedor utilizado, quando se usava o α-naftilfosfato como substrato o tempo de incubação não modificava as percentagens das duas fracções. O mesmo não sucedeu, no entanto, com o β-naftilfosfato, observando-se, para os dois amortecedores, uma ligeira variação nas percentagens das duas fracções. O aumento do tempo de incubação provocava uma ligeira diminuição ou um ligeiro aumento da primeira fracção conforme se usava o 2A2M1P ou o carbonato/bicarbonato como amortecedor, respectivamente. Em consequência deste facto usámos o mesmo tempo de incubação

na quantificação da FA sérica e na sua revelação electroforética com o β-naftilfosfato. Por uma questão de simplicidade adoptou-se o mesmo tempo de incubação na revelação electroforética com o α-naftilfosfato. O suporte electroforético e os métodos de revelação acima descritos foram então usados nos trabalhos experimentais apresentados nos Artigos II e III. Com base no efeito inibidor diferencial da hArg-L e da Phe-L relativamente à FAtne e à FAint (7, 9 - 11, 16, 20, 27, 28, 34, 36, 37, 42, 43, 45, 49, 50, 55, 64, 67, 70, 73, 77, 85 - 88, 90, 93, 96, 98, 100 - 102), as fracções electroforéticas por nós separadas foram parcialmente caracterizadas (Artigo II). Os estudos de inibição efectuados, assim como a observação dos padrões obtidos na separação electroforética para os três grupos de ratos, utilizados no Artigo II (ver à frente), e a comparação com outros tipos de resultados da literatura (referentes à separação electroforética da FA em acetato de celulose e à observação, por electroforese, do aumento da FAtne hepática sérica na colestase) (10, 42, 92, 95, 102, 118), permitiram-nos concluir que a primeira fracção era principalmente constituída por FAtne (maior percentagem de inibição pela hArg-L) e que a segunda fracção era principalmente constituída por FAint (maior percentagem de inibição pela Phe-L) (*Tabela 2 do* **Artigo II**).

Após a escolha do tempo de incubação mais adequado à utilização quer do α- quer do β-naftilfosfato no doseamento da actividade sérica total da FA, e na revelação e quantificação das suas fracções electroforéticas, estudou-se o efeito de três diferentes condições de nutrição nas actividades em análise. Concretamente, foi estudada e comparada a actividade da FA do soro de ratos em jejum completo, em jejum mas com acesso a fibras de celulose (serrim) e alimentados normalmente (Artigo II). Aplicaram-se os métodos anteriores, ou seja as mesmas condições de ensaio nas duas técnicas: os dois naftilfosfatos foram usados como substrato e o 2A2M1P como amortecedor, pH 10,4. No doseamento da actividade sérica total da FA foi também incluído o p-nitrofenilfosfato, por ser o substrato mais frequentemente utilizado e recomendado para a quantificação da FA (3 - 9, 12 - 14, 16, 18, 21, 23, 25, 29, 31, 33 - 35, 39, 41 - 43, 46 - 48, 53, 58, 59, 62, 63, 66, 68, 73 -81, 85 - 93, 95, 97 - 103, 106, 108 - 110, 112 - 115, 118, 131, 132, 141, 142, 152 - 154, 157, 166 - 169).

Em geral, a actividade das enzimas depende das condições de ensaio, incluindo a natureza do substrato utilizado. Os resultados obtidos, quer na

quantificação da actividade total quer na visualização e quantificação das fracções electroforéticas com os diferentes substratos, reflectiram essa dependência.

Em todos os grupos de ratos, os valores mais elevados para a actividade sérica total e para as actividades das fracções electroforéticas foram obtidos com o β-naftilfosfato (*Tabelas 1 e 3 do* **Artigo II**). Observámos que, usando quer o α- quer o β-naftilfosfato como substrato, a actividade sérica total era maior nos ratos alimentados normalmente e só ligeiramente maior nos ratos em jejum com acesso a serrim relativamente ao grupo em jejum completo. O efeito da ingestão de alimentos no aumento da FA sérica total foi confirmado quando se usou o p-nitrofenilfosfato como substrato; contudo, nos ensaios com este substrato não foi possível observar qualquer aumento da FA no grupo dos ratos em jejum com acesso a fibras de celulose (*Tabela 1 do* **Artigo II**). A análise electroforética mostrou que o aumento observado na actividade da FA sérica total era sobretudo da responsabilidade da segunda fracção, que aumentava mais marcadamente que a primeira (*Tabela 3 do* **Artigo II**). O aumento da segunda fracção electroforética no grupo dos ratos alimentados normalmente e no grupo com acesso a fibras de celulose foi observado com os dois naftilfosfatos, sendo no entanto mais marcado com o α-naftilfosfato (*Tabela 3 do* **Artigo II**).

Os nossos resultados confirmaram estudos anteriormente publicados mostrando que a ingestão de alimentos provoca um aumento da FAint no soro e da FA sérica total (10, 11, 17, 22, 25, 29, 32, 35, 40, 42, 44, 45, 47, 49, 56, 60, 63, 69, 70, 72, 73, 75, 76, 96, 98, 100, 105, 118, 121, 128, 154 - 156; DH Alpers 2001, comunicação pessoal). Tanto quanto sabemos o efeito da ingestão de fibras de celulose (serrim), sem acesso a alimentos, na FA sérica total não tinha sido antes objecto de estudo. De acordo com os nossos resultados, embora o aumento observado tenha sido quantitativamente diferente com os dois nafilfosfatos utilizados, o efeito da ingestão de fibras na modulação da FAint sérica era semelhante ao da ingestão de alimentos.

O protocolo experimental desenvolvido (Artigos I e II) foi também utilizado para mostrar o efeito do uso de diferentes substratos, amortecedores de pH e valores de pH na actividade sérica total da FA e na actividade das suas fracções electroforéticas (mas mantendo o mesmo conjunto de condições experimentais nas duas quantificações) (Artigo III). Neste estudo foram comparados os α- e β-naftilfosfatos, diferentes amortecedores de pH (veronal, carbonato/bicarbonato e

2A2M1P, quer isolados quer em mistura) e dois valores de pH (9,4 e 10,4). Independentemente do amortecedor ou da mistura de amortecedores utilizados, assim como do valor do pH, o valor da actividade fosfatásica alcalina sérica total foi sempre muito mais elevado quando o substrato era o β-naftilfosfato (*Tabela II do* **Artigo III**). Esta preferência pelo β-naftilfosfato relativamente ao α- também foi observada quando se calcularam as actividades das fracções electroforéticas da FA (*Tabelas V e VI do* **Artigo III**). A única excepção a esta regra foi observada quando se utilizou como amortecedor o carbonato/bicarbonato a pH 10,4. Observámos também que: **a**) o efeito da mudança do valor de pH dependia do amortecedor (ou mistura de amortecedores) e do substrato; **b**) a adição de um segundo amortecedor a um outro em uso não foi sempre acompanhada da mesma alteração nos resultados; e **c**) os resultados obtidos com diferentes amortecedores (ou mistura de amortecedores) variavam com o substrato e com o valor do pH (*Tabelas II a VI do* **Artigo III**).

Este trabalho, adicionalmente ao Artigo II, permitiu-nos confirmar que a quantificação da FA sérica total e a das suas fracções electroforéticas depende das condições do meio de incubação e, pela primeira vez, mostrar que as condições de revelação das fracções da FA, para esse propósito, são pelo menos tão importantes quanto a sua separação electroforética. É conveniente o uso de condições idênticas no estudo da FA sérica total e das suas fracções uma vez que a atribuição de um determinado peso a uma dada fracção electroforética, reflectindo correctamente a sua contribuição para a actividade total, depende das condições experimentais utilizadas. Assim, as diferenças observadas nos valores da actividade total e de cada uma das fracções, com as variações introduzidas nas condições de ensaio, permitem sugerir o aproveitamento das condições de ensaio para a individualização e/ou conhecimento bioquímico e semiológico das fracções electroforéticas.

Nos Artigos II (*Tabelas 1 e 3*) e III (*Tabelas II, V e VI*) as actividades totais e as das fracções electroforéticas, obtidas nas mesmas condições de ensaio, eram semelhantes, o que mostra que a metodologia descrita é consistente e reprodutível.

É importante realçar que os métodos apresentados poderão ser utilizados no aprofundamento do conhecimento simultâneo da modulação da actividade sérica total da FA e da modulação da actividade das fracções electroforéticas não só em diferentes condições de nutrição mas em muitas outras situações.

O interesse do esclarecimento do papel fisiológico da FA já foi focado na Introdução e dando continuidade ao trabalho anteriormente desenvolvido procuramos contribuir para esse esclarecimento.

Como já referido, a alimentação modula a FAint sérica. De acordo com vários estudos parece ser o componente lipídico da dieta o responsável pelo aumento observado (10, 11, 17, 22, 25, 29, 32, 35, 40, 42, 44, 45, 47, 49, 56, 60, 63, 69, 70, 72, 73, 75, 76, 96, 98, 100, 105, 118, 121, 128, 154 - 156; DH Alpers 2001, comunicação pessoal). Os sais biliares são necessários para a digestão e a absorção de gorduras e libertam a FA de membranas celulares. Todos estes dados estiveram na base do nosso interesse na investigação do efeito dos sais biliares na FAtne hepática e na FAint, isozimas que, fisiologicamente, estão em contacto com elevadas concentrações de sais biliares (ver por exemplo: 17, 22, 40, 42, 177). Com esse objectivo em mente, estudámos o efeito directo dos sais biliares conjugados na actividade da FA de homogeneizados de fígado e intestino delgado (Artigo IV). Neste estudo utilizou-se um método (adaptado de 63; 108, 109, 169; C Calhau 1999, comunicação pessoal) em que o substrato é o p-nitrofenilfosfato e o amortecedor o Tris-HCl, pH 10,2, em substituição dos métodos de ensaio descritos nos Artigos I, II e III. Dado que não havia, no trabalho apresentado no Artigo IV, necessidade de proceder à separação electroforética das isozimas da FA, o método escolhido para este trabalho é adequado aos objectivos que se pretendiam. Razões de natureza metodológica, como a simplicidade de execução e a ausência de recurso a solventes orgânicos, também justificam esta nossa escolha.

Observou-se que sais biliares conjugados (taurocólico, taurodesoxicólico, tauroquenodesoxicólico, glicocólico e glicoquenodesoxicólico) eram inibidores da FAtne hepática (*Figura 1 do* **Artigo IV**) e que, curiosamente, não tinham qualquer efeito na actividade da FAint. Este efeito diferencial, tanto quanto sabemos descrito por nós pela primeira vez, foi confirmado de forma indirecta através do estudo do efeito do jejum na acção inibidora dos mesmos sais biliares conjugados sobre a actividade fosfatásica alcalina sérica total. Tendo conhecimento prévio que o jejum induz um aumento da razão entre a FAtne hepática e a FAint (10, 11, 17, 22, 25, 29, 32, 35, 40, 42, 44, 45, 47, 49, 56, 60, 63, 69, 70, 72, 73, 75, 76, 96, 98, 100, 105, 118, 121, 128, 154 - 156; DH Alpers 2001, comunicação pessoal) observámos, como se esperava, que a acção inibidora dos sais biliares era maior no soro que tinha origem em animais em jejum do que no soro de animais alimentados (*Figura 2 do* **Artigo IV**).

A acção inibidora dos sais biliares na FAtne poderá fazer pensar na possibilidade de ocorrer interferência no doseamento da FA sérica total de doentes com patologias que aumentam a concentração de sais biliares na corrente sanguínea. Embora tenhamos presente a diferença entre as concentrações de sais biliares que provocavam inibição *in vitro* da FAtne hepática (Artigo IV) e as concentrações séricas dos sais biliares nos referidos doentes (177, 178) não podemos excluir, em definitivo, a possibilidade de tal interferência ocorrer. Como já referido, a FAtne hepática sérica aumenta na colestase. Está descrito que na *diabetes mellitus*, em situações de cirrose hepática e em doentes com insuficiência renal em hemodiálise a FAint aumenta no soro humano (ver por exemplo, para o aumento das duas isozimas: 10, 11, 17, 27, 31, 32, 36, 40, 42, 55, 56, 73, 88, 141, 151, 156). Embora os resultados que obtivemos no Rato não possam ser automaticamente extrapolados para o Homem, é possível que a um aumento da FAint e a um aumento da FAtne hepática corresponda, respectivamente, uma diminuição e um aumento da acção inibidora dos sais biliares na actividade da FA sérica total.

As diferentes isozimas da FA podem ser especificamente inibidas por certos compostos. Esta característica tem sido explorada na quantificação específica e na diferenciação das isozimas da FA na Bioquímica Clínica. Refira-se, a título de exemplo, que a distinção entre a FApl e a FAcg (duas isozimas estruturalmente muito parecidas e que são usadas no estudo e no controle de doentes com tumores do testículo e do ovário) se pode fazer tirando partido da inibição selectiva da FAcg pela Leu-L. A hArg-L, o levamisole e o bromotetramisole são potentes inibidores da FAtne. Os aminoácidos Phe-L e Trp-L são potentes inibidores da FAint e da FAint fetal (7, 9 - 11, 14, 16, 20, 24, 27, 28, 34 - 37, 39, 42, 43, 45, 49, 50, 55, 58, 59, 62, 64, 67, 68, 70, 73, 74, 77, 80 - 82, 85 - 90, 93, 96 - 98, 100 - 108, 114, 116, 117). Os resultados descritos no Artigo IV permitem-nos então sugerir que os sais biliares conjugados possam também ser usados na discriminação *in vitro* entre a FAtne hepática e a FAint, por exemplo na identificação de situações fisiológicas e patológicas em que se modifique a proporção sérica entre as duas isozimas da FA (como referido atrás).

A descoberta da acção diferencial dos sais biliares na FAtne hepática *versus* FAint (Artigo IV) despertou o nosso interesse para o estudo da modulação de diferentes tipos de fosfátases alcalinas. Embora, como já referido, o efeito de inibidores seja frequentemente usado para identificar diferentes isozimas, o estudo da

possível modulação diferencial das isoformas teciduais da FAtne tem sido objecto de pouca atenção.

Tendo conhecimento prévio que as FAtne hepática e renal têm glicosilações diferentes e que o padrão de glicosilação influencia a actividade das enzimas (10, 19, 45, 52, 83, 85, 94), decidimos investigar a possibilidade destas duas isoformas poderem responder de forma distinta à acção de fármacos (Artigo V).

A escolha dos fármacos a usar teve por base os resultados descritos no Artigo IV assim como resultados de outros estudos da nossa equipa (116, 117, 179). A hipotética relação funcional, já referida na Introdução, entre a FA e sistemas de transporte é um dos temas centrais em estudo no nosso laboratório. Os primeiros trabalhos em que participámos nesta linha de investigação sugeriam que não só a modulação da actividade da FA (por alteração do valor de pH do meio de incubação e pela presença de inibidores) como também a separação física entre a FA e a Pgp afectavam a actividade deste transportador (116, 117, 179). Assim, estudámos nas FAtne hepática e renal moduladores da Pgp, da MRP (180 - 184) e da captação hepática do taurocolato (185, 186).

Na investigação que descrevemos no Artigo V, testamos o efeito da lidocaína, da quinidina, da bupivacaína, do verapamil, do "kaempferol", da genisteína, da corticosterona e da progesterona em homogeneizados de fígado e de rim. Complementando a selecção dos fármacos acima referidos, também se estudou o efeito de moduladores clássicos da FAtne (levamisole, teofilina e IBMX) (7, 9 , 10, 14, 20, 27, 28, 35, 42, 43, 55, 58, 59, 62, 68, 80 - 82, 89, 90, 93, 97, 101 - 108, 114) nos dois tecidos. As condições de ensaio para o doseamento da actividade da FA foram idênticas às do Artigo IV com excepção do valor de pH (10,4 em vez de 10,2) (adaptado de 63; 108, 109, 169; C Calhau 1999, comunicação pessoal).

Com nenhum dos fármacos testados se observou activação da FAtne renal e, quando se observou inibição das duas isoformas, a inibição da FAtne renal foi sempre muito mais acentuada que a inibição da FAtne hepática. O levamisole e a teofilina inibiram quase totalmente a actividade da FAtne renal, tendo o efeito na FAtne hepática sido menos intenso. O levamisole foi um inibidor mais potente da FAtne hepática que a teofilina (*Figuras 1 e 2a do* **Artigo V**, respectivamente). O IBMX e a lidocaína produziram efeitos contrários nas duas isoformas: activaram a FAtne hepática e inibiram a isoforma renal (*Figuras 2b e 3a do* **Artigo V**, respectivamente). O levamisole foi o inibidor mais potente da FAtne renal, seguido pela teofilina e pelo

IBMX (*Figuras 1 e 2 do* **Artigo V**). A quinidina inibiu a FAtne renal não tendo tido qualquer efeito na isoforma hepática (*Figura 3b do* **Artigo V**). A inibição da FAtne renal pela bupivacaína foi ligeiramente mais elevada do que a da FAtne hepática (*Figura 3c do* **Artigo V**). O verapamil teve um efeito diferente nas duas amostras, reduzindo ligeiramente a actividade da FAtne renal e não afectando a hepática. O "kaempferol" teve um efeito activador mais pronunciado na FAtne hepática que na renal (*Figura 4 do* **Artigo V**). A genisteína teve um efeito ligeiramente diferente nas duas isoformas, reduzindo a renal e não afectando a hepática. A corticosterona inibiu a FAtne renal e a progesterona não produziu qualquer efeito nas duas isoformas.

Estes resultados permitiram-nos mostrar que os dados experimentais obtidos com uma determinada isoforma da FAtne não são automaticamente extrapoláveis para outra isoforma da mesma isozima.

A FAtne renal não aparece no soro de indivíduos saudáveis, mas pode estar presente em determinadas situações patológicas como carcinomas renais e rejeição de transplantes renais (11, 150, 151). Sugerimos, assim, que, embora com graus de eficácia distintos, o levamisole, a teofilina, o IBMX, a lidocaína, a quinidina, a bupivacaína, o verapamil, o "kaempferol" e a genisteína possam ser usados na discriminação *in vitro* entre as isoformas hepática e renal da FAtne, por exemplo na identificação de situações patológicas em que se registe uma alteração das quantidades séricas relativas destas isoformas.

Tanto quanto sabemos o estudo do efeito dos moduladores de sistemas de transporte acima referidos na actividade da FA de homogeneizados de tecidos nunca tinha sido antes objecto de publicação.

A observação de efeitos diferentes, e até mesmo opostos, de vários fármacos nas duas isoformas apoia a hipótese formulada que o diferente padrão de glicosilação das FAtne hepática e renal determina diferenças no seu comportamento funcional.

Dados do nosso laboratório, obtidos em homogeneizados de culturas primárias de hepatócitos (108) mostraram, para alguns dos moduladores acima referidos, efeitos diferentes dos por nós descritos na actividade da FAtne hepática (Artigo V). Embora desconheçamos as razões desta diferença, já que a solução usada na preparação dos homogeneizados foi a mesma nos dois estudos, admitimos como hipótese explicativa que a uma possível indução da FAtne nas culturas primárias de hepatócitos esteja associada um diferente padrão de glicosilação da enzima. Sabe-se, nomeadamente,

que o padrão de glicosilação da FA em tumores é distinto do observado nos tecidos normais (19).

A modulação diferencial das FAtne hepática e renal por fármacos poderá estar relacionada com diferentes funções da FAtne nestes dois órgãos, abrindo a possibilidade da manipulação farmacológica da FAtne *in vivo*.

Desconhece-se como é que a ligação de cadeias de poliarginina à ciclosporina aumenta a sua captação pelas células (187). De acordo com o nosso próprio trabalho (115), cadeias polipeptídicas com seis resíduos de Arg-L inibiam a FAtne quando testadas em homogeneizados de rim do Rato. Este resultado é compatível com a hipótese que na base da activação do transporte do referido fármaco ligado à cadeia de poliarginina esteja a acção inibidora desta cadeia na actividade da FA.

Dados da literatura apoiam a ideia que a FA possa ter um papel na regulação da actividade biológica de proteídos. A fosforilação e a desfosforilação de proteídos por acção catalítica de, respectivamente, cínases e fosfátases, é importante em muitos processos celulares. De acordo com diversos autores, a FA pode catalisar a hidrólise de resíduos fosfato ligados a proteídos tendo sido sugerido que a FA participe em reacções de desfosforilação de proteídos e que, concretamente, tenha uma função específica na regulação da diferenciação celular e do CFTR (14, 58, 93, 103, 104, 107, 130, 188).

O facto de a FA ter sido inicialmente descrita como tendo actividade máxima para valores de pH superiores ao pH fisiológico e a diversidade de substratos sobre os quais actua tem dificultado o esclarecimento do seu papel biológico. Contudo, foi demonstrado com alguns substratos (por exemplo o p-nitrofenilfosfato, o fenilfosfato, a fosfoetanolamina e o fosfato de piridoxal) que o pH óptimo diminui quando se testam baixas concentrações de substrato (2, 7, 9, 10, 20, 25, 28, 34, 39, 58, 80, 161).

Estudos em células de osteosarcoma (linha celular SAOS-2) e em fibroblastos humanos mostram que a FAtne na superfície celular é capaz de actuar eficazmente sobre a fosfoetanolamina e o fosfato de piridoxal, substratos naturais da FA, testados em concentrações fisiológicas a pH fisiológico (20, 28). A FA de cartilagem em mineralização de Galinha e de Porco, a FA de intestino bovino e a FA de fígado humano desfosforilam aminoácidos e proteídos a valores de pH fisiológico (14, 93, 130). Resultados recentemente publicados mostram que, para valores fisiológicos de pH, a FA presente em células de teratocarcinoma do Ratinho (linha celular P19) e de

leucemia mieloblástica humana (linha celular HL-60) pode desempenhar um papel na desfosforilação de proteídos da superfície celular e ter uma função específica na diferenciação daquelas linhas celulares (58).

A actividade do CFTR é regulada pela acção de cínases e fosfátases de proteídos sendo activado por fosforilação. Foi sugerido que a acção activadora da teofilina, IBMX e levamisole no CFTR possa ser uma consequência da acção inibidora daqueles fármacos na FA endógena. De acordo com essa hipótese a FA seria uma das fosfátases envolvidas na inactivação do CFTR. Este canal é desactivado por adição de FA exógena (103, 104, 107, 188 - 192).

Para além da teofilina, do IBMX e do levamisole, também a genisteína activa o CFTR. O modo de acção da genisteína é ainda controverso, tendo sido sugeridos diversos mecanismos, incluindo a interacção directa com o CFTR (107, 192, 193). Os nossos resultados (Artigo V) mostram que a genisteína inibe significativamente a FAtne renal, o que nos leva a levantar a hipótese que a inibição da actividade da FA pela genisteína seja um mecanismo a considerar no efeito deste fármaco no CFTR.

O facto de moduladores da Pgp, da MRP e da captação hepática do taurocolato terem efeito na actividade das FAtne hepática e renal é compatível com a ideia que estas isoformas possam estar relacionadas com sistemas de transporte. Esta hipótese é também apoiada por outros dados obtidos no nosso laboratório (169). Foi observada uma correlação positiva entre a actividade das FAtne hepática e renal e a extensão de superfície de membrana por unidade de volume nos respectivos tecidos (169).

Os resultados obtidos no estudo do efeito de sais biliares (Artigo IV) e de vários moduladores de diferentes sistemas de transporte (Artigo V) na FA assim como o facto de os sais biliares regularem a sua circulação entero-hepática e a sua própria síntese (194, 195) levou-nos a considerar a hipótese de a enzima poder, de alguma forma, estar envolvida na regulação do transporte transmembranar de sais biliares. É interessante referir que, neste contexto, fármacos que no Homem aumentam a incidência de cálculos biliares, nomeadamente a progesterona e o octreotídeo, são activadores da FA (108, 109, 111, 112, 114). A progesterona activou a FA na linha celular RBE4 (111, 112, 114) e o octreotídeo activou a enzima em homogeneizados de culturas primárias de hepatócitos e de cérebro e de jejuno do Rato (108, 109).

Realizámos então o trabalho experimental descrito no Artigo VI, estudando-se o efeito do pH e de inibidores (levamisole, Edta, vanadato e teofilina) (7, 9, 10, 14, 20, 24, 27, 28, 35, 36 , 39, 42, 43, 55, 58, 59, 62, 68, 74, 80 - 82, 89, 90, 93, 97, 101 - 108, 111, 112, 114, Artigo V) ou activadores (octreotídeo e "kaempferol") (108, 109, 111, 112, 114, Artigo V) da FAtne na captação de taurocolato por hepatócitos isolados.

Observámos que a acumulação de taurocolato nos hepatócitos diminuía na presença de octreotídeo e "kaempferol" (*Figuras 6 e 7 do* **Artigo VI**, *respectivamente*) e quando se aumentava o valor de pH do meio de incubação de 7,4 para 8,2 (*Figura 3 do* **Artigo VI**). A activação da FA pela presença de fármacos inibia o transporte. Do mesmo modo, nas nossas condições de ensaio, o aumento do valor de pH inibia o transporte o que pode ser facilmente entendido se a referida mudança de pH aumentar a actividade da FA relativamente a um seu putativo substrato modificando-o de modo a fazer-se sentir uma acção inibidora no transporte. Inversamente, o mesmo tipo de raciocínio poderá ser feito relativamente à diminuição do pH para 6,2 pois observou-se um aumento da captação de taurocolato (*Figura 3 do* **Artigo VI**). No entanto, a presença de levamisole, Edta, vanadato e teofilina (*Figura 5 do* **Artigo VI**) não interferia no transporte. Face a este resultado, embora a concentração de taurocolato fosse baixa (25 μM), admitimos que a actividade da FA estivesse inibida pela presença do sal biliar prejudicando o efeito de outros fármacos inibidores da FA no transporte em estudo. A possibilidade de fármacos inibidores da FA poderem activar o transporte do taurocolato foi confirmado testando o efeito da adição de teofilina na presença de octreotídeo e de "kaempferol": nestas condições experimentais, a teofilina funcionou como activador desse transporte (*Figuras 6 e 7 do* **Artigo VI**, *respectivamente*).

Os nossos resultados sugerem uma relação entre a actividade FA e a captação de taurocolato nos hepatócitos: um aumento da actividade enzímica diminui o transporte do sal biliar e a inibição da FA aumenta esse transporte.

Está descrito que a lidocaína, a quinidina e a bupivacaína activam a captação celular de taurocolato (186). Sendo que o efeito destes fármacos na FA não tinha sido ainda descrito admitimos que pudessem ser inibidores desta enzima. De facto, a bupivacaína inibia a FAtne hepática mas a lidocaína activava e a quinidina não tinha qualquer efeito (*Figura 3C, A e B do* **Artigo V**, *respectivamente*). Estes dois fármacos parecem pois regular o transporte de taurocolato por mecanismos que aparentemente não envolvem a FA.

Os possíveis mecanismos de acção da FA nos transportadores não foram ainda investigados e poderão ser de natureza diversa. Existem dados na literatura, já referidos (14, 20, 28, 58, 93, 130), que mostram que a FA poderá actuar sobre proteídos ou outros substratos naturais a pH fisiológico. Assim, a FA poderá modificar a actividade dos transportadores de forma directa catalisando a sua desfosforilação, ou de forma indirecta modificando o estado de fosforilação de moduladores alostéricos ou de enzimas que directa ou indirectamente condicionem a actividade desses transportadores.

Foi sugerido o envolvimento da FA na modulação do transporte de catiões orgânicos em hepatócitos isolados do Rato e em duas linhas celulares, Caco-2 (derivada de um adenocarcinoma de cólon humano) e RBE4 (110 - 113, 116) e na modulação da internalização da insulina na linha celular RBE4 (114). Um catião orgânico cujo transporte em células RBE4 parece ser modulado pela FA activa a FA na mesma linha celular (112).

O conjunto dos resultados apresentados nos Artigos V e VI assim como outros obtidos no nosso laboratório, e já referidos anteriormente (108 - 114, 116, 117, 169, 179), apontam de forma consistente para a participação da FA em sistemas de transporte.

5 – RESUMO

Nesta dissertação é, pela primeira vez, descrito e caracterizado: **(i)** um conjunto de condições experimentais simultaneamente adequadas à quantificação da actividade fosfatásica alcalina sérica total e à das suas fracções electroforéticas (Artigos I, II e III); **(ii)** o efeito da mesma variação das condições experimentais nessas quantificações (Artigos II e III); **(iii)** o efeito da ingestão única de fibras de celulose (serrim) na actividade fosfatásica alcalina sérica total e na das suas fracções electroforéticas (Artigo II); **(iv)** o efeito diferencial de sais biliares nas actividades da fosfátase alcalina tecidual não específica (FAtne) hepática, da fosfátase alcalina intestinal (FAint) e da fosfátase alcalina (FA) sérica total (Artigo IV); **(v)** o efeito diferencial de moduladores da FA, da glicoproteína-P, da "multidrug resistance protein" e da captação hepática de taurocolato nas actividades das FAtne hepática e renal (Artigo V); e **(vi)** o efeito de moduladores da actividade da FAtne na captação hepática de taurocolato (Artigo VI).

A importância do doseamento da actividade da FA sérica total assim como a separação electroforética, a identificação e a quantificação das suas isozimas na investigação Bioquímica Básica e Aplicada justifica o nosso interesse no estudo destas técnicas. A actividade da FA sérica total reflecte a actividade das diversas isozimas séricas e a actividade de cada uma dessas isozimas pode ser diferente e independentemente influenciada por uma ou mais condições de ensaio. Conscientes desta realidade, desenvolvemos um método que permite utilizar o mesmo substrato (α- ou β-naftilfosfatos) e o mesmo amortecedor de pH [2-amino-2-metil-1-propanol (2A2M1P), carbonato/bicarbonato ou veronal (isolados ou em mistura), pH 9,4 ou 10,4] no doseamento da actividade da FA sérica total e na revelação e quantificação das fracções enzímicas obtidas por separação electroforética em acetato de celulose. O efeito da variação do valor do pH e do uso de diferentes substratos e amortecedores de pH na actividade da FA sérica total do Rato e na das suas fracções electroforéticas foi também estudado (mas mantendo o mesmo conjunto de condições experimentais nas duas quantificações), observando-se que o efeito da variação de uma determinada condição de ensaio podia depender das outras condições numa complexa rede de factores interdependentes. A utilização das mesmas condições experimentais na determinação da actividade fosfatásica alcalina total do soro e na revelação das

fracções electroforéticas séricas da FA assegura o cálculo do contributo específico dessas fracções para a actividade total. Esta metodologia permitiu minorar equívocos gerados pela interferência diferencial que as condições de ensaio têm na actividade de cada uma das fracções. Com este trabalho mostrou-se que, na quantificação da actividade das fracções electroforéticas da FA, as condições de revelação dessas fracções são tão importantes quanto a sua separação.

Baseando-nos na metodologia desenvolvida, usando o α- e o β- naftilfosfato como substrato e o 2A2M1P (pH 10,4) como amortecedor, mostrámos que, no Rato, a ingestão única de fibras grosseiras de celulose (serrim) tinha um efeito semelhante ao da alimentação normal: em ambos os casos a actividade da FA sérica total aumentava, principalmente em consequência do aumento da fracção electroforética que correspondia predominantemente à FAint. Adicionalmente, mostrámos também que a intensidade dos aumentos observados dependia do substrato utilizado.

A existência e a distribuição da FA nos seres vivos são conhecidas há muitas décadas mas o seu papel fisiológico permanece em grande parte desconhecido. A sua localização na face externa da membrana citoplasmática sugere uma função associada a sistemas de transporte transmembranar.

O nosso interesse no estudo do efeito dos sais biliares na actividade da FA teve como ponto de partida o conhecimento prévio do papel destes compostos na digestão e na absorção de gorduras, da sua acção indutora da libertação da FA de membranas celulares e do aumento da FAint no soro após a ingestão de gorduras. Observámos em homogeneizados de fígado e intestino delgado do Rato que, em concentrações fisiológicas, todos os sais biliares conjugados incluídos no estudo inibiam a FAtne hepática não interferindo na FAint. O efeito diferencial dos sais biliares nestas duas isozimas foi confirmado comparando o seu efeito na actividade da FA sérica total de ratos em jejum completo e alimentados normalmente: em consequência do aumento da razão entre as actividades da FAtne hepática e da FAint no soro, induzido pelo jejum, a inibição da actividade da FA sérica total pelos sais biliares era maior nesta situação metabólica. Estes resultados sugerem que os sais biliares possam ser usados na discriminação *in vitro* entre as isozimas FAtne hepática e FAint.

A acção diferencial dos sais biliares na FAtne hepática *versus* FAint conduziu-nos ao estudo da modulação de isoformas teciduais da FAtne. Testando o efeito de moduladores da FA, da glicoproteína-P, da "multidrug resistance protein" e da

captação hepática do taurocolato em homogeneizados de fígado e de rim do Rato observámos efeitos diferentes e, até mesmo opostos, nas isoformas hepática e renal da FAtne. Com nenhum dos fármacos testados se observou activação da FAtne renal e, quando se observou inibição das duas isoformas, a inibição da FAtne renal foi sempre muito mais acentuada que a inibição da FAtne hepática. Os resultados obtidos mostraram que não é indiferente a escolha de uma determinada isoforma nos estudos de modulação da actividade da isozima FAtne e sugerem que a modulação diferencial das isoformas hepática e renal pelo levamisole, teofilina, 3-isobutil-1-metilxantina, lidocaína, quinidina, bupivacaína, verapamil, "kaempferol" e genisteína possa ser usada na discriminação *in vitro* das referidas isoformas. A observação de que as actividades da FAtne hepática e renal podiam ser afectadas por conhecidos moduladores de sistemas de transporte apoia a possibilidade do envolvimento da FA nesses sistemas.

Os resultados obtidos no estudo do efeito de sais biliares e moduladores de sistemas de transporte na FA e o conhecimento prévio de que os sais biliares regulam a sua circulação entero-hepática e a sua própria síntese levou-nos a investigar, em hepatócitos isolados do Rato, o efeito de moduladores da actividade da FAtne no transporte transmembranar do taurocolato. Os resultados obtidos no trabalho experimental subsequente são compatíveis com a ideia de que a activação da FA modula negativamente o transporte de taurocolato. Observou-se que a modulação da FA pela adição de octreotídeo ou "kaempferol" ou por alcalinização do meio de incubação inibia o transporte do taurocolato. Reciprocamente, a acidificação do meio activava o referido transporte. Embora a presença de levamisole, Edta, vanadato ou teofilina não interferisse no transporte em estudo, a adição de teofilina na presença de octreotídeo ou de"kaempferol" tinha um efeito activador desse transporte.

No seu conjunto, os nossos resultados apontam para um papel da FA na modulação de sistemas de transporte. Esta hipótese é apoiada por outros dados do nosso laboratório.

6 – RESUME

Dans cette dissertation, nous décrivons et caractérisons pour la première fois: **(i)** un ensemble de conditions expérimentales simultanément adaptées à la quantification de l'activité phosphatasique alcaline sérique totale et à celle de ses fractions électrophorétiques (Articles I, II et III); **(ii)** l'effet de la même variation des conditions expérimentales sur ces quantifications (Articles II et III); **(iii)** l'effet de l'ingestion uniquement de fibres de cellulose (sciure de bois) sur l'activité phosphatasique alcaline sérique totale et sur l'activité des ses fractions électrophorétiques (Article II); **(iv)** l'effet différentiel des sels biliaires sur les activités de la phosphatase alcaline tissulaire non spécifique (PhAtns) hépatique, de la phosphatase alcaline intestinale (PhAint) et de la phosphatase alcaline (PhA) sérique totale (Article IV); **(v)** l'effet différentiel de modulateurs de la PhA, de la glycoprotéine-P, de la "multidrug resistance protein" et de la captation hépatique de taurocholate sur les activités des FAtne hépatique et rénale (Article V); et **(vi)** l'effet de modulateurs de l'activité de la FAtne sur la captation hépatique de taurocholate (Article VI).

L'importance du dosage soit de l'activité de la PhA sérique totale soit de la séparation électrophorétique, de l'identification et de la quantification de ses isoenzymes dans la recherche Biochimique Basique et Appliquée justifie notre intérêt à l'étude de ces techniques. L'activité de la PhA sérique totale reflète l'activité des différentes isoenzymes sériques et l'activité de chacune de ces isoenzymes peut être différemment et indépendamment influencée par une seul ou par plus qu'une des conditions d'essai. Conscientes de cette réalité, nous avons développé une méthodologie qui permet d'utiliser le même substrat (α- ou β- naphtylphosphates) et le même tampon de pH [2-amino-2-méthyl-1-propanol (2A2M1P), carbonate/bicarbonate ou véronal (isolés ou mélangés), pH 9,4 ou 10,4] dans le dosage de l'activité de la PhA sérique totale et dans le développement et la quantification des fractions de la PhA sérique obtenues par séparation électrophorétique sur l'acétate de cellulose. Nous avons aussi étudié l'effet de la variation de la valeur du pH et de l'utilisation de différents substrats et tampons de pH sur l'activité de la PhA sérique totale de Rat et sur celle de ses fractions électrophorétiques (mais employant le même ensemble de conditions expérimentales

dans les deux quantifications) et nous avons observé que l'effet de la variation d'une certaine condition d'essai pouvait être dépendant des autres conditions dans un complexe réseau de facteurs interdépendants. L'utilisation des mêmes conditions expérimentales lors du dosage de l'activité phosphatasique alcaline totale du sérum et lors du développement des fractions électrophorétiques sériques de la PhA a permis d'estimer la contribution spécifique de ces fractions pour l'activité totale. Cette méthodologie a permis d'amoindrir des équivoques engendrées par l'interférence différentielle que les conditions d'essai ont dans l'activité de chacune des fractions. Ce travail a permis de montrer que, pour la quantification de l'activité des fractions électrophorétiques de la PhA, les conditions de développement de ces fractions sont aussi importantes que leur séparation.

En nous appuyant sur la méthodologie développée, utilisant l' α- et le β-naphtylphosphate comme substrat et le 2A2M1P (pH 10,4) comme tampon, nous avons démontré que, chez le Rat, l'ingestion uniquement de fibres de cellulose (sciure de bois) avait un effet pareil à celui de l'alimentation normale: dans les deux cas l'activité de la PhA sérique totale augmentait, principalement en résultat de l'augmentation de la fraction électrophorétique qui correspondait surtout à la PhAint. Nous avons également démontré que l'intensité des augmentations observées dépendait du substrat utilisé.

L'existence et la distribution de la PhA chez les êtres vivants sont connues depuis des décades mais son rôle physiologique reste presque inconnu. Sa localisation dans le côté extérieur de la membrane cytoplasmique suggère un rôle associé à des systèmes de transport transmembranaire.

Notre intérêt à l'étude de l'effet des sels biliaires sur l'activité de la PhA a eu comme point de départ la connaissance préalable du rôle joué par ses composants dans la digestion et l'absorption des matières grasses, de leur action inductrice de la libération de la PhA de membranes cellulaires et de l'augmentation de la PhAint dans le sérum après l'ingestion de matières grasses. En utilisant des homogénats de foie et d'intestin grêle de Rat nous avons observé que, dans des concentrations physiologiques, tous les sels biliaires conjugués inclus dans l'étude inhibaient la PhAtns hépatique mais pas la PhAint. L'effet différentiel des sels biliaires sur ces deux isoenzymes a été vérifié tout en comparant leur effet sur l'activité de la PhA sérique totale de Rats en jeun totale et normalement nourris: en conséquence de l'augmentation du "ratio" entre les activités de la PhAtns hépatique et de la PhAint

dans le sérum, provoquée par le jeun, l'inhibition de l'activité de la PhA sérique totale par les sels biliaires était plus significative dans cette situation métabolique. Ces résultats suggèrent que les sels biliaires puissent être utilisés pour la discrimination *in vitro* entre les isoenzymes PhAtns hépatique et la PhAint.

L'action différentielle des sels biliaires sur la PhAtns hépatique *versus* PhAint nous a fait étudier la modulation d'isoformes tissulaires de la PhAtns. En étudiant l'effet de modulateurs de la PhA, de la glycoprotéine-P, de la "multidrug resistance protein" et de la captation hépatique de taurocholate dans des homogénats de foie et de rein de Rat, nous avons observé des effets différents, voire opposés, sur des isoformes hépatique et rénale de la PhAtns. Avec aucune des drogues utilisées nous n'avons jamais observé d'activation de la PhAtns rénale et, quand nous avons observé de l'inhibition des deux isoformes, l'inhibition de la PhAtns rénale a toujours été plus importante que l'inhibition de la PhAtns hépatique. Les résultats obtenus ont démontré que le choix d'une certaine isoforme pour les études de modulation de l'activité de l'isozyme PhAtns n'est pas sans conséquence et ils suggèrent que la modulation différentielle des isoformes hépatiques et rénales par le lévamisole, théophylline, 3-isobutyl-1-méthylxanthine, lidocaïne, quinidine, bupivacaïne, verapamil, "kaempferol" et génistéïne puisse être utilisée pour leur discrimination *in vitro*. L'observation que les activités de la PhAtns hépatique et rénale pouvaient être affectées par des modulateurs de systèmes de transport bien connus soutient la possibilité de l'implication de la PhA dans ces systèmes.

Les résultats obtenus lors de l'étude de l'effet de sels biliaires et modulateurs de systèmes de transport sur la PhA et la connaissance préalable que les sels biliaires règlent leur circulation enterohépatique et leur synthèse nous a fait étudier, tout en utilisant des hépatocytes isolés de Rat, l'effet de modulateurs de l'activité de la PhAtns sur le transport transmembranaire de taurocholate. Les données obtenues lors du travail expérimental suivant s'accordent avec l'idée que l'activation de la PhA influence négativement le transport de taurocholate. Nous avons observé que la modulation de la PhA en résultat de l'addition d'octréotidéo ou de "kaempferol" ou de l'alcalinisation du milieu d'incubation inhibait le transport de taurocholate. Réciproquement, l'acidification du milieu d'incubation activait le transport cité. Quoique l'inhibition de la PhA engendrée par le lévamisole, vanadate, Edta ou théophylline n'eût pas d'effet sur le transport en étude, l'addition de théophylline en

présence de l' octréotidéo ou du "kaempferol" jouait un rôle activateur sur le transport cité.

En tout, nos résultats donnent des indices d'un rôle de la PhA dans la modulation de systèmes de transport. Cette hypothèse est soutenue par d'autres résultats obtenus dans notre laboratoire.

7 – SUMMARY

This thesis describes and characterizes, for the first time: **(i)** a set of experimental parameters that are simultaneously suitable for quantifying total serum alkaline phosphatase (ALP) activity and the activity of its fractions after electrophoretic separation (Articles I, II and III); **(ii)** the effect of varying the same experimental parameter(s) on both quantifications (Articles II and III); **(iii)** the effect of starvation with access to cellulose fibre (sawdust) on total serum ALP and its electrophoretic fractions activities (Article II); **(iv)** the differential effect of bile salts on the activity of hepatic tissue-nonspecific ALP (TNALP), of intestinal ALP (IntALP) and of total serum ALP (Article IV); **(v)** the differential effect of modulators of ALP, of P-glycoprotein, of multidrug resistance protein and of hepatic taurocholate uptake on hepatic and renal TNALP activities (Article V); and **(vi)** the effect of TNALP activity modulators on taurocholate hepatic uptake (Article VI).

The quantification of total serum ALP activity and the electrophoretic separation of its enzymic fractions, their identification and their quantification, are important research topics in Basic and Applied Biochemistry. The relevance of these techniques in biochemical research largely justifies our interest in their study. Total serum ALP activity is the sum of all the isozymes activities present in the serum, and the activity of each one of these isozymes may be differentially and independently affected by one or more experimental parameters. We developed a method that allowed us to use the same substrate (α- or β-naphthylphosphates) and the same pH buffer [2-amino-2-methyl-1-propanol (2A2M1P), carbonate/bicarbonate or veronal (alone or in a mixture), pH 9.4 or 10.4] to quantify both total serum ALP and its enzymic fractions (obtained after electrophoresis in cellulose acetate) activities. The effects of varying the pH value and of using different substrates and pH buffers on the quantification of total ALP, in rat serum, and of its electrophoretic fractions (but using the same set of experimental conditions on both quantifications) was evaluated. We observed that the effect of the variation of a particular assay parameter would be dependent on the other parameters, in a complex net of inter-dependent factors. So, in order to correctly estimate the specific contribution of each electrophoretic fraction to the overall total serum ALP activity, the same set of experimental parameters should be used when quantifying both total serum ALP and ALP electrophoretic fractions

activities. By using this approach errors generated by the differential interference of assay conditions on the activity of the different ALP electrophoretic fractions were minimized. Our work clearly showed that the assay conditions used for visualisation, identification and quantification of the ALP electrophoretic fractions are as crucial and important as its separation on the electrophoresis support medium.

Using our experimental methodology, with α- and β-naphthylphosphates as substrate and 2A2M1P (pH 10.4) as buffer, we showed that, in the rat, starvation with access to cellulose fibre (sawdust) had a similar effect to normal feeding: in both nutritional conditions total serum ALP activity increased, owing mainly to the increase of the electrophoretic fraction mainly corresponding to IntALP. Additionally, we showed that the magnitude of the observed increases was dependent on the substrate used.

The physiological role of ALP is still largely unknown, although this enzyme was first described many decades ago. ALP cellular localization on the external domain of the cytoplasmic membrane suggests a function associated with trans-membrane transport systems.

We became interested in studying the effect of bile salts on ALP after learning that bile salts participate in fat digestion and absorption, that bile salts can release ALP from cellular membranes and that fat ingestion increases IntALP activity in serum. Conjugated bile salts, used at physiological concentrations in rat liver and small intestine homogenates, inhibited hepatic TNALP but had no effect on IntALP. ALP inhibition induced by conjugated bile salts was significantly higher in the serum of starved rats than in the serum of fed animals: these results are in good agreement with the known higher proportion of hepatic TNALP and lower proportion of IntALP in the serum of starved rats than in the serum of fed rats. Our data suggest that bile salts could be used, *in vitro*, to help discriminating between hepatic and intestinal ALP isozymes.

The differential effect of bile salts on hepatic TNALP *versus* IntALP led us to study the modulation of TNALP isoforms. So, the effect of modulators of ALP, of P-glycoprotein, of multidrug resistance protein and of hepatic taurocholate uptake on TNALP activity on rat liver and kidney homogenates was investigated. Distinct and, in some cases, opposite effects were observed on hepatic and renal TNALP isoforms. None of the drugs used in our experiments resulted in the activation of renal TNALP. In cases where inhibition of the two isoforms was observed, renal TNALP was always

more sensitive to inhibition than hepatic TNALP. Our results suggest that TNALP activity modulation studies should take into account the source of TNALP isoform and that differential modulation of hepatic and renal TNALP isoforms by levamisole, theophylline, 3-isobutyl-1-methylxanthine, lidocaine, quinidine, bupivacaine, verapamil, kaempferol and genistein could be used, *in vitro*, to help discriminating between these two TNALP isoforms. The observation that hepatic and renal TNALP activities could be affected by known modulators of transport systems supports the possibility of ALP association with those systems.

Our results on the effect of bile salts and transport systems modulators on ALP activity and the reports that bile salts regulate their own enterohepatic circulation and their own synthesis, led us to investigate, in freshly isolated rat hepatocytes, the effect of TNALP activity modulators on taurocholate uptake. Our results support the hypothesis that TNALP activation modulates negatively taurocholate uptake. Indeed, we observed that TNALP modulation by adding octreotide or kaempferol or by making the incubation medium more alkaline inhibited taurocholate transport. Inversely, acidifying the incubation medium resulted in activation of the mentioned uptake. Although levamisole, Edta, vanadate or theophylline did not interfere on bile salt uptake, the addition of theophylline, in the presence of octreotide or kaempferol, activated the trans-membrane transport of taurocholate.

Overall our results are supportive of a physiological role for ALP in the modulation of transport systems. This hypothesis is also supported by other data from our laboratory.

8 – BIBLIOGRAFIA

1 - MOSS DW (1960). Kinetics of phosphatase action on naphthyl phosphates, determined by a highly sensitive spectrofluorimetric technique. *Biochem J* 76: 32P.

2 - BABSON AL, Greeley SJ, Coleman CM, Phillips GE (1966). Phenolphthalein monophosphate as a substrate for serum alkaline phosphatase. *Clin Chem* 12: 482-90.

3 - BOWERS GN Jr, Kelley M-L, McComb RB (1967). Continuous spectrophotometric measurement of serum alkaline phosphatase activity. Relative molecular activity and sensitivity of nine self-indicating phenolic substrates in 2-amino-2-methyl-1-propanol buffer. *Clin Chem* 13: 608-10.

4 - McCOMB RB, Bowers GN Jr (1972). Study of optimum buffer conditions for measuring alkaline phosphatase activity in human serum. *Clin Chem* 18: 97-104.

5 - SSCC (1974). Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology. Recommended methods for the determination of four enzymes in blood. *Scand J Clin Lab Invest* 33: 291-306.

6 - ELLIS G (1976). Quality control of serum alkaline phosphatase assays: project report and discussion of some factors affecting the assay. *Ann Clin Biochem* 13: 327-35.

7 - VAN BELLE H (1976). Alkaline phosphatase. I. Kinetics and inhibition by levamisole of purified isoenzymes from humans. *Clin Chem* 22: 972-6.

8 - BRETAUDIÈRE J-P, Vassault A, Amsellem L, Pourci M-L, Thieu-Phung H *et al* (1977). Criteria for establishing a standardized method for determining alkaline phosphatase activity in human serum. *Clin Chem* 23: 2263-74.

9 - McCOMB RB, Bowers GN Jr, Posen S (1979). Alkaline phospatase. New York, Plenum Press.

10 - CROFTON PM (1982). Biochemistry of alkaline phosphatase isoenzymes. *Crit Rev Clin Lab Sci 16: 161-94.*

11 - MOSS DW (1982). Alkaline phosphatase isoenzymes. *Clin Chem* 28: 2007-16.

12 - AACC (1983). A reference method for measurement of alkaline phosphatase activity in human serum. Enzyme working group of the subcommittee on standards, American Association for Clinical Chemistry, study group on alkaline phosphatase. *Clin Chem* 29: 751-61.

13 - IFCC (1983). International Federation of Clinical Chemistry, scientific committee, analytical section, expert panel on enzymes. IFCC methods for the mesurement of catalytic concentration of enzymes. Part 5. IFCC method for alkaline phosphatase (orthophosphoric-monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1). *Clin Chim Acta* 135: 339F-67F e *J Clin Chem Clin Biochem* 21: 731-48.

14 - CHAN JRA, Stinson RA (1986). Dephosphorylation of phosphoproteins of human liver plasma membranes by endogenous and purified liver alkaline phosphatase. *J Biol Chem* 261: 7635-9.

15 - HADA T, Imanishi H, Yamamoto T, Amuro Y, Higashino K *et al* (1986). Microheterogeneity of Kasahara isozyme. *Clin Chim Acta* 159: 37-43.

16 - MOSS DW, Parmar CR, Whitaker KB (1986). Comparison of a tumor-derived form of intestinal alkaline phosphatase with foetal and adult intestinal alkaline phosphatases. *Clin Chim Acta* 158: 165-72.

17 - WULKAN RW, Leijnse B (1986). Alkaline phosphatase and cholestasis. *Ann Clin Biochem* 23: 405-12.

18 - KALIMANOVSKA V, Whitaker KB, Moss DW (1987). Effect of bromelain on alkaline phosphatases of intestinal and non-intestinal tissues and serum. *Clin Chim Acta* 170: 219-26.

19 - KOYAMA I, Miura M, Matsuzaki H, Sakagishi Y, Komoda T (1987). Sugar-chain heterogeneity of human alkaline phosphatases: differences between normal and tumour-associated isozymes. *J Chromatogr* 413: 65-78.

20 - FEDDE KN, Lane CC, Whyte MP (1988). Alkaline phosphatase is an ectoenzyme that acts on micromolar concentrations of natural substrates at physiologic pH in human osteosarcoma (SAOS-2) cells. *Arch Biochem Biophys* 264: 400-9.

21 - THIEDE MA, Yoon K, Golub EE, Noda M, Rodan GA (1988). Structure and expression of rat osteosarcoma (ROS 17/2.8) alkaline phosphatase: product of a single copy gene. *Proc Natl Acad Sci USA* 85: 319-23.

22 - ALPERS DH, Eliakim R, DeSchryver-Kecskemeti K (1989). Physiologic release of tissue alkaline phosphatases. Secretion of hepatic and intestinal alkaline phosphatases: similarities and differences. *Clin Chim Acta* 186: 211-24.

23 - HAMILTON BA, McPhee JL, Hawrylak K, Stinson RA (1989). Alkaline phosphatase releasing activity in human tissues. *Clin Chim Acta* 186: 249-54.

24 - HIGASHINO K, Muratani K, Hada T, Imanishi H, Amuro Y *et al* (1989). Purification and some properties of the fast migrating alkaline phosphatase in FL-amnion cells (the Kasahara isozyme) and its cDNA cloning. *Clin Chim Acta* 186: 151-64.

25 - HIPÓLITO-REIS C (1989). Fosfátases alcalinas. Variações das actividades séricas e hepática com o jejum, no Rato. *Arq Med* 3: 19-24.

26 - TOH Y, Yamamoto M, Endo H, Misumi Y, Ikehara Y (1989). Isolation and characterization of a rat liver alkaline phosphatase gene. A single gene with two promoters. *Eur J Biochem* 182: 231-7.

27 - FAVRE G, Canal P, Soula G, Bagnoux JP, Jardillier JC *et al* (1990). Alkaline phosphatase isoenzymes. Genetic aspects – identification and clinical interpretation. *Ann Biol clin* 48: 119-25.

28 - FEDDE KN, Whyte MP (1990). Alkaline phosphatase (tissue-nonspecific isoenzyme) is a phosphoethanolamine and pyridoxal-5'-phosphate ectophosphatase: normal and hypophosphatasia fibroblast study. *Am J Hum Genet* 47: 767-75.

29 - HIPÓLITO-REIS C (1990). Estudo bioquímico da intoxicação alcoólica. Reversão pelo etanol em dose elevada dos efeitos do jejum sobre a glicemia e actividades fosfatásicas alcalinas séricas. *Arq Med* 4: 303-8.

30 - MATSUURA S, Kishi F, Kajii T (1990). Characterization of a 5'-flanking region of the human liver/bone/kidney alkaline phosphatase gene: two kinds of mRNA from a single gene. *Biochem Biophys Res Commun* 168: 993-1000.

31 - VAN HOOF VO, Hoylaerts MF, Geryl H, Van Mullem M, Lepoutre LG *et al* (1990). Age and sex distribution of alkaline phosphatase isoenzymes by agarose electrophoresis. *Clin Chem* 36: 875-8.

32 - DOMAR U, Hirano K, Stigbrand T (1991). Serum levels of human alkaline phosphatase isozymes in relation to blood groups. *Clin Chim Acta* 203: 305-14.

33 - FOUCAULT P, Foucalt MH, Kucharewicz B, Bureau F, Alix M *et al* (1991). Bone and total alkaline phosphatase measurements in an osteoporotic population. *Ann Biol clin* 49: 477-81.

34 - KIHN L, Rutkowski D, Nakatsui T, Stinson RA (1991). Properties of amphiphilic and hydrophilic forms of alkaline phosphatase from human liver. *Enzyme* 45: 155-64.

35 - DENG JT, Hoylaerts MF, Van Hoof VO, De Broe ME (1992). Differential release of human intestinal alkaline phosphatase in duodenal fluid and serum. *Clin Chem* 38: 2532-8.

36 - MOSS DW (1992). Perspectives in alkaline phosphatase research. *Clin Chem* 38: 2486-92.

37 - NARISAWA S, Hofmann M-C, Ziomek CA, Millán JL (1992). Embryonic alkaline phosphatase is expressed at M-phase in the spermatogenic lineage of the mouse. *Development* 116: 159-65.

38 - HUI M, Hu M, Tenenbaum HC (1993). Changes in cell adhesion and cell proliferation are associated with expression of tissue non-specific alkaline phosphatase. *Cell Tissue Res* 274: 429-37.

39 - SARROUILHE D, Lalegerie P, Baudry M (1993). Alkaline phosphatase activity at physiological pH: kinetic properties and biological significance. *Cell Mol Biol* 39: 13-9.

40 - MOSS DW (1994). Release of membrane-bound enzymes from cells and the generation of isoforms. *Clin Chim Acta* 226: 131-42.

41 - RAYMOND FD, Moss DW, Fisher D (1994). Separation of alkaline phosphatase isoforms with and without intact glycan-phosphatidylinositol anchors in aqueous polymer phase systems. *Clin Chim Acta* 227: 111-20.

42 - VAN HOOF VO, De Broe ME (1994). Interpretation and clinical significance of alkaline phosphatase isoenzyme patterns. *Crit Rev Clin Lab Sci* 31: 197-293.

43 - WHYTE MP (1994). Hypophosphatasia and the role of alkaline phosphatase in skeletal mineralization. *Endocr Rev* 15: 439-61.

44 - ALPERS DH, Zhang Y, Ahnen DJ (1995). Synthesis and parallel secretion of rat intestinal alkaline phosphatase and a surfactant-like particle protein. *Am J Physiol* 268: E1205-14.

45 - MILLÁN JL, Fishman WH (1995). Biology of human alkaline phosphatases with special reference to cancer. *Crit Rev Clin Lab Sci* 32: 1-39.

46 - RINDI G, Ricci V, Gastaldi G, Patrini C (1995). Intestinal alkaline phosphatase can transphosphorylate thiamin to thiamin monophosphate during intestinal transport in the rat. *Arch Physiol Biochem* 103: 33-8.

47 - WHYTE MP, Landt M, Ryan LM, Mulivor RA, Henthorn PS *et al* (1995). Alkaline phosphatase: placental and tissue-nonspecific isoenzymes hydrolyze phosphoethanolamine, inorganic pyrophosphate, and pyridoxal-5'-phosphate. Substrate accumulation in carriers of hypophosphatasia corrects during pregnancy. *J Clin Invest* 95: 1440-5.

48 - DENG JT, Hoylaerts MF, Nouwen EJ, De Broe ME, Van Hoof VO (1996). Purification of circulating liver plasma membrane fragments using a monoclonal antileucine aminopeptidase antibody. *Hepatology* 23: 445-54.

49 - WADA H, Niwa N, Hayakawa T, Tsuge H (1996). Changes of serum alkaline phosphatase isoenzymes in fasted rats. *J Nutr Sci Vitaminol* 42: 435-47.

50 - FUKUI K, Hada T, Imanishi H, Liu W, Iwasaki A *et al* (1997). The tumor-derived fetal-intestinal alkaline phosphatase cDNA is identical in sequence to the adult intestinal alkaline phosphatase isozyme gene. *Clin Chim Acta* 265: 57-63.

51 - MOSS DW (1997). Physicochemical and pathophysiological factors in the release of membrane-bound alkaline phosphatase from cells. *Clin Chim Acta* 257: 133-40.

52 - NOSJEAN O, Koyama I, Goseki M, Roux B, Komoda T (1997). Human tissue non-specific alkaline phosphatases: sugar-moiety-induced enzymic and antigenic modulations and genetic aspects. *Biochem J* 321: 297-303.

53 - POELSTRA K, Bakker WW, Klok PA, Hardonk MJ, Meijer DKF (1997). A physiologic function for alkaline phosphatase: endotoxin detoxification. *Lab Invest* 76: 319-27.

54 - POELSTRA K, Bakker WW, Klok PA, Kamps JA, Hardonk MJ *et al* (1997). Dephosphorylation of endotoxin by alkaline phosphatase in vivo. *Am J Pathol* 151: 1163-9.

55 - PONCE A, Peris P, Álvarez L, Muñoz-Gómez J (1997). Significado clínico de un aumento de fosfatasa alcalina. *Rev Clin Esp* 197: 176-9.

56 - VAN HOOF VO, Deng JT, De Broe ME (1997). How do plasma membranes reach the circulation? *Clin Chim Acta* 266: 23-31.

57 - ZIMMERMANN H, Braun N (1999). Ecto-nucleotidases-molecular structures, catalytic properties, and functional roles in the nervous system. *Prog Brain Res* 120: 371-85.

58 - SCHEIBE RJ, Kuehl H, Krautwald S, Meissner JD, Mueller WH (2000). Ecto-alkaline phosphatase activity identified at physiological pH range on intact P19 and HL-60 cells is induced by retinoic acid. *J Cell Biochem* 76: 420-36.

59 - OHKUBO S, Kimura J, Matsuoka I (2000). Ecto-alkaline phosphatase in NG108-15 cells: a key enzyme mediating P1 antagonist-sensitive ATP response. *Br J Pharmacol* 131: 1667-72.

60 - XIE Q, Alpers DH (2000). The two isoenzymes of rat intestinal alkaline phosphatase are products of two distinct genes. *Physiol Genomics* 3: 1-8.

61 - DEPIERRE JW, Karnovsky ML (1974). Ecto-enzymes of the guinea pig polymorphonuclear leukocyte. 1. Evidence for an ecto-adenosine monophosphatase, -adenosine triphosphatase, and -p-nitrophenyl phosphatase. *J Biol Chem* 249: 7111-20.

62 - SAKIYAMA T, Robinson JC, Chou JY (1978). Multiple forms of alkaline phosphatase in untreated and 5-bromo-2'-deoxyuridine-treated choriocarcinoma cells. *Arch Biochem Biophys* 191: 782-91.

63 - YOUNG GP, Friedman S, Yedlin ST, Alpers DH (1981). Effect of fat feeding on intestinal alkaline phosphatase activity in tissue and serum. *Am J Physiol* 241: G461-8.

64 - FISHMAN WH (1987). Clinical and biological significance of an isozyme tumor marker - PLAP. *Clin Biochem* 20: 387-92.

65 - LOW MG (1987). Biochemistry of the glycosyl-phosphatidylinositol membrane protein anchors. *Biochem J* 244: 1-13.

66 - HAWRYLAK K, Stinson RA (1988). The solubilization of tetrameric alkaline phosphatase from human liver and its conversion into various forms by phosphatidylinositol phospholipase C or proteolysis. *J Biol Chem* 263: 14368-73.

67 - MILLÁN JL (1988). Oncodevelopmental expression and structure of alkaline phosphatase genes. *Anticancer Res* 8: 995-1004.

68 - ZACKSON SL, Steinberg MS (1988). A molecular marker for cell guidance information in the axolotl embryo. *Dev Biol* 127: 435-42.

69 - ELIAKIM R, Seetharam S, Tietze CC, Alpers DH (1990). Differential regulation of mRNAs enconding for rat intestinal alkaline phosphatase. *Am J Physiol* 259: G93-8.

70 - FISHMAN WH (1990). Alkaline phosphatase isoenzymes: recent progress. *Clin Biochem* 23: 99-104.

71 - MANES T, Glade K, Ziomek CA, Millán JL (1990). Genomic structure and comparison of mouse tissue-specific alkaline phosphatase genes. *Genomics* 8: 541-54.

72 - ELIAKIM R, Mahmood A, Alpers DH (1991). Rat intestinal alkaline phosphatase secretion into lumen and serum is coordinately regulated. *Biochim Biophys Acta* 1091: 1-8.

73 - PANTEGHINI M (1991). Benign inherited hyperphosphatasemia of intestinal origin: report of two cases and a brief review of the literature. *Clin Chem* 37: 1449-52.

74 - WATANABE T, Wada N, Kim EE, Wyckoff HW, Chou JY (1991). Mutation of a single amino acid converts germ cell alkaline phosphatase to placental alkaline phosphatase. *J Biol Chem* 266: 21174-8.

75 - DAY AP, Feher MD, Chopra R, Mayne PD (1992).Triglyceride fatty acid chain length influences the post prandial rise in serum intestinal alkaline phosphatase activity. *Ann Clin Biochem* 29: 287-91.

76 - TIETZE CC, Becich MJ, Engle M, Stenson WF, Mahmood A *et al* (1992). Caco-2 cell transfection by rat intestinal alkaline phosphatase cDNA increases surfactant-like particles. *Am J Physiol* 263: G756-66.

77 - BUBLITZ R, Armesto J, Hoffmann-Blume E, Schulze M, Rhode H *et al* (1993). Heterogeneity of glycosylphosphatidylinositol-anchored alkaline phosphatase of calf intestine. *Eur J Biochem* 217: 199-207.

78 - ENGLE MJ, Mahmood A, Alpers DH (1995). Two rat intestinal alkaline phosphatase isoforms with different carboxyl-terminal peptides are both membrane-bound by a glycan phosphatidylinositol linkage. *J Biol Chem* 270: 11935-40.

79 - SOUVANNAVONG V, Lemaire C, De Nay D, Brown S, Adam A (1995). Expression of alkaline phosphatase by a B-cell hybridoma and its modulation during cell growth and apoptosis. *Immunol Lett* 47: 163-70.

80 - ANAGNOSTOU F, Plas C, Forest N (1996). Ecto-alkaline phosphatase considered as levamisole-sensitive phosphohydrolase at physiological pH range during mineralization in cultured fetal calvaria cells. *J Cell Biochem* 60: 484-94.

81 - ANAGNOSTOU F, Plas C, Nefussi J-R, Forest N (1996). Role of β-GP-derived Pi in mineralization via ecto-alkaline phosphatase in cultured fetal calvaria cells. *J Cell Biochem* 62: 262-74.

82 - SOUVANNAVONG V, Lemaire C, Brown S, Adam A (1997). UV irradiation of a B-cell hybridoma increases expression of alkaline phosphatase: involvement in apoptosis. *Biochem Cell Biol* 75: 783-8.

83 - MCCARTHY AD, Cortizo AM, Segura GG, Bruzzone L, Etcheverry SB (1998). Non-enzymatic glycosylation of alkaline phosphatase alters its biological properties. *Mol Cell Biochem* 181: 63-9.

84 - GOLOVASTOV VV, Mikhaleva NI, Kadyrova LY, Nesmeyanova MA (2000). The major phospholipid of Escherichia Coli, phosphatidylethanolamine, is required for efficient production and secretion of alkaline phosphatase. *Biochemistry (Mosc)* 65: 1097-104.

85 - MUELLER WH, Kleefeld D, Khattab B, Meissner JD, Scheibe RJ (2000). Effects of retinoic acid on N-glycosylation and mRNA stability of the liver/bone/kidney alkaline phosphatase in neuronal cells. *J Cell Physiol* 182: 50-61.

86 - HIPÓLITO-REIS C, Pereira-Leite L (1973). As actividades fosfatásicas alcalinas em obstetrícia. *Jornal do médico* LXXXII: 1065-72.

87 - HIPÓLITO-REIS C, Cardoso V, Justiniano A (1974). Actividades fosfatásicas alcalinas séricas em patologia vascular hepáticas. *Rev Bras Pesq Med Biol* 7: 539-47.

88 - LEE LMY, Kenny MA (1975). Electrophoretic method for assessing the normal and pathological distribution of alkaline phosphatase isoenzymes in serum. *Clin Chem* 21: 1128-35.

89 - VINET B, Zizian L, Gauthier B (1978). Characteristics of the inhibition of serum alkaline phosphatase by theophylline. *Clin Biochem* 11: 57-61.

90 - FARLEY JR, Ivey JL, Baylink DJ (1980). Human skeletal alkaline phosphatase. Kinetic studies including pH dependence and inhibition by theophylline. *J Biol Chem* 255: 4680-6.

91 - MOSS DW, Edwards RK (1984). Improved electrophoretic resolution of bone and liver alkaline phosphatases resulting from partial digestion with neuraminidase. *Clin Chim Acta* 143: 177-82.

92 - ROSALKI SB, Foo AY (1984). Two new methods for separating and quantifying bone and liver alkaline phosphatase isoenzymes in plasma. *Clin Chem* 30: 1182-6.

93 - BURCH WM, Hamner G, Wuthier RE (1985). Phosphotyrosine and phosphoprotein phosphatase activity of alkaline phosphatase in mineralizing cartilage. *Metabolism* 34: 169-75.

94 - MOSS DW, Whitaker KB (1985). Modification of alkaline phosphatases by treatment with glycosidases. *Enzyme* 34: 212-6.

95 - ROSALKI SB, Foo AY (1985). Incubation with neuraminidase and affinity electrophoresis with wheat-germ lectin compared for separating and quantifying alkaline phosphatase isoenzymes in plasma. *Clin Chem* 31: 1198-200.

96 - SUSSMAN NL, Seetharam S, Blaufuss MC, Alpers DH (1986). Translation of rat intestinal RNA yields two alkaline phosphatases. Biochem J 234: 563-8.

97 - AMACHER DE, Higgins CV, Schomaker SJ, Clay RJ (1989). Further charaterization of serum alkaline phosphatase from male and female beagle dogs. *Enzyme* 42: 1-7.

98 - MIURA S, Yoshioka M, Hamada Y, Morita A, Asakura H *et al* (1989). Alkaline phosphatase of rat intestinal lymph: its characterization and the effect of fat administration. *Clin Chim Acta* 186: 239-48.

99 - WALLINDER H, Angbäck K, Kahan J, Onica D, Waldenlind L (1989). Measurement of alkaline phosphatase activity by Kodak Ektachem 700 XR analyzer compared with other routine analytical methods. *Clin Chem* 35: 328-9.

100 - BENTOUATI L, Baboli MS, Hachem H, Hamza M, Canal P *et al* (1990). Hyperphosphatasemia related to three intestinal alkaline phosphatase isoforms: biochemical study. *Clin Chim Acta* 193: 93-102.

101 - HUMMER C, Millán JL (1991). Gly^{429} is the major determinant of uncompetitive inhibition of human germ cell alkaline phosphatase by L-leucine. *Biochem J* 274: 91-5.

102 - GRIFFITHS WC, Camara PD, Rosner M, Lev R, Brooks EM (1992). Prevalence and properties of the intestinal alkaline phosphatase identified in serum by cellulose acetate electrophoresis. *Clin Chem* 38: 507-11.

103 - BECQ F, Fanjul M, Merten M, Figarella C, Hollande E *et al* (1993). Possible regulation of CFTR-chloride channels by membrane-bound phosphatases in pancreatic duct cells. *FEBS* 327: 337-42.

104 - BECQ F, Jensen TJ, Chang X-B, Savoia A, Rommens JM *et al* (1994). Phosphatase inhibitors activate normal and defective CFTR chloride channels. *Proc Natl Acad Sci USA* 91: 9160-4.

105 - HOFFMANN WE, Everds N, Pignatello M, Solter PF (1994). Automated and semiautomated analysis of rat alkaline phosphatase isoenzymes. *Toxicol Pathol* 22: 633-8.

106 - HUI M-Z, Tenebaum HC, McCulloch CAG (1997). Collagen phagocytosis and apoptosis are induced by high level alkaline phosphatase expression in rat fibroblasts. *J Cell Physiol* 172: 323-33.

107 - ILLEK B, Yankaskas JR, Machen TE (1997). cAMP and genistein stimulate HCO_3^- conductance through CFTR in human airway epithelia. *Am J Physiol* 272: L752-61.

108 - CALHAU C, Martel F, Hipólito-Reis C, Azevedo I (2000). Effect of P-glycoprotein modulators on alkaline phosphatase activity in cultured rat hepatocytes. *Cell Physiol Biochem* 10: 195-202.

109 - CALHAU C, Martel F, Hipólito-Reis C, Azevedo I (2000). Differences between duodenal and jejunal rat alkaline phosphatase. Clin Biochem 33: 571-577.

110 - CALHAU C, Martel F, Hipólito-Reis C, Azevedo I (2001). Regulation of intestinal absorption of organic cationic drugs in Caco-2 cells by an ecto-alkaline phosphatase activity. *Artigo submetido.*

111 - CALHAU C, Martel F, Soares-da-Silva P, Hipólito-Reis C, Azevedo I (2001). Modulation of ^{3}H-MPP^{+} uptake in RBE4 cells by an ecto-phosphatase activity. *Faseb J*, 15: A130.

112 - CALHAU C, Martel F, Soares-da-Silva P, Hipólito-Reis C, Azevedo I (2001). Regulation of of ^{3}H-MPP^{+} transport by phosphorylation/dephosphorylation pathways in RBE4 cells: role of ecto-alkaline phosphatase. *Artigo submetido.*

113 - CALHAU C, Martel F, Hipólito-Reis C, Azevedo I (2001). Effect of thiamine on ^{3}H-MPP^{+} uptake by Caco-2 cells. *Artigo submetido.*

114 - CALHAU C, Martel F, Pinheiro-Silva S, Pinheiro H, Soares-da-Silva P, Hipólito-Reis C, Azevedo I (2001). Modulation of insulin transport in rat brain microvessel endothelial cells by an ecto-alkaline phosphatase. *In press.*

115 - MARTINS MJ, Negrão MR, Hipólito-Reis C, Azevedo I (2001). Arginine and a polyarginine peptide inhibit alkaline phosphatase activity: possible consequences for cellular transport systems. *Clin Biochem* 34: 435-7.

116 - MARTEL F, Martins MJ, Hipólito-Reis C, Azevedo I (1996). Inward transport of [^{3}H]-1-methyl-4-phenylpyridinium in rat isolated hepatocytes: putative involvement of a P-glycoprotein transporter. *Br J Pharmacol* 119: 1519-1524.

117 - MARTEL F, Calhau C, Hipólito-Reis C (1998). Effect of bile duct obstruction on hepatic uptake of 1-methyl-4-phenylpyridinium in the rat. *Pharmacol Res* 37: 497-504.

118 - BAYER PM, Hotschek H, Knoth E (1980). Intestinal alkaline phosphatase and the ABO blood group system – a new aspect. *Clin Chim Acta* 108: 81-7.

119 - GOLDSTEIN DJ, Harris H (1981). Mammalian brain alkaline phosphatase: expression of liver/bone/kidney locus. Comparison of fetal and adult activities. *J Neurochem* 36: 53-7.

120 - KOMODA T, Koyama I, Nagata A, Sakagishi Y, DeSchryver-Kecskemeti K *et al* (1986). Ontogenic and phylogenic studies of intestinal, hepatic, and placental alkaline phosphatases. Evidence that intestinal alkaline phosphatase is a late evolutionary development. *Gastroenterology* 91: 277-86.

121 - SEETHARAM S, Ovitt C, Strauss AW, Rubin D, Alpers DH (1987). Fat feeding stimulates only one of the two mRNAs encoding rat intestinal membranous and secreted alkaline phosphatase. *Biochem Biophys Res Commun* 145: 363-8.

122 - WEISS MJ, Ray K, Henthorn PS, Lamb B, Kadesh T *et al* (1988). Structure of the human liver/bone/kidney alkaline phosphatase gene. *J Biol Chem* 263: 12002-10.

123 - DICTUS-VERMEULEN C, Gueuning C, Graff GL (1989). Alkaline phosphatase activities in normal and denervated, slow and fast rat muscles. Bearing on the possible participation of the enzyme in the transmembrane transport of phosphate ions. *Arch Int Physiol Biochim* 97: 13-23.

124 - KILEDJIAN M, Kadesch T (1990). Analysis of the human liver/bone/kidney alkaline phosphatase promoter in vivo and in vitro. *Nucleic Acids Res* 18: 957-61.

125 - TERAO M, Studer M, Gianni M, Garattini E (1990). Isolation and characterization of the mouse liver/bone/kidney-type alkaline phosphatase gene. *Biochem J* 268: 641-8.

126 - TOH Y, Yamamoto M, Endo H, Misumi Y, Ikehara Y (1990). Duplicate leader exons in the mouse liver-type alkaline phosphatase gene. *Biochem Int* 22: 213-8.

127 - ZERNIK J, Thied MA, Twarog K, Stover ML, Rodan GA *et al* (1990).Cloning and analysis of the 5'region of the rat bone/liver/kidney/placenta alkaline phosphatase gene. A dual-function promoter. *Matrix* 10: 38-47.

128 - YEH K-y, Yeh M, Holt PR, Alpers DH (1994). Development and hormonal modulation of postnatal expression of intestinal alkaline phosphatase mRNA species and their encoded isoenzymes. *Biochem J* 301: 893-9.

129 - OTTO VI, Fried R, Wiederkehr F, Hänseler E (1995). Separation of the two most closely related isoenzymes of alkaline phosphatase by two-dimensional electrophoresis. *Electrophoresis* 16: 1284-8.

130 - SHINOZAKI T, Watanabe H, Arita S, Chigira M (1995). Amino acid phosphatase activity of alkaline phosphatase. A role of protein phosphatase. *Eur J Biochem* 227: 367-71.

131 - VAN BELLE H (1976). Alkaline phosphatase. II. Conditions affecting determination of total activity in serum. *Clin Chem* 22: 977-81.

132 - VAN HOOF VO, Van Mullem M, De Broe ME, Lepoutre LG (1993). Comparison of two commercially available systems for the electrophoretic separation of alkaline phosphatase isoenzymes. *J Chromatogr* 646: 235-43.

133 - HODSON AW, Latner AL, Raine L (1962). Iso-enzymes of alkaline phosphatase. *Clin Chim Acta* 7: 255-261.

134 - HAIJE WG, De Jong M (1963). Iso-enzyme patterns of serum alkaline phosphatase in agar-gel electrophoresis and their clinical significance. *Clin Chim Acta* 8: 620-3.

135 - FISHMAN WH, Inglis NI, Stolbach LL, Krant MJ (1968). A serum alkaline phosphatase isoenzyme of human neoplastic cell origin. *Cancer Res* 28: 150-4.

136 - FISHMAN WH, Inglis NR, Green S, Anstiss CL, Gosh NK *et al* (1968). Immunology and biochemistry of Regan isoenzyme of alkaline phosphatase in human cancer. Nature (London) 219: 697-9.

137 - NAKAYAMA T, Yoshida M, Kitamura M (1970). L-Leucine sensitive, heat-stable alkaline phosphatase isoenzyme detected in a patient with pleuritis carcinomatosa. *Clin Chim Acta* 30: 546-8.

138 - HIGASHINO K, Hashinotsume M, Kang KY, Takahashi Y, Yamamura Y (1972). Studies on a variant alkaline phosphatase in sera of patients with hepatocellular carcinoma. *Clin Chim Acta* 40: 67-81.

139 - HIGASHINO K, Kudo S, Ohtani R, Yamamura Y (1976). Further observation of Kasahara isoenzyme in patients with malignant diseases. *Gann* 67: 909-11.

140 - HOFMANN MC, Jeltsch W, Brecher J, Walt H (1989). Alkaline phosphatase isozymes in human testicular germ cell tumors, their precancerous stage, and three related cell lines. *Cancer Res* 49: 4696-700.

141 - VAN HOOF VO, Van Campenhout CM, De Broe ME, Lepoutre LG (1990). Variations in measured alkaline phosphatase activity: influence of isoenzymes and buffer systems. *Clin Chem* 36: 2012-3.

142 - WEI JS, Shih CL, Chung NC, Wang J-Y (1990). Quantitative determination of high molecular weight alkaline phosphatase in patients with colorectal cancer by polyacrylamide gel electrophoresis. *Enzyme* 43: 188-91.

143 - MURTHY VV, Malikin G, Karmen A (1991). Agarose gel electrophoresis of alkaline phosphatase isoenzymes with wheat germ lectin and fluorogenic substrate. *Clin Chem* 37: 1309-10.

144 - HOFMANN MC, Millán JL (1993). Developmental expression of alkaline phosphatase genes; reexpression in germ cell tumours and in vitro immortalized germ cells. *Eur Urol* 23: 38-45.

145 - FUKUI K, Hada T, Imanishi H, Iwasaki A, Hirano K *et al* (1996). A fetal intestinal-type alkaline phosphatase produced in Caco-2 cells. *Tumour Biol* 17: 320-4.

146 - BROYLES DL, Nielsen RG, Bussett EM, Lu WD, Mizrahi IA *et al* (1998). Analytical and clinical performance characteristics of Tandem-MP Ostase, a new immunoassay for serum bone alkaline phosphatase. *Clin Chem* 44: 2139-47.

147 - WOLFF JM, Ittel TH, Borchers H, Boekels O, Jakse G (1999). Metastatic workup of patients with prostate cancer employing alkaline phosphatase and skeletal alkaline phosphatase. *Anticancer Res* 19: 2653-5.

148 - DEMERS LM, Costa L, Lipton A (2000). Biochemical markers and skeletal metastases. *Cancer* 88: 2919-26.

149 - JOHNSON RB Jr (1969). A new fluorometric method for the estimation or detection of total and fractionated alkaline phosphatase. *Clin Chem* 15:108-23.

150 - WHITAKER KB, Eckland D, Hodgson HJ, Saverymuttu S, Williams G *et al* (1982). A variant alkaline phosphatase in renal cell carcinoma. *Clin Chem* 28: 374-7.

151 - MOSS DW (1988). Elimination of enzymes from the circulation. *Adv Clin Enzymol* 6: 102-10.

152 - VAN HOOF VO, Lepoutre LG, Hoylaerts MF, Chevigné R, De Broe ME (1988). Improved agarose electrophoretic method for separating alkaline phosphatase isoenzymes in serum. *Clin Chem* 34: 1857-62.

153 - FRANZINI C, Cattozzo G, Ceriotti F, Ferrero CA (1991). Measurement of alkaline phosphatase activity in serum with N-methyl-D-glucamine as a buffer: evaluation of the method for routine use. *Eur J Clin Chem Clin Biochem* 29: 759-65.

154 - MAHMOOD A, Yamagishi F, Eliakim R, DeSchryver-Kecskemeti K, Gramlich TL *et al* (1994). A possible role for rat intestinal surfactant-like particles in transepithelial triacylglycerol transport. *J Clin Invest* 93: 70-80.

155 - ZHANG Y, Shao J-S, Xie Q-M, Alpers DH (1996). Immunolocalization of alkaline phosphatase and surfactant-like particle proteins in rat duodenum during fat absorption. *Gastroenterology* 110: 478-88.

156 - OGNIBENE A, Pala L, Messeri G, Rotella CM, Berti P (1997). Relations between intestinal alkaline phosphatase activity and insulin secretion in obese patients. *Clin Chem* 43: 1672-3.

157 - BLOM E, Ali MM, Mortensen B, Huseby N-E (1998). Elimination of alkaline phosphatases from circulation by the galactose receptor. Different isoforms are cleared at various rates. *Clin Chim Acta* 270: 125-37.

158 - MORNET E (2000). Hypophosphatasia: the mutations in the tissue-nonspecific alkaline phosphatase gene. *Hum Mutat* 15: 309-15.

159 - SELIGMAN AM, Chauncey HH, Nachlas MM, Manheimer LH, Ravin HA (1951). The colorimetric determination of phosphatases in human serum. *J Biol Chem* 190: 7-15.

160 - KIND PRN, King EJ (1954). Estimation of plasma phosphatase by determination of hydrolysed phenol with amino-antipyrine. *J Clin Path* 7: 322-6.

161 - MORTON RK (1957). The kinetics of hydrolysis of phenyl phosphate by alkaline phosphatases. *Biochem J* 65: 674-82.

162 - BABSON AL, Read PA (1959). A new assay for prostatic acid phosphatase in serum. *Amer J Clin Path* 32: 88-91.

163 - ROMEL WC, LaMancusa SJ, Dufrene JK (1968). Detection of serum alkaline phosphatase isoenzymes with phenolphthalein monophosphate following cellulose acetate electrophoresis. *Clin Chem* 14: 47-57.

164 - DEMETRIOU JA, Beattie JM (1971). Electrophoretic separation on agarose thin film of isoenzymes of alkaline phosphatase from human serum and tissue. *Clin Chem* 17: 290-5.

165 - STEVENS J, Thomas F (1972). Alkaline phosphatase: reaction rate analysis at 340 nm. *Clin Chim Acta* 37: 541-3.

166 - HAIJE WG (1973). Influence of buffer conditions on the activities of some iso-enzymes of alkaline phosphatase in the serum. *Clin Chim Acta* 48: 23-6.

167 - HEALY PJ (1974). Quantitative analysis of serum alkaline phosphatase isoenzyme activity. *Clin Chim Acta* 55: 407-9.

168 - LAMB EJ, Browne M, John WG, Price CP (1998). Alkaline phosphatase activity measurement in the UK by AMP-buffered methods: an appraisal of current practice. *Ann Clin Biochem* 35: 120-7.

169 - CALHAU C, Hipólito-Reis C, Azevedo I (1999). Alkaline phosphatase and exchange surfaces. *Clin Biochem* 32: 153-4.

170 - MOSS DW (1997). Enzyme standardization – the way forward? *Ann Clin Biochem* 34: 13-6.

171 - GLOMP I, Schafer D, Hess B (1985). Cytochemical localization of alkaline phosphatase in the cell membrane of Dictyostelium discoideum amebae. *Histochemistry* 83: 251-5.

172 - WEISS MJ, Cole DE, Ray K, Whyte MP, Lafferty MA *et al* (1989). First identification of a gene defect for hypophosphatasia: evidence that alkaline phosphatase acts in skeletal mineralization. *Connect Tissue Res* 21: 99-106.

173 - HUI M, Tenenbaum HC (1998). New face of an old enzyme: alkaline phosphatase may contribute to human tissue aging by inducing tissue hardening and calcification. *Anat Rec* 253: 91-4.

174 - FEDDE KN, Blair L, Silverstein J, Coburn SP, Ryan LM *et al* (1999). Alkaline phosphatase knock-out mice recapitulate the metabolic and skeletal defects of infantile hypophosphatasia. *J Bone Miner Res* 14: 2015-26.

175 - DEHGHANI H, Narisawa S, Millán JL, Hahnel AC (2000). Effects of disruption of the embryonic alkaline phosphatase gene on preimplantation development of the mouse. *Dev Dyn* 217: 440-8.

176 - MORRIS DC, Masuhara K, Takaoka K, Ono K, Anderson HC (1992). Immunolocalization of alkaline phosphatase in osteoblasts and matrix vesicles of human fetal bone. *Bone Miner* 19: 287-98.

177 - COLEMAN R (1987). Bile salts and biliary lipids. *Biochem Soc Trans* 15: 68S-80S.

178 - ABUKAWA D, Nakagawa M, Iinuma K, Nio M, Ohi R *et al* (1998). Hepatic and serum bile acid compositions in patients with biliary atresia: a microanalysis using gas chromatography-mass spectrometry with negative ion chemical ionization detection. *Tohoku J Exp Med* 185: 227-37.

179 - MARTEL F, Martins MJ, Calhau C, Hipólito-Reis C, Azevedo I (1998). Postnatal development of organic cation transport in the rat liver. *Pharmacol Res* 37: 131-6.

180 - FORD JM, Hait WN (1990). Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* 42: 155-99.

181 - ELBLING L, Berger W, Rehberger A, Waldhör T, Micksche M (1993). P-Glycoprotein regulates chemosensititivity in early developmental stages of the mouse. *Faseb J* 7: 1499-1506.

182 - CHIELI E, Romiti N, Cervelli F, Tongiani R (1995). Effects of flavonols on P-glycoprotein activity in cultured rat hepatocytes. *Life Sci* 57: 1741-51.

183 - HOOIJBERG JH, Broxterman HJ, Heijn M, Fles DLA, Lankelma J *et al* (1997). Modulation by (iso)flavonoids of the ATPase activity of the multidrug resistance protein. *FEBS Lett* 413: 344-8.

184 - SHAROM FJ (1997). The p-glycoprotein efflux pump: how does it transport drugs? *J Membrane Biol* 160: 161-75.

185 - ZIMMERLI B, Valantinas J, Meier PJ (1989). Multispecificity of Na^+-dependent taurocholate uptake in basolateral (sinusoidal) rat liver plasma membrane vesicles. *J Pharmacol Exp Ther* 250: 301-8.

186 - KIM RB, Leake B, Cvetkovic M, Roden MM, Nadeau J *et al* (1999). Modulation by drugs of human hepatic sodium-dependent bile acid transporter (sodium taurocholate cotransporting polypeptide) activity. *J Pharmacol Exp Ther* 291: 1204-9.

187 - ROTHBARD JB, Garlington S, Lin Q, Kirschberg T, Kreider E *et al* (2000). Conjugation of arginine oligomers to cyclosporin A facilitates topical delivery and inhibition of inflammation. *Nat Med* 6: 1253-7.

188 - TABCHARANI JA, Chang X-B, Riordan JR, Hanrahan JW (1991). Phosphorylation-regulated Cl^- channel in CHO cells stably expressing the cystic fibrosis gene. *Nature* 352: 628-31.

189 - PICCIOTTO MR, Cohn JA, Bertuzzi G, Greengard P, Nairn AC (1992). Phosphorylation of the cystic fibrosis transmembrane conductance regulator. *J Biol Chem* 267: 12742-52.

190 - CHANG X-B, Tabcharani JA, Hou Y-X, Jensen TJ, Kartner N *et al* (1993). Protein kinase A (PKA) still activates CFTR chloride channel after mutagenesis of all 10 PKA consensus phosphorylation sites. *J Biol Chem* 268: 11304-11.

191 - HWANG TC, Horie M, Gadsby DC (1993). Functionally distinct phospho-forms underlie incremental activation of protein kinase-regulated Cl^- conductance in mammalian heart. *J Gen Physiol* 101: 629-50.

192 - RAMAN S, Shorten S, Yurko-Mauro K, Dam A, Reenstra WW (1996). Peptide mapping of *in vivo* phosphorylated CFTR. *FASEB J* 10: A92.

193 - WANG F, Zeltwanger S, Yang IC, Nairn AC, Hwang TC (1998). Actions of genistein on cystic fibrosis transmembrane conductance regulator channel gating. Evidence for two binding sites with opposite effects. *J Gen Physiol* 111: 477-90.

194 - MAKISHIMA M, Okamoto AY, Repa JJ, Tu H, Learned RM *et al* (1999). Identification of a nuclear receptor for bile acids. *Science* 284: 1362-5.

195 - PARKS DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG *et al* (1999). Bile acids: natural ligands for an orphan nuclear receptor. *Science* 284: 1365-8.

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