BACTERIAL CONTAMINATION AS A SOURCE OF ERROR IN D-GLUCOSE-BINDING STUDIES USING INTESTINAL BRUSH BORDER MEMBRANE PREPARATIONS

Sixto GARCÍA-CASTIÑEIRAS, Ramon TORRES-PINEDO and Francisco ALVARADO

Department of Physiology and Biophysics, University of Puerto Rico Medical School, San Juan, Puerto Rico 00936

Received 24 November 1972

1. Introduction

Considerable advances have been made in the last few years concerning the mechanism of membrane transport at the phenomenological level, but an understanding of this mechanism in molecular terms is still lacking. Substrate binding proteins have been isolated from bacteria and attempts have been made to demonstrate their role in the initial (recognition) step of transport, but with limited success [1]. Similar attempts have been made using tissues from mammals, particularly the small intestine, from which particles have been purified that, it is claimed, specifically bind D-glucose (for review see [2]). This binding has been identified as representing either the initial step in sugar transport [3] or "a different property of the intestine", nevertheless related somehow to the sugar transport mechanism [4]. The present paper deals with the so-called D-glucose-binding capabilities of guinea pig small intestinal brush borders and fractions thereof, and specifically refers to the possibility that the methods thus far used in these studies do not ascertain how much of the "binding" actually represents glucose metabolism by bacteria contaminating either the extracts of some of the reagents used to study it. A preliminary account of this work has appeared [5].

2. Methods and materials

The entire jejunum and ileum of non-fasted guinea pigs killed by a blow on the head was removed, everted, cut into 2–5 cm pieces, washed in 0.2% KI for 5 min and then with saline for 10 min with the aid of a magnetic stirrer. After this preliminary washing, brush borders were extracted by stirring the tissues for 1 hr at room temp. in 5 mM EDTA pH 7.2, containing 500 mg/ℓ ampicillin. Under these conditions, 80 to 100% of the total content of sucrose (used here as a marker for brush border material) was extracted in a form that could be sedimented, together with brush borders recognizable by light and electron microscopy, at low-speed centrifugation (about 1 000 $g \times 10$ min). After washing three times by resuspension in EDTA and recentrifugation, the brush borders were fragmented by homogenization in a Waring blender (3 min at top speed); 9 ml of the resulting preparation were placed on top of a 26-ml discontinuous Ficoll gradient (40%, 4 ml; 35%, 4 ml; 30%, 3 ml; 20%, 3 ml; 15%, 3 ml; 10%, 5 ml; and 5%, 4 ml; the Ficoll was dissolved in a mixture of 5 mM EDTA and 25 mM sodium phosphate, pH 7.2) and centrifuged for 1 hr at 25 000 rpm (about 80 000 g at the midpoint of the tubes, rotor SW 30 of the Beckman L3-50 ultracentrifuge).

After centrifugation, 12 to 14 fractions were separated by aspiration, the Ficoll was removed by sedimentation of particulate material at 25 000 rpm × 60 min and resuspension in fresh buffer or 5 mM EDTA, and the final suspension was analyzed for protein [6], phospholipid [7], total carbohydrate [8], sucrase [9] and D-glucose binding (see later). The fractions were also studied by electron microscopy*

* Thanks are due to Dr. F. González-Aguilar of this Department and to Miss Ulla Jarfors for their help in making these electron micrographs.

(the results of which will be published elsewhere). Bacteria did not appear to contaminate any of these preparations to any significant extent, although bacterial forms could occasionally be observed. It should be mentioned that bacterial contamination of our material (as revealed by colony counting in agar) was considerable before introducing ampicillin in the brush border extraction procedure. Also, contamination of the brush borders with eggs from parasites was initially observed: to avoid this, we now always wash the tissue in 0.2% KI for at least 5 min prior to the 10-min washing of the tissues in saline (see above).

Evolution of CO_2 from [^{14}C]glucose was determined by incubating the appropriate membrane fraction in a stoppered Warburg flask for 2 hr at 37°, with shaking. The reaction was stopped by mixing with 0.1 ml of 4 N HCl located in the side arm, and shaking continued for 2 more hr. CO_2 absorbed into a KOH-impregnated piece of filter paper, located in the inner well, was determined using a modified Bray's mixture [10]. Transport was determined by the tissue-accumulation method, as previously described [11].

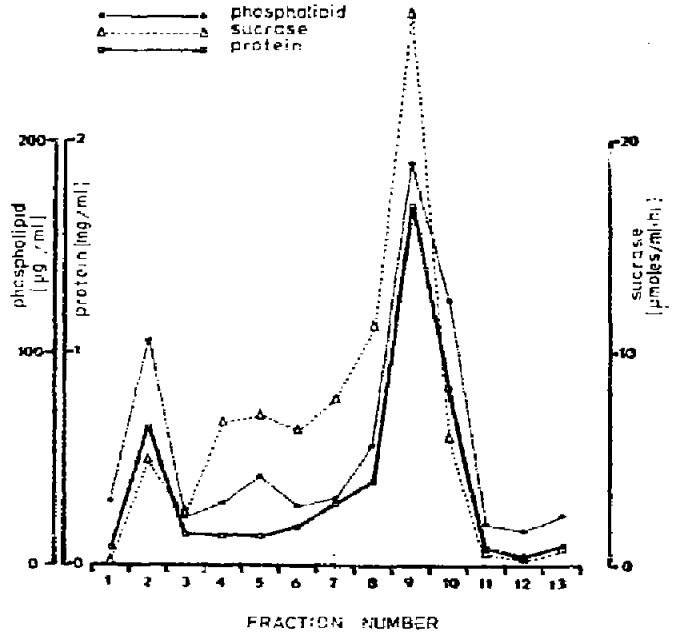

Fig. 1. Density-gradient fractionation of fragmented brush borders. The points represent the mean of 10–12 separate experiments. The peaks correspond to the fractions (2, 5 and 9) mentioned in the text.

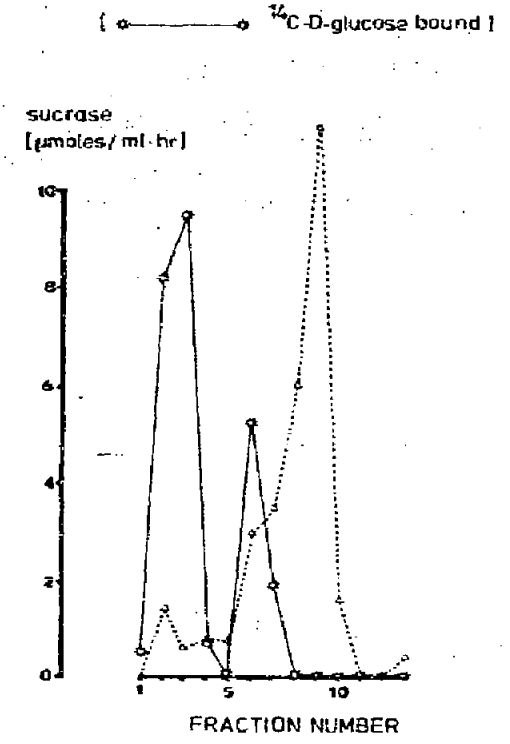

Fig. 2. Typical pattern of sucrase and D-glucose "binding" activities after density-gradient fractionation of fragmented brush borders. Binding results in arbitrary units.

3. Results and discussion

As shown in fig. 1, three main sucrase peaks are obtained, which we call fractions 2, 5 and 9. The peaks shown refer to absolute sucrase activity. If we refer to the protein content of the fractions (specific activity), the purest fraction is no. 8, located at the 10% Ficoll layer, a fraction revealed by electron microscopy to be composed of a very pure population of membrane vesicles, presumably derived from the microvilli: it might be considered equivalent to the fraction "C" of Eichholz et al. [12, 13]. Our fraction 2, with a high absolute amount of sucrase at low specific activity, may to some extent correspond to the "D" fraction of Eichholz since it includes the heaviest components of the original preparation. It discloses unbroken brush borders interspersed with other uncharacterized material.

Using the above fractions, D-glucose binding was studied by incubating aliquots in the presence of

Table 1

Effect of sugars and analogues on the "binding" of, and evolution of CO_2 from, D glucose, by a preparation (fraction no. 2, see text) of fragmented brush borders.

Inhibitor	Our results		Binding results, literature			
	CO_2 production	"Binding"	Faust et al. [3]		Parsons [15]	Eichholz et al. [4]
Concentration (mM)	5.35	5.35	1	10	0.1	0.05
Glucose	96	100	100	–	100	99
Mannose	93	89	–	–	92	90
2-Deoxyglucose	–	–	–	–	75	93
Glucosamine	77	98	93.9	–	66	–
Xylose	80	72	18.8	46.7	–	–
3-O-Methylglucose	75	87	45.5	91.7	18	93
Galactose	72	79	1.7	46.8	28	32
Fructose	45	34	–	–	4	–
Galactosamine	33	41	(9.8)	–	–	–
Mannitol	20	11	–	–	–	6

Our results are directly compared with those published by others (only data on the inhibition of binding are available). All results are expressed as per cent inhibitions, the reference (0% inhibition) being a control incubated in parallel, without added sugar or analogue. A figure in parenthesis indicates stimulation rather than inhibition. A dash indicates sugar not tested. All inhibitors belonged to the D-series.

Table 2

Effect of D-glucose, D-galactose, and their respective 2-deoxy-2-amino derivatives on the transport of methyl-α-D-glucopyranoside by rings of guinea-pig small intestine, *in vitro*.

Effector (30 mM)	Initial velocity	Relative velocity (%)
Mannitol	0.160 ± 0.054	"100"
Glucosamine	0.212 ± 0.028	132.5
Galactosamine	0.191 ± 0.040	119.4
Glucose	0.015 ± 0.003	9.4
Galactose	0.047 ± 0.012	29.4

Incubations were for 5 min in oxygen atmosphere, at 37°, in a mixture of 4.5 ml phosphate buffer containing 1 mM ^{14}C-substrate and 0.5 ml of a 0.3 M solution of the indicated effectors. Results are expressed as initial and relative velocities (V = μmoles substrate accumulated per ml tissue water per minute, corrected for the extracellular space). To calculate the relative velocities, a control in presence of the inert polyalcohol, D-mannitol, was used. Figures are the means of 8 determinations ± S.D.

[^{14}C]D-glucose in a suitable buffer. After incubation, the particles were sedimented by centrifugation at 25 000 rpm. Radioactive glucose that disappeared from the supernatants was in principle assumed to be bound to the particles. As a reference, the levels of tubes incubated under similar conditions, in the presence of excess ^{12}C-D-glucose, were used. According to this criterion, the binding activity of these gradients was localized in discrete bands the most reproducible and quantitatively most important band coinciding with fraction number 2 (see fig. 2). A second peak of binding that appears in the experiment of fig. 2 (around fraction number 6) was not very reproducible.

Using this technique, the binding properties of fraction 2 were investigated and found to be similar to those described by others in brush border fractions [2]. For instance, the binding exhibited a K_m on the order of 10 μmolar and was inhibited by SH reagents and sugar analogues, as will be shown later. But when the binding was studied by equilibrium dialysis, it was observed that [^{14}C]glucose disappeared steadily: further exploration indicated that a large proportion but not all of the radioactivity could be recovered as [^{14}C]CO_2. It became obvious to us that our prepara-

tions were contaminated, probably by bacteria, not necessarily derived only from the intestine, but also perhaps from the reagents used which, following common practice, had not been sterilized.

Table 1 shows the specificity of the "binding" activity of our preparations, as evidenced by the inhibition produced on it by the presence of sugars and analogues. The results are directly compared with the effects observed on the production of CO_2 (first column); and also with the results of "binding" inhibition published by others. Although there are small differences in detail, the parallelism is striking.

Particularly interesting is the strong inhibition by mannose, 2-deoxy-glucose and glucosamine, all of them good hexokinase substrates, but none of them having any appreciable affinity for the sugar carrier. Although Faust et al. [3] claim that D-glucosamine is a powerful inhibitor of intestinal sugar transport, our results (table 2), in support of earlier observations [14], clearly demonstrate that neitner glucosamine nor galactosamine has any significant effect on sugar transport. In his 1969 study, Parsons [15] called attention to the similarity in specificity between the so-called "binding" activity of brush borders and the enzyme, hexokinase. In the light of our observations, it can be argued that the "binding" reflects glucose metabolism to CO_2 and non-volatile products, the rate-limiting step in the overall reaction being perhaps at the level of the microbial hexokinase, thus explaining the hexokinase-like specificity of the "binding". Other workers [3, 4] claim that their preparations are free from hexokinase contamination. However, a very small amount of bacterial contamination could also explain their results, the hexokinase activity present in such a small mass of bacteria being practically undetectable by ordinary methods. Nevertheless, it should be considered that other factors are probably also involved, as evidenced by the fact that both galactose and 3-O-methylglucose, both of them poor hexokinase substrates [16], significantly inhibit the "binding".

At this point, a technical comment should be made. In our binding studies, we only used radioactive D-glucose, whereas other workers used a mixture of D-glucose (labelled with either ^{14}C or ^{3}H) and a supposedly inert substrate (labelled with either ^{3}H or ^{14}C) such as D-mannose [3] or L-glucose [4]. They express their results as a difference, apparently to distinguish between specific (preferential) and non-specific "binding". It is our contention that such a direct comparison is not sufficient to demonstrate the "preferential binding" of D-glucose. In the case of L-glucose, if there is bacterial metabolism, it seems obvious that the D-isomer will be preferentially metabolized. In the case of D-mannose, and if the hexokinase reaction were rate-limiting, both glucose and mannose should have similar affinities for the system. However, if D-glucose is present at a concentration 100 times higher (10^{-3} mM glucose versus 10^{-5} mM mannose in the experiments of Faust et al. [3]), it appears likely that D-glucose will be preferentially metabolized. It seems regrettable that Faust and his colleagues have apparently failed to test D-mannose

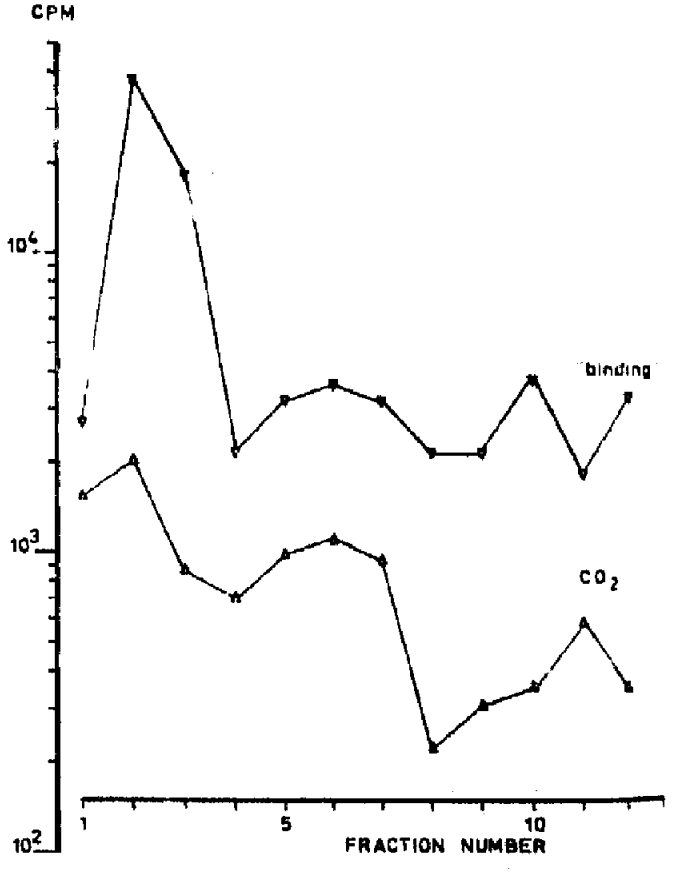

Fig. 3. Pattern of D-glucose "binding" and CO_2 evolution in fractions of a "blind" Ficoll gradient (run in the absence of added brush border material). The Ficoll was removed as described in Methods, after adding to each fraction a constant amount of a heat-denatured protein preparation as carrier.

as a competitor of their D-glucose "binding" (see table 1 in this paper).

Bacterial contamination could in principle also be invoked to explain the glucose binding described in brush border preparations obtained from germ-free rats [4, 17]. In this case, a source of contamination might perhaps be found in some of the unsterilized reagents used. For instance, Ficoll solutions prepared and kept for a few days in the refrigerator, without taking special precautions for sterility, are typically contaminated by bacteria. Ficoll gradients made using this material, run and fractionated as described before, but to which no brush border material had been added, gave a pattern of peaks of glucose "binding" and CO_2 production similar to those described above for brush border material, that is, the bacteria in the Ficoll tend to collect in discrete bands, the main one coinciding with our fraction 2 (fig. 3).

In conclusion, although our experiments do not prove that all prior studies of binding of glucose to brush border fractions can be explained in terms of glucose metabolism by contaminating bacteria, the inference is very strong. Use of non-metabolizable substrates for this type of investigation is strongly recommended.

Acknowledgements

Aided by NIH grants AM-13118; 5 S01 RR 05419 and 5D08 ME 00048. One of us (F.A.) was also supported by Research Career Development Award 5-KO-AM 42383 from the National Institute of Arthritis and Metabolic Diseases, U.S.P.H.S. The skilful technical assistance of Miss Violeta Napoleón is greatly appreciated.

References

[1] H.R. Kaback, Ann. Rev. Biochem. 39 (1970) 561.
[2] A. Eichholz and K.E. Howell, Gastroenterology 62 (1972) 647.
[3] R.G. Faust, M.G. Leadbetter, R.K. Plenge and A.J. McCaslin, J. Gen. Physiol. 52 (1968) 482.
[4] A. Eichholz, K.E. Howell and R.K. Crane, Biochim. Biophys. Acta 193 (1969) 179.
[5] R. Torres-Pinedo, S. Garcia-Castiñeiras and F. Alvarado, Federation Proc. 31 (1972) 239.
[6] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.L. Randall, J. Biol. Chem. 193 (1951) 265.
[7] J. Folch, M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226 (1957) 497.
[8] T.A. Scott and F.H. Melvin, Anal. Chem. 1953 (1956) 25.
[9] A. Dahlqvist, Anal. Biochem. 7 (1964) 18.
[10] G.A. Bray, Anal. Biochem. 7 (1964) 279.
[11] J.W.L. Robinson and F. Alvarado, Pflugers Arch. 326 (1971) 48.
[12] A. Eichholz and R.K. Crane, J. Cell Biol. 26 (1965) 687.
[13] A. Eichholz, Biochim. Biophys. Acta 135 (1967) 475.
[14] R.K. Crane, Physiol. Revs. 40 (1960) 789.
[15] B.J. Parsons, Life Sciences 8 (1969) 939.
[16] A. Sols, Biochim. Biophys. Acta 19 (1956) 144.
[17] W.A. Olsen and L. Rogers, Comp. Biochem. Physiol. 39B (1971) 617.