Biochimica et Biophysica Acta, 1106 (1992) 311–316

BBAMEM 75635

Uptake of tyramine cellobiose by rat liver

Zhi-Duan Zhong, Michel Jadot, Simone Wattiaux-De Coninck and Robert Wattiaux

Laboratoire de Chimie Physiologique, Facultés Universitaires Notre-Dame de la Paix, Namur (Belgium)

(Received 30 December 1991)

Key words: Acidotropism; Lysosome; Tyramine cellobiose; (Rat liver)

The uptake of ^{125}I-tyramine cellobiose (TC) by isolated rat hepatocytes and by total rat liver is markedly higher than that of ^{14}C-sucrose and ^{125}I-PVP, suggesting that TC does not enter the cells by fluid phase endocytosis. The distribution of radioactivity after differential centrifugation shows that the compound is shared out amongst sedimentable structures and unsedimentable fraction. Analysis by isopycnic centrifugation indicates that quickly after its penetration into the cells, most of sedimentable ^{125}I-TC is associated with lysosomes. Such an intracellular localization is confirmed by the distributions observed after free flow electrophoresis and by the fact that radioactivity and cathepsin C, a lysosomal hydrolase, are simultaneously released from a mitochondrial fraction treated with glycyl-L-phenylalanine-2-naphthylamide. Pretreatment of the rats with chloroquine, an acidotropic drug that accumulates in lysosomes, prevents to some extent the entry of ^{125}I-TC into these organelles. Experiments performed with purified lysosomes show that ^{14}C-sucrose does not cross the lysosomal membrane when ^{125}I-TC accumulates linearly with time in the fractions. These results are explained by supposing that the linkage of tyramine to cellobiose allow the disaccharide to diffuse through the plasma and the lysosome membranes, and that the accumulation of the molecule in these organelles results from its weak basic properties. ^{125}I-TC could be an interesting molecule with which to study acidotropism in the whole animal and in isolated and cultured cells.

Introduction

Some lysosomotropic substances, such as aliphatic amine molecules, can cross the lysosome membrane and accumulate within these organelles as a result of the low pH of the lysosomal matrix [1]. These molecules, often referred to as acidotropic, are also able to accumulate in other acidic subcellular structures such as endosomes. In general, the acidotropic properties of a compound is inferred from morphological observations on culture cells, indicating that it induces a swelling of lysosomes (vacuolation) resulting from the osmotic imbalance caused by its accumulation in these organelles. Obviously, such a morphologic approach gives only qualitative information on the penetration of the substance into lysosomes, it does not allow one to study the kinetics of the process, the intracellular distribution of the molecule, its concentration in lysosomes, etc. On the other hand, biochemical investigations that could bring such information, particularly on the whole animal, are scarce for two reasons. (1) Many acidotropic molecules such as alphatic amines that could be used as probes, are metabolized to a large extent before entering lysosomes and therefore detection in these structures is difficult. (2) Some substances that could be particularly suitable, for example chloroquine, are not readily available in a radioactive form; therefore to be detected in subcellular fractions by less sensitive methods they must be injected in high quantities. Such high amount can perturb the cells where the molecule accumulates and even have general toxic effects.

We were aware that tyramine-cellobiose (TC) frequently used as a label for protein, in endocytosis studies [2] could be a convenient molecule to study acidotropism. Indeed, it is a relatively hydrophobic secondary amine with a pK'_1 of 9.8, is not metabolized and can be labelled with ^{125}I. In the work presented here, we have investigated the uptake of ^{125}I-TC by rat liver and its intracellular distribution. The results indicate that TC accumulates in lysosomes, probably after diffusion through the plasma and lysosomal membranes, and behaves like an acidotropic compound.

Materials and Methods

Experiments were performed with male Wistar rats weighting about 200 g. ^{125}I-TC was injected intravenously with an increasing amount of time before

Correspondence: R. Wattiaux, Laboratoire de Chimie Physiologique, Facultés Universitaires Notre-Dame de la Paix, 61, rue de Bruxelles, B-5000 Namur, Belgium.

killing. Rat livers perfused with cold 0.9% NaCl, were removed and homogenized in ice-cold 0.25 M sucrose. Differential centrifugation was carried out according to De Duve et al. [3]. A nuclear fraction N, a heavy mitochondrial fraction M, a light mitochondrial fraction L, a microsomal fraction P and an unsedimentable fraction S were isolated. Isopycnic centrifugation was achieved according to Beaufay et al. [4]. Free flow electrophoresis was performed with an Elphor Vap 22 apparatus (Bender & Hobein, München, Germany), according to the procedure described by Stahn et al. [5] with slight modifications. Briefly, L fraction was prepared and the pellet resuspended with cold TEA (10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, adjusted to pH 7.4 with NaOH)-0.25 M sucrose. The granule preparation was then centrifuged for 5 min at 1500 rpm in order to remove the small amount of aggregated material. The supernatant was introduced (1.5 ml/h) into the separation chamber eluated with the same medium. The electrode buffer was composed of 100 mM triethanolamine, 100 mM acetic acid, 0.25 M sucrose, brought to pH 7.4 with NaOH. The electrophoresis conditions were: 5°C, 117 mamps (±1150 volts), 2 ml/h per fraction. 90 fractions were collected and analysed. Rat hepatocytes were isolated according to the procedure of Seglen [6] except that both Ca^{2+} free and collagenase perfusion were carried out at 34°C for 6 min. Purified lysosomes were prepared by the method of Wattiaux and Wattiaux-De Coninck [7]. Cathepsin C was assayed according to Jadot et al. [8] and proteins according to Bradford et al. [9]. The method of Hysing et al. [10] was used to iodinate tyramine-cellobiose (TC).

TC was synthesized according to the method of Pittman et al. [11]. ^{125}I-Tyramine and ^{14}C-sucrose were obtained from Amersham (UK); collagenase types I and IV from Sigma (St Louis, MO).

Results

Uptake of ^{125}I-TC by isolated hepatocytes and by liver

Disaccharides such as cellobiose and sucrose do not diffuse through the plasma membrane but can enter the cell by fluid phase endocytosis. To see if this is also the case for TC, we compared its uptake by isolated hepatocytes with that of sucrose. Sucrose was selected because, like cellobiose, it is not metabolized. Moreover ^{14}C-sucrose is commercially available while radioactive cellobiose is not. As shown by Fig. 1(A), the amount of TC taken up by hepatocytes is markedly higher than that of sucrose, suggesting that TC enters the cells by a mechanism other than fluid phase endocytosis.

This is confirmed by observations made on the whole liver, reported in Fig. 1 (B). In these experiments the marker for fluid phase endocytosis was ^{125}I-PVP, since

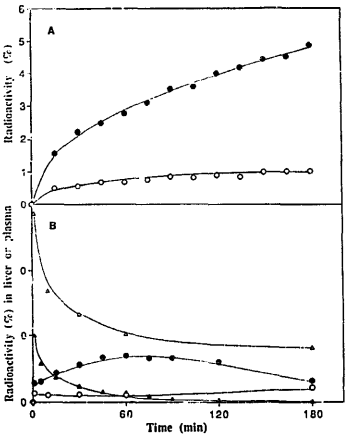

Fig. 1. Uptake of ^{125}I-TC and ^{14}C-sucrose by isolated rat hepatocytes (A) ^{125}I-TC and ^{125}I-PVP by rat liver (B). (A) Freshly isolated rat hepatocytes were incubated with ^{125}I-TC (660 nM) or ^{14}C-sucrose (300 nM) at 37°C in PRMI 1640 medium. At indicated time, 100 µl mixture containing $2.7 \cdot 10^5$ cells were sampled and laid in a Eppendorf microtube, on the surface of 150 µl of oil (a mixture of dibutyl phthalate and dioctyl phthalate of 1.20 g/ml density), and centrifuged in an Eppendorf centrifuge for 30 s. After that cell's pellet was cut off, the pellet was counted in a γ-counter (^{125}I-TC) or (^{14}C-sucrose) added to 0.5 ml boiling SDS solution (0.1%) in scintillation counting vial; after 5 min, 4 ml liquid-scintillation cocktail (Aqualuma Lumac, Landgraaf, Netherlands) was added. Counting was performed in a Beckman β counter. ●, ^{125}I-TC; ○, ^{14}C-sucrose. (B) Radioactivity was measured in rat liver and plasma at increasing times after injection of ^{125}I-TC (0.4 µg/100 g body wt.) or ^{125}I-PVP (0.5 µg/100 g body wt.). Total value in the plasma was estimated by supposing it to represent 3% of animal mass. ^{125}I-TC in liver (●) and plasma (▲); ^{125}I-PVP in liver (○) and plasma (△). Results are given as percentages of the amount added to the culture (A) or injected to the rat (B).

sucrose is quickly eliminated by the kidney after injection. Obviously, the amount of TC taken up by the liver greatly exceeds the amount of PVP, although the net plasma concentration of PVP remains higher than that of TC at any time after injection. The liver concentration of TC reaches a maximum value 30 to 45 min after injection and remains constant for at least 2 h.

Distribution after differential and isopycnic centrifugation

Livers were fractionated by differential centrifugation according to de Duve et al. [3]. The distributions of radioactivity in the fractions at increasing times after

TC injection are given in Table I. At any time, the radioactivity is shared out between sedimentable structures and unsedimentable fraction. The proportion of sedimentable radioactivity associated with the mitochondrial fractions (M + L), where most of the lysosomes are recovered, is high. Even 1 min after injection it reaches about 50%. Analysis of M + L fractions by isopycnic centrifugation in a sucrose gradient shows (Fig. 2) that the radioactivity distribution is similar to that of cathepsin C, a lysosome marker. Moreover, when a specific perturbant of lysosomes, Triton WR 1339 [12] is injected into rats 4 days before TC administration, most of the TC-loaded structures are shifted towards low density regions like cathepsin C. Thus as early as 1 min after injection, sedimentable structures containing ^{125}I-TC behave like lysosomes.

Distribution after free flow electrophoresis

Distributions after free flow electrophoresis are in agreement with the centrifugation results. Fig. 3 shows the distributions of radioactivity and cathepsin C after free flow electrophoresis of an L fraction isolated at 1

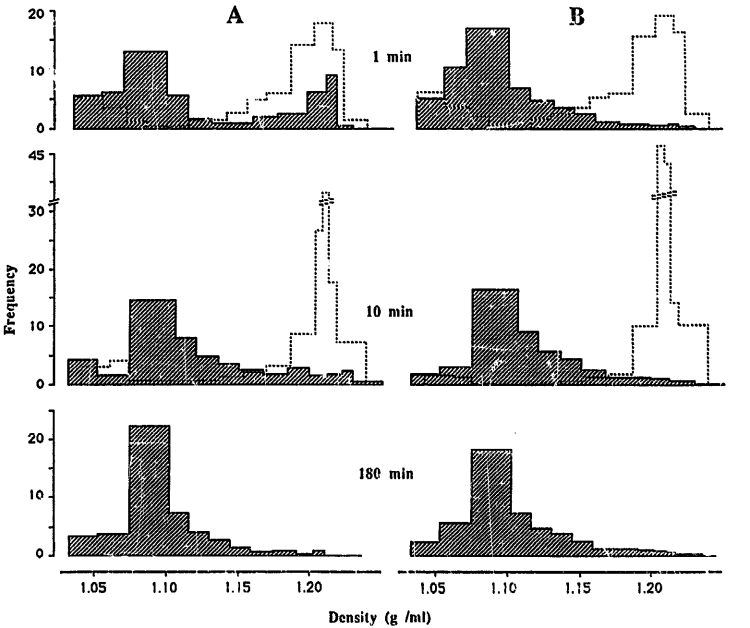

Fig. 2. Density distribution histograms of radioactivity and cathepsin C after isopycnic centrifugation of a total mitochondrial fraction in a sucrose gradient. Experiments were performed with normal rats (□) or rats preinjected with Triton WR 1339 (□, 170 mg in 1 ml saline/rat) i.v. 4 days before killing [12]. Total mitochondrial (M + L) fraction isolated 1, 10 and 180 min after injection of ^{125}I-TC was laid on a sucrose gradient extending from 1.09 g/ml to 1.26 g/ml. Centrifugations were performed at 39000 rev./min in the SW 65 Spinco rotor. The time integral of the square angular velocity was 144 rad^2/ns. In ordinate: frequency i.e. $Q/\Sigma Q \cdot \Delta l$ where Q represents the activity found in the fraction, ΣQ, the total recovered activity and Δl the increment of density from top to bottom of the fraction. (A) Radioactivity; (B) cathepsin C.

TABLE I

Distribution of radioactivity after differential centrifugation according to the procedure of De Duve et al. [3]

Distributions of radioactivity in rat livers were obtained at increasing times after injection of ^{125}I-TC into the animals. N, nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, final supernatent.

Fractions	Radioactivity (% of total); time (min) after injection				
	1	10	30	60	180
N	10.7	11.7	4.3	7.1	5.7
M	16.1	17.4	17.7	29.5	25.6
L	4.8	6.2	17.4	15.1	18.0
P	11.2	12.2	13.1	9.3	7.5
S	57.0	52.6	47.6	39.0	43.2

and 5 min after ^{125}I-TC injection. In both cases, the radioactivity distribution coincides with that of the lysosomal enzyme.

Effect of glycyl-L-phenylalanine-2-naphthylamide (GPN)

As shown by Jadot et al. [8], when lysosomes are incubated with GPN, the membrane is disrupted and their content released into the medium. That results from the penetration of GPN into lysosomes and its hydrolysis by cathepsin C. Therefore, a convenient method to see if a compound is located within lysosomes is to determine if it can be released by GPN from the structures it is associated with. Mitochondrial fractions isolated 1 min, 5 min and 10 min after ^{125}I-TC injection were incubated with GPN and the release of radioactivity and of cathepsin C was measured. Results

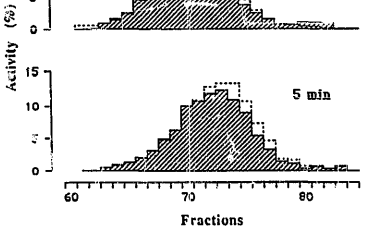

Fig. 3. Distribution of radioactivity and cathepsin C after free flow electrophoresis of L fraction. Light mitochondrial (L) fraction was isolated 1 min or 5 min after injection of ^{125}I-TC, and subjected to free flow electrophoresis. Ordinate: percentage recovered in fractions. ■, radioactivity; ○, cathepsin C.

TABLE II

Effect of glycyl-L-phenylalanine-2-naphthylamide

Mitochondrial fractions, originating from livers of rats killed 1 min and 10 min after injection of ^{125}I-TC, were incubated at 37°C for 10 min in the absence or the presence of 0.25 mM glycyl-L-phenylalanine-2-naphthylamide in a medium containing 0.05 M acetate buffer pH 5, 0.25 M sucrose, 5 mM NaCl. After that, the mixture was chilled by adding 2 ml of ice-cold 0.25 M sucrose, then centrifuged at 25000 rev./min for 25 min in a Spinco 40.3 rotor. The cathepsin C and radioactivity released in supernatant were measured. Values are given as percentages of the amounts released under the same conditions in the presence of 0.05% Triton X-100.

Time (min) after injection	Activity released (% of total)			
	control		treated	
	cathepsin C	radioactivity	cathepsin C	radioactivity
1	0	18.4	98.9	91.5
10	0	24.9	80.0	106.0

presented in Table II demonstrate that radioactivity and the lysosomal hydrolase are released to the same extent even 1 min after injection.

Effect of chloroquine administration

Chloroquine is an acidotropic drug that accumulates in lysosomes to a very large extent and is able to increase the lysosomal pH [1]. We were interested to see if a pretreatment of rats with chloroquine could affect the distribution of ^{125}I-TC in the liver. Rats received chloroquine (50 μg/g of body weight) 50 min before injection of ^{125}I-TC and were killed at increasing times after injection. There was no significant dif-

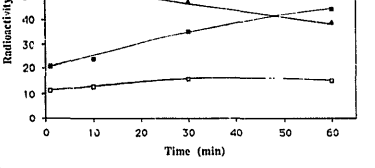

Fig. 4. Distribution of radioactivity and cathepsin C after differential centrifugation of a chloroquine pretreated rat liver. Chloroquine (50 μg/g of body weight) was injected i.p. 50 min before injecting ^{125}I-TC. ^{125}I-TC was given at increasing times before killing. The livers were fractionated and radioactivity of the different fractions measured. The percentages recovered in total mitochondrial fractions (M + L) and in the S fraction as a function of the amount found in the homogenate are given in the figure. ^{125}I-TC in normal liver M + L (■) and S (▲) fractions or in chloroquine treated liver M + L (□) and S (△) fractions.

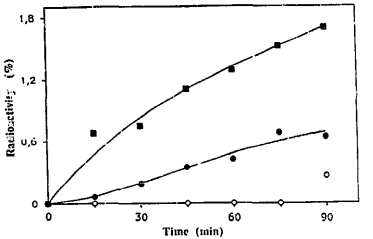

Fig. 5. Accumulation of ^{125}I-TC, ^{14}C-sucrose in isolated lysosomes. Purified lysosomes (300 μg) were incubated at 37°C in a medium containing 0.25 M sucrose and 10 mM Hepes (pH 7.4) in the presence of ^{125}I-TC (660 nM) or ^{14}C-tyramine (6.6 μM) or ^{14}C-sucrose (300 nM). At indicated time, 100 μl mixture were sampled and immediately filtered through a Whatman GF/C glass filter (24 mm diameter) after being diluted by 1 ml ice-cold PBS buffer (pH 7.4). Filter was rapidly washed twice with 2 ml ice-cold PBS, placed in a scintillation counting vial containing 4 ml liquid-scintillation cocktail. Radioactivity was counted in a Beckman β counter. Results are given as percentages of the amount added to the medium. ■, ^{14}C-tyramine; ●, ^{125}I-TC; ○, ^{14}C-sucrose

ference between the amount of ^{125}I-TC taken up by liver for chloroquine pretreated and for normal rats (not shown). However, as indicated in Fig. 4. the intracellular distribution of radioactivity is modified by chloroquine. In normal rats, the radioactivity associated with the mitochondrial fractions (M + L) increases with time when that present in the S fraction decreases. In chloroquine treated animals, the radioactivity recovered in M + L is significantly lower than in the normal rats and does not increase with time. Thus chloroquine pretreatment prevents the entry of ^{125}I-TC into lysosomes to some extent.

Uptake of ^{14}C-sucrose, ^{14}C-tyramine and ^{125}I-TC by isolated lysosomes

To see whether ^{125}I-TC can freely enter lysosomes, these organelles were purified in a metrizamide gradient [7] and incubated with ^{125}I-TC or with ^{14}C-sucrose. As expected, ^{14}C-sucrose does not cross the lysosomal membrane when ^{125}I-TC linearly accumulates with time in the lysosomes (Fig. 5). We found (not shown) that ^{125}I-TC uptake is not inhibited by high concentration of cellobiose, tyramine and unlabelled TC. The important fact is that tyramine is readily taken up by isolated lysosomes (Fig. 5). TC and unlabelled tyramine do not affect the kinetics of the process (not shown).

Discussion

The rate of TC uptake by hepatocytes and by the whole liver is several times higher than that of sucrose and PVP. As the three substances can not be metabolized, such a difference must result from a difference in the rate of penetration of these molecules into the cells. Sucrose and PVP enter the cells by fluid phase endocytosis, thus another process is involved in the case of TC uptake.

Two possibilities have to be considered: TC is either taken up by adsorptive endocytosis or it enters the cells by diffusion. Distributions observed after centrifugation are in favour of the second mechanism. At any time after differential centrifugation, TC is distributed amongst sedimentable and soluble fractions. Isopycnic centrifugation, particularly after Triton WR 1339 treatment and the effect of GPN make it clear that the organelles TC is associated with are lysosomes. Accordingly, as early as 1 min after injection most of TC is located in lysosomes and in cytosol which is not consistant with uptake by endocytosis. Therefore TC probably enters these cells by diffusion and after reaching the cytosol, accumulates in lysosomes. If we suppose that these organelles and the cytosol occupy, respectively, 1% and 25% of the total volume of the liver [13], when 45% of TC is located in lysosomes (after 180 min), its concentration in these structures is more than 20-times higher than that in cytosol. On the other hand, we have shown that TC but not sucrose is able to enter isolated lysosomes. The more plausible explanation is that TC can diffuse through the lysosomal membrane and remains trapped in the organelle matrix like an acidotropic molecule. The effect of chloroquine strengthens this hypothesis. Indeed, when the drug is injected into animal before giving TC, it does not significantly inhibit the uptake of that compound by the liver, but prevents to a large extent its accumulation in lysosomes. Such a distribution is to be expected if TC accumulates in lysosomes as a result of the low pH prevailing in these particles, since chloroquine is a lysosomotropic weak base [1] and the increase of lysosomal pH it can induce will lower the concentration of TC which can be achieved inside lysosomes.

One can not exclude the possibility that TC may also accumulate in acidic compartments other than lysosomes. However, if it is the case, such an accumulation involves only a minor proportion of TC. For example, as shown by Table I, after 60 or 180 min about 80% of the sedimentable radioactivity is recoved in M and L fractions. At this time, analysis of these fractions by isopycnic centrifugation clearly shows that TC is associated with lysosomes. It is particularly obvious when experiments were performed with rats injected with Triton WR 1339, a specific lysosome density perturbant.

Apparently adding a tyramine moiety to cellobiose allows the disaccharide to cross the plasma membrane and the lysosome membrane. We have recently found

that tyramine enters hepatocytes by facilitated and by free diffusion (unpublished results). TC does not inhibit tyramine diffusion (unpublished results) and therefore does not use the tyramine carrier to enter hepatocytes. On the other hand, the results reported here indicate that ^{14}C-tyramine probably crosses the lysosome membrane by free diffusion since no inhibition is observed by unlabelled tyramine. The same is true for TC. These observations suggest that the difference between cellobiose and TC, with respect to their capacity to step across the plasma and the lysosome membrane, results from the hydrophobicity gained by the disaccharide when it is linked to tyramine, in spite of the increase in molecular size caused by such an addition. In fact we have found that the partition coefficient of TC between octanol and water, although low (0.0206), is 11-times higher than that of sucrose and close to that of tyramine (0.033).

TC resulting from intralysosomal hydrolysis of endocytosed proteins labelled with that substance remains inside these organelles for a relatively long time [2,10]. This is not inconsistent with our discoveries that TC can cross lysosomal membrane. On the contrary, we show that after entering lysosomes, TC accumulates suggesting that it can not easily diffuse from inside to outside these particles, probably because it is more protonated.

From a practical point of view, owing to the facts that it can be labelled with ^{125}I and that it is not metabolized, TC could be an interesting molecule to study acidotropism in the whole animal and in isolated and cultured cells.

Acknowledgements

This work was supported by the Fonds de la Recherche Scientifique, the Fonds de la Recherche Scientifique Medicale (contract No. 452391) and the ASBL Air Escargot.

References

1 De Duve, C., De Barsy, T., Poole, B., Trouet, A., Tulkens, P. and Van Hoof, F. (1974) Biochem. Pharm. 20, 2847–2860.
2 Misquith, S., Wattiaux-De Coninck, S. and Wattiaux, R. (1988) Eur. J. Biochem. 174, 691–697.
3 De Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) Biochem. J. 60, 604–617.
4 Beaufay, H., Jacques, P., Baudhuin, P., Sellinger, O.Z., Berthet, J. and De Duve, C. (1964) Biochem. J. 92, 184–205.
5 Stalin, R., Maier, K.P. and Hannig, K. (1970) J. Cell Biol. 46, 576–591.
6 Seglen, P.O. (1976) Methods Cell Biol. 13, 29–83.
7 Wattiaux, R. and Wattiaux-De Coninck, S. (1983) in Iodinated Density Gradient Medium. A practical approach (Rickwood, D., ed.), 119–137, I.R.L. Press, Oxford.
8 Jadot, M., Colmant, C., Wattiaux-De Coninck, S. and Wattiaux, R. (1984) Biochem. J. 219, 965–970.
9 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
10 Hysing, J. and Tolleshaug, H. (1986) Biochim. Biophys. Acta 887, 42–50.
11 Pittman, R.C., Carew, J.E., Glass, C.K., Green, S.R., Tayol, C.A. and Attie, A.D. (1983) Biochem. J. 212, 791–800.
12 Wattiaux, R., Wibo, M. and Baudhuin, P. (1963) Ciba Found. Symp., Lysosomes, pp. 176–200.
13 Wattiaux, R., Wattiaux-De Coninck, S., Ronveaux-Dupal, M.F. and Dubois, F. (1978) J. Cell Biol. 78, 349–365.