External Ca^{2+} Concentrations Associated with Membrane Alkalinization in Mitochondria*

Britton Chance and Tamiko Yoshioka

ABSTRACT: Electrometric measurements of calcium concentration external to rat liver mitochondria in the absence of permeant anions show three distinct phases of the accumulation reaction. In the first phase, at amounts of calcium less than 20 mμmoles/mg of protein, the external calcium concentration is maintained at an undetectable level, less than 5 μM. Measurements by the bromothymol blue technique indicate that the membrane buffer capacity maintains membrane alkalinization at a low level (<0.1 pH). The H^+/Ca^{2+} value is 1.5–2.0.

In the second phase, at amounts of added calcium of approximately 40 mμmoles/mg of protein, the external calcium concentration reaches 12 μM and the indicator technique indicates half-maximal alkalinization of the membrane (approximately 0.5 pH unit). The H^+/Ca^{2+} value remains high. In the third phase, at amounts of calcium of 80 mμmoles/mg of protein, all of the added calcium appears externally; no more is taken up, and the membrane alkalinization has reached a plateau value of approximately 1 unit. The H^+/Ca^{2+} value now falls toward zero. The effectiveness of phosphate and acetate as permeant anions in calcium accumulation is compared, and at amounts of calcium of 40 mμmoles/mg of protein, both maintain the external calcium levels of less than 5 μM. Under these conditions the ratio of the internal to the external calcium exceeds a value of 10^3.

Previous studies of cation-stimulated respiration show a progressive alkalinization of the mitochondrial membrane in response to sequential additions of calcium in the absence of permeant anions (Chance, 1966; Chance and Mela, 1966a,b; Mela, 1966). Because of the inherent instability of the pH gradient across the mitochondrial membrane, measurements of cation accumulation by conventional methods (centrifugation, filtration, etc.) are less satisfactory than direct measurements. The possibility that Ca^{2+} could be continuously monitored by an electrometric method now seems possible in view of the availability of calcium electrodes from various manufacturers (*Chem. Eng. News*, 1966).

This paper describes the determinations of the extramitochondrial Ca^{2+} concentrations by two types of calcium electrodes. Extramitochondrial and intramitochondrial pH changes caused by the Ca^{2+} accumulation are recorded, respectively, by the glass electrode and by a bound indicator (BTB)[1] which indicates intramitochondrial alkalinity caused by proton ejection in Ca^{2+} accumulation. Under these special conditions of Ca^{2+} accumulation in the absence of a permeant anion, proton ejection is accompanied by an extensive alkalinization of the mitochondrial membrane as indicated by the bound indicator (BTB). This paper describes correlations of the three quantities, external Ca^{2+} concentration, ejection of protons externally to the mitochondria, and the accumulation of hydroxyl ions in the mitochondrial membrane. Such results are of particular interest in evaluating the relationship between a membrane pH gradient and cation accumulation (Chance and Mela, 1966b).

Experimental Methods

Preparations. Rat liver mitochondria were prepared in the presence of 5×10^{-5} M EDTA in order to ensure their being Ca^{2+} deficient; Ca^{2+} concentrations of approximately 5 mμmoles/mg of protein were routinely observed. Exact protein concentrations in the extramitochondrial membrane are indicated in the figure legends; usually 5 mg/ml was employed. Under these conditions, the BTB is tightly bound to the mitochondrial membrane.

In order to obtain optimal operation of the Ca^{2+} electrode, the reaction medium contained a minimal complement of other cations. For example, Tris–chloride is used as a buffer and sodium or potassium succinate as a substrate. In a particular case endogenous substrate was present in sufficient concentrations to allow cation accumulation.

Spectrophotometry. Absorbance changes of BTB were measured by a double-beam spectrophotometer equipped with interference filters transmitting at 618 and 680 mμ. The light beam from this spectrophotometer had a diameter of approximately 5 mm and passed through the bottom of the 5-ml cuvet just below the calcium electrode. The absorbance changes were recorded on an Esterline Angus strip-chart recorder with a response time of approximately 1 sec.

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[1] Abbreviations used: BTB, bromothymol blue (3,3-dibromothymolsulfonphthalein); EGTA, ethylene glycol (2-aminoethyl)-tetracetic acid.

Apparatus. The apparatus for combined multiple electrode and spectrophotometric recording is similar to that described previously (Chance, 1955). The potential of the electrodes was measured by a Radiometer Model 22 pH meter used as a millivoltmeter and connected to a suitable amplifier.

Calcium Activity Electrodes. Although no complete publication is available on these electrodes, a general description of the type available is included in a recent summary (*Chem. Eng. News*, 1966). Some information on the operation of the electrode is obtained from the instruction manual for the Orion Research Corp. Ca^{2+} activity electrode, Model 92-20. The electrode assembly apparently consists of a silver–silver chloride reference electrode in contact with a 1 mM calcium chloride solution. This solution contacts the inside surface of a thin, porous membrane, which itself is in contact with a calcium-specific liquid ion exchanger, a calcium salt of an organophosphoric acid. Thus, the electrode gives zero potential with millimolar calcium on the outside and responds to differences of the activity of the external and internal calcium solutions with a rather typical Nernst slope of 30 mv/decade. The ion exchanger itself is said to provide a background level of about 5 μM calcium. However, the leakage rate seems to be sufficiently low that we have not observed any stimulation of mitochondrial respiration due to this source of calcium. Thus, increments of calcium above this level are readily detectable.

The Corning electrode[2] differs from the Orion electrode in that the $CaCl_2$ reference solution is not used. The reference is apparently the calcium content of the ion-exchange material itself. The porous membrane is of sintered glass or a replaceable cellophane film in the prototype electrodes.

Calibration of the Electrode. The plots of Figures 2 and 4 include an electrode calibration, namely a profile of added Ca^{2+} concentration *vs.* change of electrode potential, measured in the reaction medium but in the absence of mitochondria. At low Ca^{2+} concentrations, the relation deviates from the Nernst equation, and an approximately linear response is obtained. Since the nature of this linear response and its constancy have not been proved in detail, all of the experiments reported here contain a calibration curve. A Ca^{2+} concentration scale based upon the mean slope of the calibration curve is placed on the ordinates of Figures 2 and 4. At these low Ca^{2+} concentrations, slopes of 15 μM Ca^{2+}/mv are observed. At higher concentrations (in excess of 200 μM added Ca^{2+}), the Nernst equation is applicable. For a more accurate evaluation, the external Ca^{2+} concentrations can be read directly off the calibration curve.

The zero point of the scales for the potential changes differs with the particular type of electrode. With the Orion electrode and a calomel electrode as reference, zero potential is obtained at approximately 10^{-3} M Ca^{2+}. With the Corning electrode, zero potential is obtained at high concentrations of calcium. Thus, potential changes are plotted with an arbitrary zero, usually taken with the electrode in the reaction medium and in the absence of added calcium. The potential increases with increasing calcium concentration.

Streaming potentials contribute a fluctuation to the electrode output at rapid rates of stirring. It was found that the amplitude of the vibrating electrode employed as a stirrer could be adjusted so as to give negligible electrode noise, and yet adequate mixing.

Drift. Precautions were taken to avoid shifting the electrode from one medium to another containing different amounts of cations. Under typical conditions of recordings, the drift was less than the equivalent of 5 μM calcium/min, which was not found to be objectionable.

The response speed of the electrode was adequate for the special purposes of these experiments where the presence of Ca^{2+} outside the mitochondria rather than the kinetics of its uptake was of greater interest. Usually the response began in less than 2 sec following the addition of Ca^{2+}, and a half-maximal response was observed in approximately 5 sec. The mixing time was less than 1 sec. Under certain conditions, however, some delays were observed in the electrode response up to 10 sec, particularly on adding EGTA.[1] In this respect it is appropriate to emphasize that we have employed the electrodes for stoichiometric rather than for kinetic studies.

Experimental Results

Mitochondria in the absence of a permeant anion react with low concentrations of Ca^{2+} in two distinct steps. In the first step, the endogenous phosphate and the buffer capacity of the mitochondria maintain neutrality until the capacity (40 mμmoles/mg of protein) is exceeded. In the second step, Ca^{2+} additions cause increasing alkalinity of the mitochondrial membrane until a membrane gradient of approximately 1 pH unit is established (Chance and Mela, 1966a). In order to examine critically the relationship of the BTB response and the external Ca^{2+} concentration, we have made serial additions of 40 μM Ca^{2+} (8 mμmoles/mg of protein) to a mitochondrial suspension containing a concentration of 5 mg of protein/ml. The responses of BTB and of the Ca^{2+} electrode are indicated in Figure 1. The first three or four additions of 40 μM calcium cause cyclic responses of the BTB trace (top) indicating a transient alkalinization followed by a neutralization due to the endogenous phosphate and the buffer capacity of the mitochondria. The accumulated alkalinization reaches a plateau at approximately 200 μM Ca^{2+}. In Figure 2 the experimental data are plotted in terms of optical density against the Ca^{2+} concentrations at two different times, 15 (solid circles) and 90 sec (open squares) after each separate addition. These times correspond to the transient and steady-state alkalinization of the mitochondrial membrane. An extrapolation of a straight portion of these curves to the points of their intersections with the abscissa

[2] Obtained through the courtesy of A. T. Hundert of the Corning Glass Co.

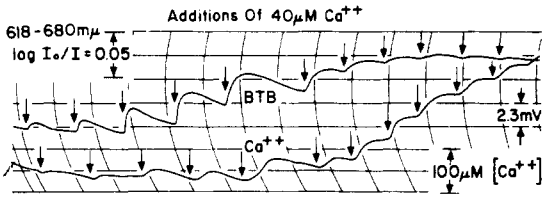

FIGURE 1: An experiment showing the responses of BTB and Ca^{2+} electrode on sequential additions of 40 μM Ca^{2+} to rat liver mitochondria, 5 mg of protein/ml, 0.3 M mannitol–sucrose, 20 mM Tris–Cl, pH 7.4, 4 μM BTB.

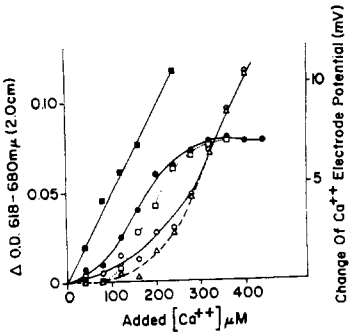

FIGURE 2: A plot of titration curves from the experiment of Figure 1, indicating Ca^{2+} electrode titration curve in the absence (■) and presence of mitochondria 15 (○) and 90 sec (△) after Ca^{2+} addition, and BTB titration curve 15 (●) and 90 sec (□) after Ca^{2+} addition.

gives 50 μM (10 mμmoles/mg of protein) at 15 sec and 100 μM (20 mμmoles/mg of protein) at 90 sec for the endogenous phosphate content and the buffer capacity of the mitochondria at the two different times.

If we now consider the traces of Figure 1 obtained with the Ca^{2+} electrode, it is observed that no significant deflections are obtained for the first two additions of Ca^{2+}. Thereafter, each addition of Ca^{2+} gives a successively larger response until a summation of the responses is obtained at added Ca^{2+} concentrations in excess of 200 μM (40 mμmoles/mg of protein). The sensitivity of Ca^{2+} calibrated as described below is 50 μM Ca^{2+}/division. Thus, the small deflections observed for the first three additions of Ca^{2+} indicate a concentration of Ca^{2+} external to the mitochondria of less than 10 μM.

These relationships are clarified by the graphs of Figure 2. This includes first the calibration curve for the electrode in the absence of mitochondria (solid squares) which gives an approximately linear slope of 17 μM/mv over the range of calcium concentrations employed. This calibration curve may be employed for an evaluation of the concentration of Ca^{2+} external to the mitochondria. In the presence of mitochondria, the two curves at 15 and 90 sec indicate a number of correlations between the BTB and the calcium electrode data. For example, when the 15- and 90-sec responses of BTB are half-maximal, the added Ca^{2+} concentration is 150 μM, and the external Ca^{2+} concentrations are 24 and 12 μM, respectively, at the two times. The Ca^{2+} uptake is 30 mμmoles/mg of protein. Near the plateau of the BTB response the external calcium concentration is 80 μM (16 mμmoles/mg of protein). The point at which no further Ca^{2+} uptake occurs, judging from the 90-sec curve, corresponds to about 90% saturation of the BTB response. Lastly, the Ca^{2+} affinity under these particular conditions, as judged roughly from the approach of the 90-sec curve to its asymptote, corresponds to approximately 150 μM Ca^{2+}. Above 300 μM Ca^{2+}, there is no further uptake of Ca^{2+} by the mitochondria, and the slope of the straight portion of the curve is equal within the experimental error to the slope obtained in calibration of the electrode in the absence of mitochondria.

Effects of Phosphate and Acetate. Figure 3A represents the alkalinization of the mitochondrial membrane upon the second addition of 100 μM Ca^{2+} to 5.0 mg of protein/ml. Also included in this trace is the concentration of extramitochondrial Ca^{2+}. It is seen that the membrane pH gradient slowly declines as the Ca^{2+} is expended. However, considerable Ca^{2+} remains outside the mitochondria for approximately 4 min.

In Figure 3B the experiment is repeated and the second of two additions of 100 μM Ca^{2+} is illustrated. At the maximum alkalinization of the membrane, 200 μM phosphate is added causing the neutralization of the membrane pH change in approximately 20 sec. The initial level of the calcium trace prior to the second addition of 100 μM Ca^{2+} has been displaced from zero due to the first addition of 100 μM Ca^{2+} (not shown). Following the second addition of Ca^{2+}, the deflection rises to a steady value but falls abruptly following the addition of 200 μM phosphate. The slope of the calcium electrode trace and the time for reaching zero calcium is slower than that for BTB. Judging from the response of the Ca^{2+} electrode to added calcium ($t_{1/2}$ approximately 3 sec) and the response of the Ca^{2+} electrode to phosphate, the kinetics of Ca^{2+} accumulation following phosphate addition are probably accurately portrayed, and the temporal discrepancy between the BTB and Ca^{2+} traces is probably valid. On this basis the rapid neutralization of the membrane pH by phosphate allows the accumulation of calcium to occur. The effect of phosphate upon the calcium electrode response at these concentrations has been controlled in separate experiments and found to be negligible.

In a similar experiment (Figure 3C) the kinetics of neutralization of the membrane pH gradient by addition of acetate are recorded. As in the above experiments, the membrane is made alkaline by two additions of 100 μM each of calcium; the effect of the second one is shown in this figure. In this case the addition of 5 mM acetate neutralizes the membrane pH gradient almost as rapidly as in the case of 200 μM phosphate. Upon addition of acetate there is no immediate change

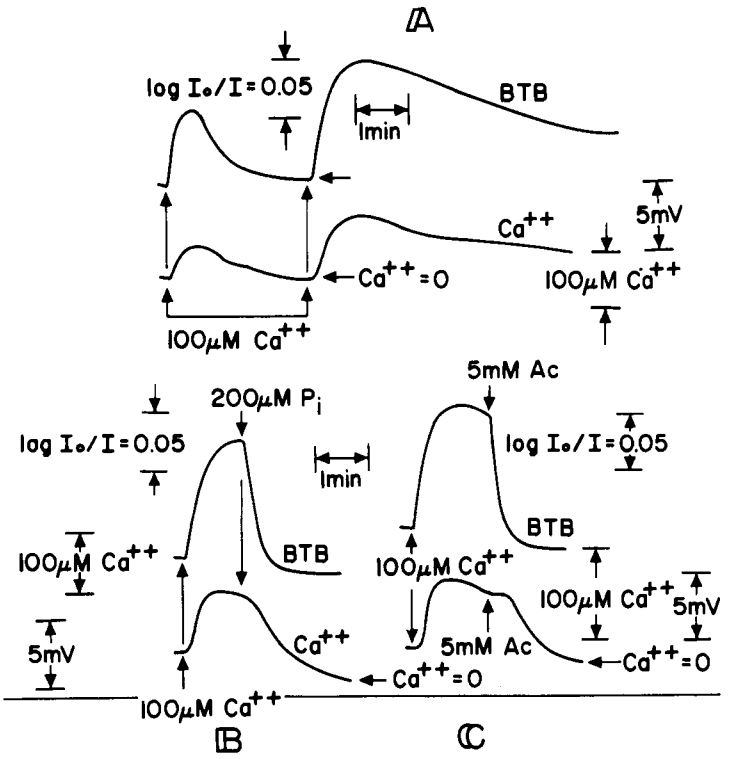

FIGURE 3: Experiments showing the effect of phosphate and acetate additions upon the responses of Ca^{2+} electrode and BTB. A is a control with two additions of 100 μM Ca^{2+}; in B, 200 μM P_i and in C, 5 mM Ac^- are added after the second addition of Ca^{2+}; rat liver mitochondria, 5 mg of protein/ml, 0.3 M mannitol-sucrose, 20 mM Tris-Cl, pH 7.4, 4 μM BTB. Additions are shown as single vertical arrows; calibrations as double enclosed arrows on the figure margins.

of the calcium electrode trace; 15 sec thereafter a decrease begins and is complete in approximately 1 min. Independent experiments have indicated the high rate of permeation of mitochondrial membrane by acetate (Rasmussen *et al.*, 1965).

In Figure 4 we have plotted the calcium electrode potential against the concentration of added Ca^{2+} to the mitochondria under three conditions: potassium succinate, solid circles; potassium succinate plus 1 mM phosphate, solid triangles; and potassium succinate plus 20 mM acetate, open circles. In addition, a calibration curve for the electrode is included (solid squares). In agreement with Figure 2 the mitochondria show appreciable external Ca^{2+} in the absence of a permeant anion. Nevertheless, the mitochondria continue to accumulate Ca^{2+} at the higher concentrations. In the presence of 1 mM phosphate, the external Ca^{2+} is less than 5 μM until 400 μM Ca^{2+} has been added. Thus, the ratio of added Ca^{2+}/extramitochondrial Ca^{2+} is approximately 100, *i.e.*, over 99% of the Ca^{2+} has been accumulated. At higher concentrations, the mitochondria swell, and Ca^{2+} is rapidly released into the external medium (dashed portion of the curve). In the presence of acetate the extramitochondrial Ca^{2+} concentration is maintained as low as in the case of phosphate. However, the ratio of the added Ca^{2+} to the free Ca^{2+} does not reach such a high value as in the case of phosphate. Nevertheless, more Ca^{2+} is taken up in the presence of acetate than in the presence of phosphate, possibly due to the progressive swelling of the mitochondria while Ca^{2+} is being accumulated in the presence of acetate (Chance and Yoshioka, 1965).

Alteration of the H^+/Ca^{2+} Value. In order to determine in more detail the nature of the reactions that occur during the two different segments of the curve for BTB response *vs.* added calcium (for example, in Figures 2 and 4), we have measured the H^+/Ca^{2+} value in some detail in order to shed some light on the nature of the membrane reaction. The basis for the experiments is afforded by the observation of Rasmussen *et al.* (1965) that the hydrogen ion ejection from mitochondrial membranes during Ca^{2+} accumulation is suppressed by a permeant anion such as acetate. Thus, the participation of a related substance would be detected by the low value of H^+/Ca^{2+}. We find under experimental conditions similar to those of Figures 2 and 4, and over a range of calcium concentrations from 33 to 200 μM added Ca^{2+}, the H^+/Ca^{2+} value ranges from 1.5 to 2.0 and thereafter falls to very nearly zero

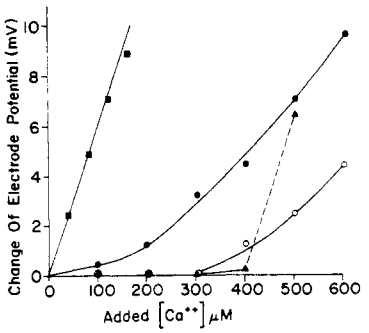

FIGURE 4: A plot of change of Ca^{2+} electrode potential against added Ca^{2+} concentration. Experimental conditions: ■, 10 mM K succinate; ●, 10 mM K succinate and mitochondria; ▲, 10 mM K succinate and mitochondria, plus 1 mM Tris–P_i; ○, 10 mM K succinate and mitochondria, plus 5 mM Tris–Ac^-; rat liver mitochondria, 6.7 mg of protein/ml, 0.3 M mannitol–sucrose, 20 mM Tris–Cl, pH 7.4.

at 700 μM added Ca^{2+}.

Discussion

Measurements of Ca^{2+} concentrations external to mitochondria during the process of membrane alkalinization allow a more detailed examination of proposals for the function of the membrane gradient in cation accumulation. Our first proposal is that the pH gradient serves as a compulsory "intermediate" in the accumulation of Ca^{2+}. The other is that it promotes an "inhibitory side reaction." In favor of the second viewpoint is the well-established observation that excessive cation accumulation leads to a state where no more cations can be accumulated and respiration is inhibited (Chance and Yoshioka, 1965; Chance, 1964). The more recent data of this paper (see also Mela, 1966; Chance, 1966; Chance and Mela, 1966b) indicated that accumulation of cations under these conditions leads to an alkalinization which other data indicate to be associated with the mitochondrial membrane (cristae), rather than the matrix spaces (Chance and Mela, 1966a,b).

An elementary kinetic analysis serves usefully to examine the possible role of the gradient as an intermediate in cation accumulation. Two situations may be considered: in the first situation, the degree of alkalinization of the mitochondrial membrane depends upon the residence time of the Ca^{2+} in the membrane, which is long in the absence of a permeant anion. With a permeant anion this time is shorter. Thus, low concentrations of Ca^{2+} are effective in alkalinizing the membrane in the absence of a permeant anion, while high concentrations are required to build up a significant membrane alkalinization in the presence of permeant anion. This simple picture does not explain the process by which the anion facilitates the movement of the cation from the membrane (cristae) to the matrix space.

Another mechanism which is somewhat simpler postulates only that Ca^{2+} uptake is slowed in the absence of permeant anions. Then there is a high concentration of Ca^{2+} outside the membrane and unneutralized calcium hydroxide exists in the membrane leading to a pH gradient in the membrane. When a permeant anion is added, the membrane pH gradient is neutralized and Ca^{2+} accumulation is reactivated. In the absence of an added permeant anion, the endogenous phosphate content and other sources of buffer capacity of the membrane serve this purpose either by neutralizing the alkalinity of the mitochondrial membrane or by providing the equivalent of a permeant anion.

On this basis we may interpret the response of the membrane to other types of cations and anions. Strontium behavior is similar to that of Ca^{2+} and the uptake of strontium by mitochondria is relatively rapid. The much slower uptake of manganese causes relatively less alkalinization of the membrane, which we attribute to the fact that permeation of anions keeps step with the uptake of manganese. For example, the slow return of the BTB traces following the initial additions of Ca^{2+} (see Figure 3) may be due to the permeation of substrate anions into the mitochondria, a process which is rapid enough to cause this slow neutralization of the membrane alkalinity, but is not nearly as effective as acetate in maintaining membrane neutrality immediately following the addition of Ca^{2+}. It is of interest that in the presence of Ca^{2+} the uptake of manganese does cause a large membrane alkalinization, but in this case the manganese uptake has been accelerated by the added Ca^{2+} (Chance and Mela, 1966c).

The preliminary measurements described here of the free Ca^{2+} concentrations external to the mitochondria verify the increased capacity of the mitochondria for cations in the presence of permeant anions. The data furthermore show that acetate is as effective as phosphate as a permeant anion up to an uptake of 40 mμmoles of Ca^{2+}/mg of protein. Above this level, phosphate leads to precipitation and to subsequent irreversible damage. It is of interest that mitochondria appear to withstand the accumulation of larger amounts of Ca^{2+} in the presence of acetate than in the presence of phosphate, at least under our experimental conditions. This may be attributed to the fact that acetate supplementation leads to an increase of mitochondrial volume (Chance and Yoshioka, 1965).

In the experiment of Figure 4, in the presence of phosphate or acetate, 43 mμmoles of Ca^{2+}/mg of protein is accumulated without the measurement of detectable external Ca^{2+} (<5 μM Ca^{2+}). If we assume Ca^{2+} is accumulated in the total mitochondrial volume, the ratio is 3000 (see also Chance, 1965; Saris, 1963). If we alternately assume that Ca^{2+} is accumulated in the protein of the mitochondria, we then conclude that the Ca^{2+} concentration is 43 mM, an outside/inside ratio of approximately 8000-fold.

The possibility that different types of membrane reactions occur at high and low calcium concentrations could result from an initial reaction with phospholipid

where one calcium can apparently bind two molecules of cephalin (Feinstein, 1964). Evidence in favor of the role of phospholipids in calcium binding is afforded by preliminary experiments (E. J. Harris, B. Chance, and L. Mela, unpublished data) on the effect of a local anesthetic, such as butacaine, on the H^+/Ca^{2+} values at both low Ca^{2+} concentrations and at higher concentrations where other materials of the membrane or soluble anions might be involved.

The H^+/Ca^{2+} values allow a tentative identification of the nature of the anion involved; values in the region of 0.8 are likely to be due to phosphate, while considerably lower values (approximately 0.2) would be due to a permeant anion such as acetate (Rasmussen *et al.*, 1965). Since we observe consistent values in excess of 1, we tend to attribute the initial binding and the "membrane buffer capacity" to phospholipid components (see also Chappell *et al.*, 1963).

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β-Aspartyl Peptides in Enzymatic Hydrolysates of Protein*

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ABSTRACT: In a search for the source of β-aspartyl oligopeptides in urine we have analyzed exhaustive enzymic hydrolysates of human hemoglobin, porcine pepsin, human and bovine Achilles tendon collagen, and egg white lysozyme. β-Aspartylglycine was present in all hydrolysates and β-aspartylalanine was found in digests of hemoglobin. There was no increase in the β-aspartylglycine content of human or bovine tendon collagen with aging *in vivo* nor of lysozyme with aging *in vitro*. The data suggest that, in intact proteins, β-aspartyl linkages do not form spontaneously from α-aspartyl or asparaginyl linkages.

We have previously described the isolation and identification of several β-aspartyl di- and tripeptides from human urine (Buchanan *et al.*, 1962a). β-Aspartylglycine (Asp(Gly))[1] was the most abundant. The belief that β-aspartyl peptides are metabolic end products, less readily cleaved by tissue peptidases and more susceptible to renal excretion than α-aspartyl peptides, was supported by results of experiments in which human subjects were given glycine-labeled Asp-Gly or Asp(Gly), or labeled glycine intravenously (Buchanan *et al.*, 1962b). Most of the β peptide was quickly excreted, while the metabolic pattern of the injected α peptide was similar to that found when free glycine was administered. An explanation of the occurrence of these urinary peptides may be the spontaneous or even catalyzed conversion of asparaginyl or α linkages during *in vivo* metabolism. Isomerization occurs with α- and β-aspartyl peptides in acid solution and the β isomer is more stable (John and Young, 1954; Swallow and Abraham, 1958; Bryant *et al.*, 1959). Our present data show that conversion of Asp-Gly to Asp(Gly) occurs spontaneously in pH 7.4 buffer

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[1] Abbreviations used: Asp-Gly, α-L-aspartylglycine; Asp(Gly), β-L-aspartylglycine; Asp(Ala), β-L-aspartylalanine; Asn-Gly, L-asparaginylglycine. It is assumed that the peptides in the protein hydrolysates are of the L configuration. Other abbreviations: TCA, trichloroacetic acid; VK, Viokase; PRN, Pronase; COL, collagenase; LAP, leucine aminopeptidase; PRL, prolidase; CA, carboxypeptidase A; CB, carboxypeptidase B.