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Peptide transport in rabbit intestinal brush-border membrane vesicles studied with a potential-sensitive dye *

Vadivel Ganapathy [a], Gerhard Burckhardt [b] and Frederick H. Leibach [a,**]

[a] *Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, GA 30912 (U.S.A.) and* [b] *Max-Planck Institute for Biophysics, Frankfurt am Main (F.R.G.)*

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Peptide transport in purified rabbit intestinal brush-border membrane vesicles has been studied using a potential-sensitive fluorescent dye, di-S-C_3(5). Transport of dipeptides is accompanied by an increase in the fluorescence of the dye in the presence and absence of Na^+, indicating electrogenic, Na^+-independent peptide transport. Dipeptides containing D-amino acids also increase the fluorescence, showing that these peptides too possess significant affinity for the peptide transport system. β-Alanylglycylglycine and prolylglycylglycine, very much like the dipeptides, increase the fluorescence even in the absence of Na^+ which demonstrates the Na^+-independent, electrogenic transport of tripeptides. However, concentrations needed for half-maximal fluorescence changes are higher for tripeptides than for dipeptides suggesting different affinities for the carriers. The studies, in addition, provide evidence for the existence of more than one carrier system for translocation of small peptides in rabbit intestinal brush-border membrane.

Introduction

Active transport of small peptides has been demonstrated in mammalian intestine either in the whole animal or in intact tissue preparations [1]. In the last few years, use of purified intestinal brush-border membrane vesicles has produced results strikingly different from earlier observations with regard to the role of Na^+ in peptide transport [2]. Peptide transport in vesicles is Na^+-independent [3–7], electrogenic [8,9] and is probably driven by an inward proton gradient [9]. The presence of such a proton gradient across the intestinal brush-border membrane in man has been recently demonstrated in vivo [10].

Current studies on peptide transport in vesicles are limited, since the number of commercially available radiolabeled peptides is small. In particular, no labeled tripeptide is available, and thus no information on tripeptide transport in vesicles has been generated. Potential-sensitive fluorescent dyes have been used to study the electrogenic transport of glucose [11–14], amino acids [12–14], and dicarboxylic acids [15] in membrane vesicles using unlabeled substrates. Recently, we have shown that a similar approach can be successfully employed to monitor dipeptide transport in intestinal brush-border membrane vesicles prepared from young rabbits [9]. In the present study, various unlabeled di- and tripeptides have been used to investigate the kinetics, substrate specificity, and

* This is contribution No. 0827 from the Department of Cell and Molecular Biology, Medical College of Georgia.

** To whom correspondence should be addressed.

Abbreviations: FCCP, carbonylcyanide *p*-trifluoromethoxyphenyl hydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino) ethanesulfonic acid; di-S-C_3(5), 3,3'-dipropylthiadicarbocyanine iodide.

multiplicity of intestinal peptide transport in purified brush-border membrane vesicles.

Methods and Materials

Preparation of membrane vesicles

Young rabbits weighing 600–900 g were used throughout this study. After the rabbits were killed by cervical dislocation, the entire small intestine was taken out and flushed thoroughly with Ringer solution. The mucosa was scraped off and brush-border membrane vesicles were prepared from the mucosal scrapings by Mg^{2+}-precipitation as previously described [16]. The final washing of the membranes was done with the preloading buffer which was, unless otherwise indicated, 10 mM Mes/Tris buffer (pH 6.0) containing 75 mM K_2SO_4. The resulting pellet was suspended in the same buffer using a 25 gauge needle and the protein concentration of the suspension was adjusted to 20 mg/ml.

Transport assay

The electrical potential difference across the brush-border membrane of the purified vesicles was measured using the potential-sensitive dye, 3,3'-dipropylthiadicarbocyanine iodide (di-S-$C_3(5)$). The measurements were carried out in a spectrofluorophotometer (Shimadzu, RF-510) with an excitation wavelength of 622 nm and emission wavelength of 669 nm. The instrument was equipped with a red-sensitive photomultiplier, R446U. The temperature of all the solutions used in the measurement (except the membrane vesicles which were kept on ice) as well as that of the sample compartment of the fluorometer was constantly maintained at 37°C. At the start of each measurement, 20 μl of stock dye solution (306 μM in ethanol) was added to 2 ml of transport buffer in disposable polyacryl cuvettes. The transport buffer contained 75 mM of either K_2SO_4 or Na_2SO_4 in 10 mM Mes/Tris buffer (pH 6.0). This pH was employed throughout the study because peptide transport is maximal at pH 5.5–6.0 [8]. The final concentration of the dye was 3 μM. After 1 min, the fluorescence reading was adjusted to 250 arbitrary units and subsequently, 20 μl of vesicles (0.4 mg protein) were added to the cuvette. An additional 3 min were allowed for stabilization of the fluorescence before 250 μl of concentrated stock solution of test substrate was injected through a small opening at the top of the sample compartment. The stock solutions of peptides, amino acids and glucose were prepared in 10 mM Mes/Tris buffer (pH 6.0). The solution in the cuvette was mixed constantly throughout the recording by magnetic stirring. The fluorescence increase due to the addition of test substrates was corrected for the small fluorescence change caused by the injection of 250 μl of the buffer alone.

Materials

The fluorescent dye was obtained from Molecular Probes, Inc. Glycylsarcosine was purchased from Bachem Inc., and β-alanylglycylglycine was from Vega Biochemicals. All other peptides were obtained from Sigma.

Results

Electrogenic peptide transport and its Na^+-independence

The effect of glycyl-L-proline and β-alanylglycylglycine on the fluorescence of di-S-$C_3(5)$ in rabbit intestinal brush-border membrane vesicles is shown in Fig. 1. Addition of glycyl-L-proline and β-alanylglycylglycine transiently increased the fluorescence of the dye when Na^+ was present in the extravesicular medium (lines a). Since the fluorescence of di-S-$C_3(5)$ increases with inside-positive and decreases with inside-negative membrane potentials, the results indicate that the transport of these peptides evokes an inside-positive membrane potential. The fluorescence increase was also observed when K^+ replaced the extravesicular Na^+ (lines b). This is in contrast to D-glucose and L-amino acids which evoke a fluorescence increase of the dye only in the presence of Na^+ [11–14]. The data indicate that the transport of glycyl-L-proline and β-alanylglycylglycine is electrogenic both in the presence and in the absence of Na^+.

In order to exclude any possible non-specific fluorescence changes due to interaction between the peptides and the dye, the effect of these peptides was studied under 'short circuit' conditions. In this experiment, K^+ was present in equimolar concentrations both inside and outside the vesicles and valinomycin was added to the

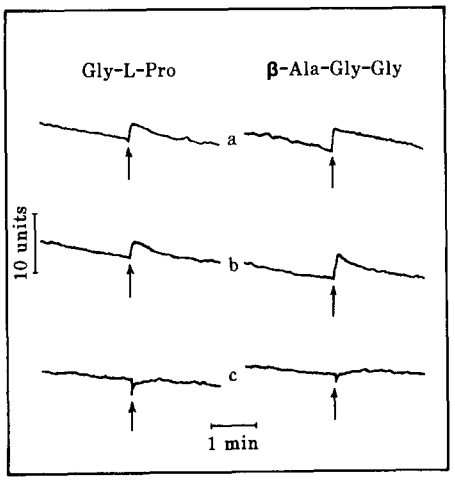

Fig. 1. Fluorescence response to the addition of glycyl-L-proline and β-alanylglycylglycine in rabbit intestinal brush-border membrane vesicles. The vesicles were preloaded with 10 mM Mes/Tris buffer (pH 6.0) containing 75 mM K_2SO_4 and the fluorescence response was monitored in 10 mM Mes/Tris buffer (pH 6.0) containing either 75 mM Na_2SO_4 (lines a) or 75 mM K_2SO_4 (lines b and c). When indicated by arrows, 250 μl of the peptide stock solution (150 mM in 10 mM Mes/Tris buffer (pH 6.0)) was injected into the cuvette. To short-circuit the membrane, valinomycin was added to the vesicles as a stock solution in ethanol 3 min prior to the addition of the peptide (lines c). Final concentration of valinomycin in the cuvette was 62.5 μg/ml and ethanol concentration was less than 1.25%.

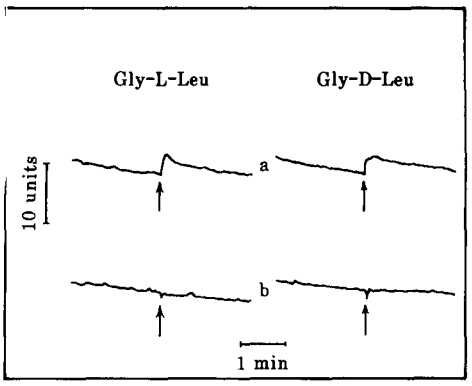

Fig. 2. Fluorescence response to the addition of glycyl-L-leucine and glycyl-D-leucine in rabbit intestinal brush-border membrane vesicles. The vesicles were preloaded with 10 mM Mes/Tris buffer (pH 6.0) containing 75 mM K_2SO_4 and the fluorescence response was monitored in the same buffer. When indicated by arrows, 250 μl of the peptide stock solution (150 mM in 10 mM Mes/Tris buffer (pH 6.0)) was injected into the cuvette. To short-circuit the membrane, valinomycin was added to the vesicles as a stock solution in ethanol 3 min prior to the addition of the peptide. Final concentration of valinomycin in the cuvette was 62.5 μg/ml and ethanol concentration was less than 1.25%. Lines a, fluorescence changes in the absence of valinomycin. Lines b, fluorescence changes in the presence of valinomycin.

incubation mixture before the addition of peptides. Under these conditions, charge transfer by electrogenic peptide transport is immediately compensated by K^+ movement across the membrane and, thus, no change in membrane potential is observed during transport. Fig. 1, lines c, show that, under 'short circuit' conditions, addition of glycyl-L-proline and β-alanylglycylglycine did not affect the fluorescence of the dye, indicating the absence of any non-specific fluorescence change.

Stereospecificity of dipeptide transport

Fig. 2 shows the result of an experiment in which the effect of glycyl-L-leucine and glycyl-D-leucine upon the fluorescence of di-S-C_3(5) was studied. The experiment was performed in the absence of Na^+ so as to exclude any fluorescence change resulting from free amino acids. Addition of glycyl-L-leucine as well as glycyl-D-leucine resulted in a transient inside-positive potential in the brush-border membrane vesicles as monitored by fluorescence increase of the dye (Fig. 2, lines a). The fluorescence change caused by glycyl-D-leucine was slightly smaller than the change caused by glycyl-L-leucine. Both peptides did not produce any change in the fluorescence under 'short circuit' conditions (Fig. 2, lines b), showing that the changes were not due to non-specific interaction between the dye and the peptides. It thus appears that glycyl-D-leucine is also transported intact to a significant extent across rabbit intestinal brush-border membranes.

Effect of peptide concentration on the fluorescence

When the dipeptide glycylsarcosine was added in increasing concentrations to the incubation mixture, there was an increase in the fluorescence change of the dye (Fig. 3). When the data were analyzed by double-reciprocal plot, it was clear that the increase in fluorescence with different concentrations of glycylsarcosine conformed to Michaelis-Menten kinetics (plot not shown). Similar results were obtained with the tripeptide β-

alanylglycylglycine (Fig. 3.) The saturation of the fluorescence increase is not due to the saturation of the dye response to the potential change. This is evident from Fig. 4 where the fluorescence increase is plotted against the K^+-diffusion potential generated by valinomycin. In this experiment, the intravesicular K^+ concentration was 1 mM. The extravesicular K^+ concentration was varied from 1 mM to 16 mM in the presence of valinomycin. Thereby, inside-positive K^+-diffusion potentials are generated (analogous to the potential orientation produced by the peptides). In contrast to peptide-dependent fluorescence increases, no saturation was observed when the fluorescence increase was plotted against log $[K^+]_e/[K^+]_i$. The relationship was linear at least up to 6 units, showing that the saturation kinetics of peptide transport is not due to limitation of the dye response, but rather due to saturation of the transport system.

The kinetic constants, ΔF_{max} (maximal fluorescence increase produced by the peptide) and K_f (peptide concentration producing 0.5 ΔF_{max}) were calculated from double-reciprocal plots of the data obtained in experiments similar to the one illustrated in Fig. 3 for three peptides and two tripeptides (Table I). All five peptides used in this experiment are highly resistant to hydrolysis by brush-border membrane peptidases. Moreover, the experiment was performed in the absence of Na^+ in order to exclude any fluorescence change caused

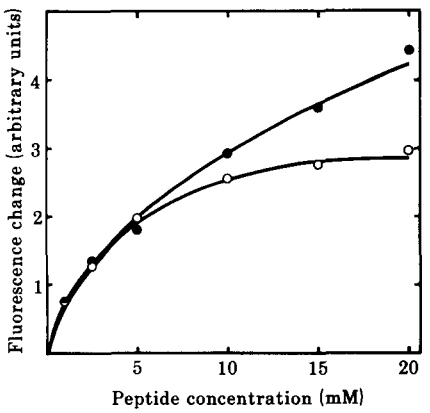

Fig. 3. Effect of peptide concentration on fluorescence increase in rabbit intestinal brush-border membrane vesicles. The experimental conditions are as given in Table I. O——O, glycylsarcosine; ●——●, β-alanylglycylglycine.

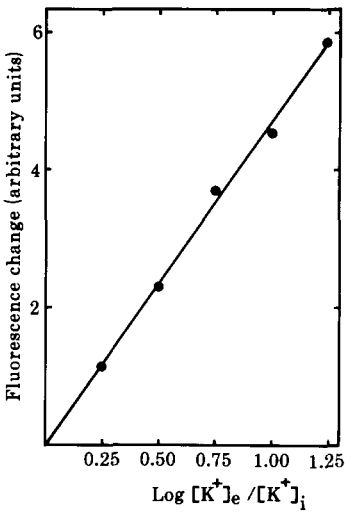

Fig. 4. Relationship between fluorescence increase and valinomycin-induced inside-positive K^+-diffusion potential in rabbit intestinal brush-border membrane vesicles. The vesicles were preloaded with 10 mM Mes/Tris buffer (pH 6.0) containing 0.5 mM K_2SO_4 and 74.5 mM Na_2SO_4 and the fluorescence increase was monitored in the same buffer containing in addition 62.5 μg/ml valinomycin. A constant volume (250 μl) of solutions containing different concentrations of K_2SO_4 in 10 mM Mes/Tris buffer (pH 6.0) (and Na_2SO_4 to maintain the osmolality) was injected into the cuvette. The maximal transient fluorescence increase following the injection of K^+ solutions was corrected for non-transient fluorescence changes due to dilution and is shown as a function of the logarithm of the ratio of extravesicular $[K^+]$ to intravesicular $[K^+]$.

TABLE I

KINETIC CONSTANTS FOR TRANSPORT OF FIVE PEPTIDES IN RABBIT INTESTINAL BRUSH-BORDER MEMBRANE VESICLES

The vesicles were preloaded with 10 mM Mes/Tris buffer (pH 6.0) containing 75 mM K_2SO_4, and transport was assayed in the same buffer. Final concentration of peptides was varied between 1 and 20 mM. K_f and ΔF_{max} were calculated from double-reciprocal plots. The data represent the means ±S.D. from three different membrane preparations.

Peptide	K_f (mM)	ΔF_{max}
Glycyl-L-proline	2.3±0.3	3.1±0.5
L-Prolylglycine	2.8±0.8	2.3±0.6
Glycylsarcosine	3.7±0.5	3.4±0.9
L-Prolylglycylglycine	5.6±1.2	3.6±0.6
β-Alanylglycylglycine	8.0±1.3	5.4±1.6

by free amino acids. Therefore, the data reflect the kinetic constants of intact peptide transport only. The transport of all five peptides exhibited saturation kinetics. The K_f values of tripeptides were higher than those for dipeptides. β-alanylglycylglycine evoked the greatest fluorescence increase among the five peptides studied. Even though there is evidence for participation of more than one transport system in the case of β-alanylglycylglycine (see below), the systems could not be dissected and analyzed individually in the double-reciprocal plot.

The increase in the fluorescence of the dye evoked by saturating concentrations of D-glucose, L-alanine, glycylsarcosine and β-alanylglycylglycine were measured under similar conditions in the presence of Na^+ and the ΔF_{max} values for these solutes were 5.1 ± 0.1, 4.1 ± 0.1, 3.3 ± 0.2 and 5.6 ± 0.2 units, respectively.

Multiplicity of intestinal peptide transport system

The number of dipeptides theoretically possible from protein amino acids is at least 400 and it is much higher for tripeptides. Are all these peptides transported in intestine by a single transport system or are multiple transport systems involved in the process? We have attempted to answer this question by studying additivity of the fluorescence changes caused by two peptides. Two peptides were added at saturating concentrations either separately or jointly to the incubation medium containing the membrane vesicles and the dye, and the magnitude of fluorescence increase was measured in each case.

As shown in Table II, the fluorescence changes for glycyl-L-proline and L-prolylglycine and for glycyl-L-proline and glycylsarcosine were not additive. This suggests that all three dipeptides share a common transport system. However, the fluorescence changes for L-prolylglycine and β-alanylglycylglycine or glycylsarcosine and β-alanylglycylglycine were partially additive, indicating that more than one system is involved in the transport of these peptides.

TABLE II

EVIDENCE FOR MULTIPLICITY OF PEPTIDE TRANSPORT SYSTEM IN RABBIT INTESTINAL BRUSH-BORDER MEMBRANE VESICLES

The experimental conditions were as given in Table I. Final concentration of each peptide was 30 mM. Since, for any given peptide, ΔF varied significantly between membrane preparations, the data represent the results from a single membrane preparation. Each datum is the mean ± S.D. of triplicate values.

Peptide		ΔF
Glycyl-L-proline	A	2.7 ± 0.2
L-Prolylglycine	B	3.1 ± 0.1
Glycylsarcosine	C	2.3 ± 0.2
β-Alanylglycylglycine	D	4.1 ± 0.2
Glycyl-L-proline + L-prolylglycine	A + B	3.4 ± 0.2
L-Prolylglycine + β-alanylglycylglycine	B + D	6.3 ± 0.3
Glycylsarcosine + β-alanylglycylglycine	C + D	4.8 ± 0.2
Glycyl-L-proline + glycylsarcosine	A + C	2.7 ± 0.1

Discussion

We have used the fluorescence response of a potential-sensitive dye on addition of peptides to membrane vesicles as a means of studying intestinal peptide transport. Since the number of commercially available labeled peptides is very limited, the dye technique offers an alternative to characterize peptide transport by the use of unlabeled peptides. In the current study, we have used small intestinal brush-border membrane vesicles from young rabbits because they show larger fluorescence changes than vesicles from older animals. It is well known that transport activities of many dipeptides exhibit a peak at the perinatal period and then decrease slowly with increase in age [17,18]. All the peptides employed in this study transiently increased the fluorescence of the dye, indicating that peptide transport generates a transient inside-positive membrane potential. This fluorescence increase was observed even in the absence of Na^+. When the membranes were short-circuited by valinomycin and potassium, peptides caused no fluorescence change, excluding non-specific interaction with the dye. Our data are in agreement with microelectrode studies on intestinal peptide transport which have shown that peptide transport is accompanied by depolarization of the brush-border membrane [7,19], and

that this depolarization is not significantly affected by Na^+ replacement. Previous studies with vesicles have provided additional evidence that peptide transport is electrogenic both in the presence and in the absence of sodium [8]. Since peptide transport in vesicles is stimulated by an inward proton gradient, it is likely that peptides are cotransported with protons, and the process results in net transfer of positive charge across the membrane.

The fluorescence increase caused by these peptides is not due to free amino acids resulting from hydrolysis of the peptides. Glycyl-L-proline and β-alanylglycylglycine are very resistant to hydrolysis by purified brush-border membrane vesicles from intestine, as well as by intact intestinal tissue preparations [20–22]. Moreover, if the transient inside-positive membrane potential observed in the presence of these peptides were due to their constituent amino acids, the fluorescence changes would have resulted only in the presence of Na^+.

All previous studies have employed only dipeptides to characterize peptide transport in intestinal brush-border membrane vesicles. The dye method has made it possible for the first time to study tripeptide transport in vesicles using unlabeled tripeptides. The results in the present study show that the transport of tripeptides in intestinal brush-border membrane vesicles, as that of dipeptides, is Na^+-independent and electrogenic. However, the tripeptides have a lower affinity for the peptide transport system than the dipeptides.

The K_f values shown in Table I are different from those obtained previously for glycyl-L-proline and glycylsarcosine with tracer techniques [3,9] and this, we believe, is due to the age differences of the rabbits used in these studies. The kinetic constants of intestinal peptide transport greatly vary with the age of the experimental animals [17].

The results in Table II show that there is more than one transport system involved in the translocation of small peptides across the intestinal brush-border membrane. These results agree with recent studies which have suggested the presence of multiple peptide transport systems in mammalian intestine [21,23–26]. Considering the total number of theoretically possible di- and tripeptides which might arise in the lumen of the intestine from protein digestion, it is very unlikely that a single transport system would be capable of recognizing all these peptides so vastly different in structure. Moreover, the existence of multiple peptide transport systems in mammalian intestine would be biologically advantageous because, if there were only one peptide transport system, its absence due to a genetic defect might prove to be fatal to the organism.

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References

1 Matthews, D.M. and Adibi, S.A. (1976) Gastroenterology 71, 151–161
2 Ganapathy, V. and Leibach, F.H. (1982) Life Sci. 30, 2137–2146
3 Ganapathy, V., Mendicino, J. and Leibach, F.H. (1981) J. Biol. Chem. 256, 118–124
4 Ganapathy, V., Mendicino, J.F. and Leibach, F.H. (1981) Life Sci. 29, 2451–2457
5 Berteloot, A., Khan, A.H. and Ramaswamy, K. (1981) Biochim. Biophys. Acta 649, 179–188
6 Berteloot, A., Khan, A.H. and Ramaswamy, K. (1982) Biochim. Biophys. Acta 686, 47–54.
7 Himukai, M., Kameyama, A. and Hoshi, T. (1983) Biochim. Biophys. Acta 732, 659–667
8 Ganapathy, V. and Leibach, F.H. (1983) J. Biol. Chem. 258, 14189–14192
9 Ganapathy, V., Burckhardt, G. and Leibach, F.H. (1984) J. Biol. Chem. 259, 8954–8959
10 Lucas, M. (1983) Gut 24, 734–739
11 Beck, J.C. and Sacktor, B. (1978) J. Biol. Chem. 253, 7158–7162
12 Kragh-Hansen, V., Jorgensen, K.E. and Sheikh, M.I. (2982) Biochem. J. 208, 359–368
13 Burckhardt, G. and Murer, H. (1980) in Advances in Physiological Sciences (Takacs, L., ed.), Vol. 11, pp. 409–418, Pergamon Press, New York
14 Schell, R.E., Stevens, B.R. and Wright, E.M. (1983) J. Physiol. (Lond.) 335, 307–318
15 Wright, S.H., Krasne, S., Kippen, I. and Wright, E.M. (1981) Biochim. Biophys. Acta 640, 767–778
16 Burckhardt, G., Kramer, W., Kurz, G. and Wilson, F.A. (1983) J. Biol. Chem. 258, 3618–3622

17 Himukai, M., Konno, T. and Hoshi, T. (1980) Pediatr. Res. 14, 1272–1275
18 Guandalini, S. and Rubino, A. (1982) Pediatr. Res. 16, 99–103
19 Boyd, C.A.R. and Ward, M.R. (1982) J. Physiol. (Lond.) 324, 411–428
20 Ganapathy, V. and Radharishnan, A.N. (1980) Biochem. Pharmacol. 29, 713–716
21 Rubino, A., Field, M. and Shwachman, A. (1971) J. Biol. Chem. 246, 3542–3548
22 Addison, J.M., Burston, D. and Matthews, D.M. (1974) Clin. Sci. Mol. Med. 46, 5P–6P
23 Lane, A.E., Silk, D.B.A. and Clark, M.L. (1975) J. Physiol. (Lond.) 248, 143–49
24 Ganapathy, V., Hellier, M.D. and Radhakrishnan, A.N. (1979) J. Biosci. 1, 1–12
25 Ganapathy, V. and Radhakrishnan, A.N. (1979) Clin. Sci. 57, 521–527
26 Matthews, D.M. and Burston, D. (1983) Clin. Sci. 65, 177–184