Do Carbonylamine Browning Reactions Occur in Vivo?

Cora J. Dillard and Al L. Tappel*

Investigations by fluorescence measurements of carbonylamine browning products in vivo and studies of the fate of exogenously produced glucose–glycine browned products injected into or fed to rats were done. Model systems of physiological concentrations of amino acids and carbohydrates at pH 7.35 undergo browning at 37 and 50°C with formation of ultraviolet absorbing compounds and compounds with fluorescence spectral characteristics of carbonylamine Schiff bases. Similar fluorophores were not detected in dialysates of fresh or incubated human plasma. Fluorescent browned glucose–glycine mixtures fed to rats via a gastric needle were not detected in plasma or urine 3 hr after dosage. [^{14}C]Glucose–glycine browned products were efficiently removed via the urine, an average of 84.7% of the injected label being removed by 48 hr. Although no direct evidence was obtained for the presence of fluorescent carbonylamine browned products in vivo, their formation has not been disproven.

Biological molecules are damaged variously by carbonylamine reactions. Reynolds (1965) has reviewed the chemistry of nonenzymic browning, including reactions between reducing sugars and amines, lipids and amines, and browning reactions of ascorbic acid. Nonenzymic browning reactions have become a major topic of food research because of the relevance of their undesirable occurrence in foods. Damage to lysine in food processing by these reactions recently has been reviewed by Carpenter (1973). Damage to biological molecules in vitro by the carbonylamine reactions occurring during lipid peroxidation has been documented for proteins, including enzymes (Roubal and Tappel, 1966; Chio and Tappel, 1969b), phospholipids (Tappel, 1973; Bidlack and Tappel, 1973b), nucleic acids (Klamerth and Levinsky, 1967; Reiss et al., 1972; Reiss and Tappel, 1973), and subcellular organelles (Dillard and Tappel, 1971; Bidlack and Tappel, 1973a). Few studies other than those that involve lipid peroxidation have been reported of the occurrence of browning reactions as they relate to possible biological damage in vivo. Many of the reactants involved in browning are concurrently present in cells and tissues of living animals, which raises the question as to whether these types of reactions occur in vivo and, if so, whether the damage is significant. This study was undertaken to determine whether evidence could be found for the natural occurrence of browning reactions in vivo by fluorescence techniques, and to determine what happens to exogenously produced browned products when they are fed to or injected into rats.

MATERIALS AND METHODS

Bio-Gel P2, 100–200 mesh, was obtained from Bio-Rad Laboratories, Richmond, Calif., and Sephadex G-10 from Pharmacia Fine Chemicals, Uppsala, Sweden. Ready Solve Solution VI, obtained from Beckman Instruments, Inc., Fullerton, Calif., was used for scintillation counting. The biochemicals used and their sources were as follows: pyridoxal-HCl, pyridoxamine-HCl, folic acid, glycine, L-lysine-HCl, and reduced nicotinamide adenine dinucleotide were obtained from Sigma Chemical Co., St. Louis, Mo.; glucuronic acid, D(+)-glucosamine, DL-glutamine, and all other amino acids, from Nutritional Biochemicals Corporation, Cleveland, Ohio; glucose, from Mallinckrodt Chemical Works, St. Louis, Mo.; dihydrostreptomycin and penicillin G, from Grand Island Biological Co., Grand Island, N.Y.; butylated hydroxytoluene, from Tennessee Eastman Company, Kingsport, Tenn.; and D-[U-^{14}C]glucose, from ICN Pharmaceuticals, Inc., Isotope and Nuclear Division, Irvine, Calif.

Preparation and Standardization of Bio-Gel P2 Column. A column (83 × 1.5 cm) of Bio-Gel P2, 100–200 mesh, with a molecular weight exclusion greater than 1500, was prepared and equilibrated with 0.05 *M* Tris buffer (pH 7.2); 2-ml fractions of eluate were collected during each chromatographic separation. The void volume was determined with blue dextran. Some fluorescent compounds known to be present in blood plasma were individually chromatographed as standards; these included pyridoxal-HCl, pyridoxamine-HCl, folic acid, reduced nicotinamide adenine dinucleotide, L-tryptophan, L-tyrosine, and L-phenylalanine.

Fluorescence Measurements. Fluorescence measurements were made with an Aminco (American Instrument Co., Inc., Silver Spring, Md.) spectrophotofluorometer with a ratio photometer. The excitation, emission, and turret slit settings were 3, 1, and 3 mm, respectively. A standard solution of 1 μg of quinine sulfate/ml of 0.1 *N* H_2SO_4 used to calibrate the instrument had a fluorescence intensity of 380 at a sensitivity setting of 1.

Browning of Synthetic Amino Acid–Carbohydrate Mixture. The reactant concentrations selected for this synthetic mixture were based upon reported values (Kiem and Lentner, 1970) for blood plasma. The following reactants, in milligrams, were mixed in 200 ml of water and adjusted to pH 7.35: L-alanine, 6.14; L-arginine, 2.84; DL-glutamine, 16.60; L-glutamic acid, 1.72; L-glycine, 3.48; isoleucine, 1.42; leucine, 2.64; lysine-HCl, 5.08; methionine, 0.64; proline, 5.42; serine, 2.36; threonine, 3.88; valine, 3.98; D(+)-glucosamine-HCl, 196; ammonium chloride, 0.6; glucose, 199; fructose, 15; glucuronic acid, 6.4; dihydrostreptomycin, 26; penicillin G, 14; and cysteine, 16.6. The cysteine level was higher than the mean level of plasma cysteine, but the excess was used to represent the balance of plasma amino acids not included in the reaction because of their own native fluorescence. One-half of the mixture was incubated at 50°C and one-half at 37°C for 7 days. Aliquots were removed at intervals for measurement of ultraviolet absorption and of fluorescence.

Human Plasma Samples. Two batches of pooled fresh human plasma were obtained from a local clinical laboratory. Ten-milliliter aliquots from each fresh plasma pool were dialyzed immediately in cellulose tubing that retained molecules with a mol wt of 3500 or higher. Dialysis was

Department of Food Science and Technology, University of California, Davis, California 95616.

for 48 hr with one change of deionized water. The dialysate from each plasma pool was concentrated to 0.5 ml by rotary evaporation, and these concentrates were applied directly to a Bio-Gel P2 column. The fluorescence excitation and emission maxima and the fluorescence intensity of each 2-ml fraction were recorded.

A 20-ml aliquot of plasma pool I was incubated at 37°C for 5 days with a continuous flow of oxygen being bubbled through the liquid. To prevent bacterial growth, dihydrostreptomycin (2.6 mg) was added, and to prevent lipid peroxidation, butylated hydroxytoluene (200 mg) was added. [^{14}C]Glucose (0.25 μCi) was added to determine whether it would be incorporated into a fluorescent product. A 10-ml aliquot of plasma pool II was similarly incubated for 4 days. Each incubated sample was dialyzed for 48 hr with one change of deionized water. The dialysates of each were pooled and reduced in volume to 0.5 ml as described above and then were chromatographed on a Bio-Gel P2 column.

Preparation of Glucose–Glycine Browning Products for Gastric Feeding to Rats. An aqueous mixture of 2 *M* glucose (36 g/100 ml) and 1 *M* glycine (7.52 g/100 ml) was heated at 100°C for 90 min; an unheated mixture was stored at −20°C. Eight 150–175 g male Sprague-Dawley rats were fasted for 24 hr. An aliquot of 1.5 ml of the browned glucose–glycine was administered by a gastric needle to each of four rats; 1.5 ml of the unheated mixture was given to each of four control rats. Each animal was placed in a metabolic cage for 3 hr to collect urine. Each rat was then anesthetized with sodium pentobarbital, blood was collected by heart puncture into a heparinized syringe, and the liver and kidneys were removed. Ten percent homogenates of the individual livers and kidneys were prepared in 0.01 *M* potassium phosphate buffer (pH 7.0). The soluble portions obtained by centrifugation at 31550*g* for 45 min and the plasma obtained from the heparinized blood were diluted 1/10 with buffer; the urine samples were diluted 1/200 with buffer and all samples were examined for fluorescence indicative of the browned glucose–glycine mixture.

Preparation of [^{14}C]Glucose–Glycine Browning Products for Injection into Rats. In a total volume of 15 ml, a mixture of 1.1 g of glycine, 5.4 g of glucose, and 0.43 mg of [^{14}C]glucose (17 μCi) was heated for 60 min at 100°C. The browned sample was cooled and applied in a desalting technique to a Sephadex G-10 column (3 × 12.5 cm). Elution was done with water. The void volume was determined with blue dextran and standardization with glucose and glycine was followed by measurements of refractive index. A 14-ml pooled fraction that contained the most highly fluorescent compounds (700–1500 mol wt) and that was free of unreacted [^{14}C]glucose was concentrated by rotary evaporation to 3 ml. This fraction contained 1.5% of the total label added to the initial reaction mixture. Aliquots that contained 105000 cpm were injected intraperitoneally into each of three rats. The urine from each rat was collected in individual metabolic cages at 24 and 48 hr and then pooled. Food was removed at 48-hr post-injection and the urine was collected for another 24 hr. The animals were killed at 72 hr post-injection. Homogenates of the pooled livers and kidneys were prepared 1:8 in 0.25 *M* sucrose. Radioactivity present in the individual urine samples and pooled liver and kidney homogenates was measured in Ready Solve Solution VI with a Beckman LS-100 liquid scintillation counter.

RESULTS

Search for Fluorescent Browning Products in Synthetic Reaction Mixtures. Figure 1 compares the uv absorption spectra of products in a synthetic mixture of amino acids and carbohydrates formed at 37 and 50°C over a period of 7 days. Browning reactions traditionally are followed by an increase in absorption at 410 nm. In these reaction systems, uv absorption changes were measured before compounds absorbing at 410 nm could be measured. The absorption peak at 265–275 nm in the 50°C reaction system indicates conjugated and unsaturated carbonyl compounds were being formed. Figure 2 compares the fluorescence spectra of the same two reaction mixtures. The fluorophores were typical of those formed by boiling glucose and glycine (Adhikari, 1973). There were two excitation maxima, one at 240–245 nm and a second at ca. 325–330 nm, and a single emission maximum at 420–425 nm. Although the fluorescence emission with excitation at the higher wavelength maximum was over 16 times greater by 7 days in the mixture at 50°C than at 37°C, it is significant that these products do form at 37°C under simulated physiological conditions of pH and concentrations. The amount of fluorescence formed per

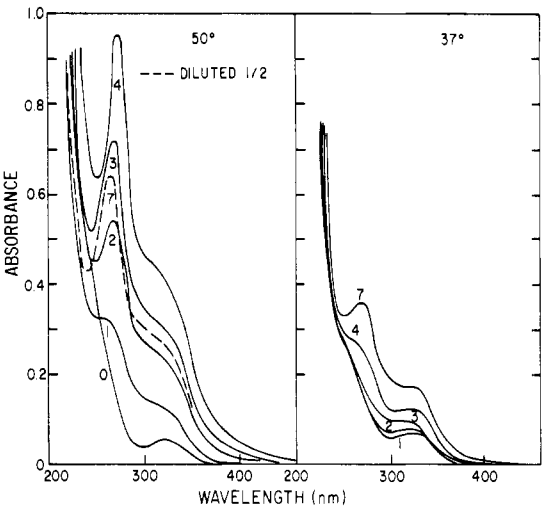

Figure 1. Absorbance spectra of amino acid-carbohydrate mixtures at 0, 1, 2, 3, 4, and 7 days.

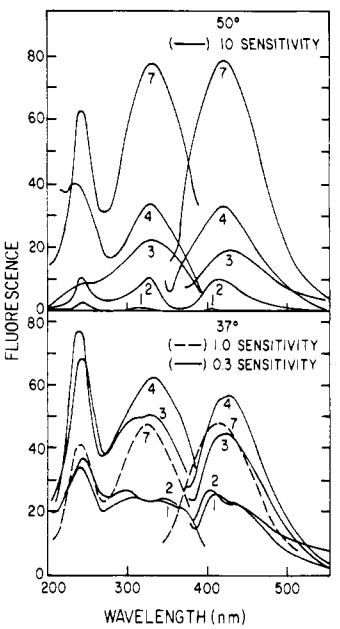

Figure 2. Complete fluorescence spectra of amino acid-carbohydrate mixtures at 1, 2, 3, 4, and 7 days with the excitation wavelength set at the higher wavelength maximum (ca. 325 nm) and the emission set at the single maximum (ca. 430 nm).

Table I. Distribution of ^{14}C Label among Rat Kidney, Liver, and Urine after Injection with [^{14}C]Glucose-Glycine Browned Products

Tissue sample	Total cpm × 10^{-3} 0-48 hr	Total cpm × 10^{-3} 48-72 hr	% injected cpm 0-48 hr	% injected cpm 48-72 hr
Liver[a]		1.9		0.6
Kidney[a]		4.4		1.4
Urine				
Animal 1	94.0	2.9	89.5	2.8
Animal 2	91.5	1.4	87.1	1.3
Animal 3	81.4	1.5	77.5	1.4

[a] Homogenates of tissues were pooled from three rats each injected with 105000 cpm of [^{14}C]glucose-glycine browned products.

milliliter of the reaction mixture at 37°C was equivalent to approximately 0.2 μmol of a standard glycin-1-amino-3-iminopropene, a fluorescent carbonylamine Schiff base synthesized by Chio and Tappel (1969a).

Search for Fluorescent Browning Products in Human Plasma. Being interested primarily in small molecular weight fluorescent products of the type produced by heating glucose and glycine (Adhikari and Tappel, 1973), the search was directed toward human plasma dialysates that contained compounds with molecular weights of less than 3500. The dialysates from the two fresh plasma pools and the dialysates from the two plasma pools that had been incubated at 37°C with oxygen to enhance any browning reactions were examined for fluorescence before they were concentrated for application to the Bio-Gel P2 column, and each fraction collected was examined. The fluorescent compounds that were detected and identified were tyrosine and tryptophan. Added [^{14}C]glucose and plasma glucose did not react with plasma amino acids during heating at 37°C to form a dialyzable fluorescent browned product. Most of the [^{14}C]glucose initially added was eluted in a series of nonfluorescent fractions that corresponded with the elution profile of unreacted glucose.

Search for Glucose–Glycine Browning Products Fed to Rats. The relative fluorescence of the glucose–glycine browned mixture fed to rats by gastric needle was 7700 units/ml at a spectrophotofluorometer sensitivity setting of 1; that of the unheated mixture was 50. The fluorescence excitation and emission maxima were 345 and 430 nm, respectively, for the heated mixture, and 335 and 420 nm, respectively, for the unheated mixture. The browned sample could still be measured fluorometrically at a 1/5000 dilution, so it was expected that any significant amount of product absorbed and circulating in the plasma or excreted in the urine should be detectable. Each urine, plasma, kidney-soluble fraction, and liver-soluble fraction was examined for the presence of fluorochromes with the same spectral characteristics of the stomach tube fed mixture, but there was no evidence of their presence in any of the samples examined 3 hr after administration of the browned mixture.

Distribution of [^{14}C]Glucose among Rat Liver, Kidney, and Urine from Rats Injected with ^{14}C-Labeled Browned Products. The distribution was determined of the ^{14}C label in the liver, kidney, and urine samples from rats after they were injected with [^{14}C]-glucose–glycine browned products. Table I shows that an average of 84.7% of the injected label was excreted during the following 48 hr; only 2% of the label was measurable in the kidneys and liver at 72-hr post-injection. No attempt was made to account for the remaining activity in other tissues of the rat. Sephadex G-10 chromatography of 48-hr rat urine showed coincidence of label and low level fluorescence with that of the injected fluorophores.

DISCUSSION

The browning phenomenon involves among other reactions the reaction of aldoses, ketoses, or uronic acids with amines, both primary and secondary. These complex reactions have been studied in vitro for many years. Although they are not generally thought of in terms of carbonylamine browning reactions, these types of reactions occur in vivo, as shown by the following examples. Dixon (1972) has hypothesized that hemoglobin A_{1c}, which comprises about 6% of the total hemoglobin A, is biosynthesized by glycosylation of hemoglobin A. In the eye, retinal is apparently bound at the ε-aminolysyl site of opsin (Anderson, 1972). Reiss (1972) described the presence in rat liver tRNA of a fluorescent compound with spectral characteristics of a carbonylamine Schiff base that was unrelated to fluorescent $tRNA^{Phe}$ (Fink et al., 1968; Nakanishi et al., 1970). Some lysine units in collagen fibrils cross-link through ε-amino groups with aldehydes (Bailey, 1968) or carboxylic groups of peptide-linked aspartic acid and glutamic acid (Steven et al., 1968, 1972). Feeney et al. (1964) found deteriorative changes to occur in egg whites during incubation at 37°C of whole eggs in the shell. They suggested that glucose–amine reactions may occur more commonly than previously suspected in biological systems. Lipid peroxidation products, including malonaldehyde, react with biological amines in vivo to form fluorescent carbonylamine Schiff bases by a browning-type reaction (Tappel, 1973).

Amino acids react with glucose to form browned products, as shown by the classical study of Lea and Hannan (1949). During the browning reaction, fluorescent products develop that can be measured before the onset of pigment formation, and which have been postulated to have the structure: $OHC(=O)CH_2N=CHCH=C(-OH)$ (Adhikari and Tappel, 1973). Hannan and Lea (1952) were able to separate products from the early stages of browning that had different uv absorption maxima. Aqueous solutions of browning pigments show general absorption with peaks at 390, 290, and 270 nm; the position of the peak depends upon the preparation conditions used (Reynolds, 1965). The products formed from amino acids and carbohydrates at 37 and 50°C in this study had increased uv absorption at 270 and 320 nm. The results of Hannan and Lea (1952) suggested to Clark and Tannenbaum (1974) that many different chromophores were possible, and different fluorophores also may be possible, as shown by the slight variations in the excitation and emission maxima recorded over the course of the 7-day reaction.

The degree to which browning occurs is temperature dependent (Lea and Hannan, 1949). Our search for fluorescent browning products formed at 37°C showed that there was about 16 times less fluorescent products formed than at 50°C; however, the fact that these products did form at 37°C at physiological concentrations encouraged a further search for their presence in animal tissue. Our past studies of fluorescent compounds in animal tissues, such as blood plasma, urine, and soluble fractions of kidney and liver, have often shown the presence of compounds with fluorescence excitation maxima at 320–340 nm and maximum emission at 430–450 nm. A study of the dialysate from human plasma, both fresh and incubated for 4 and 5 days, did not reveal the fluorochromes that are indicative of carbonylamine Schiff bases. In tissue samples it may be that mixtures of fluorescent compounds give resultant fluorescence spectra that are similar to those of the Schiff base fluorophores, or browning reactions occur

that give fluorophores attached to larger biological molecules than were dialyzed away from the plasma proteins.

A logical route of disposal of any nondegradable browned products that might be formed in vivo would be via the kidneys; thus, urine would be the body fluid most accessible for examination. Examination of human urine for fluorescent products is a difficult task because of the relatively large volume output and because of the large number of other very fluorescent compounds in the urine. Because rats eliminate urine that is more concentrated and of a smaller volume, these animals were chosen to study the fate of browned products introduced by feeding and injecting synthetic browned fluorophores. During the 3 hr following the feeding of browned glucose–glycine to rats, there was no evidence of fluorescent products in blood plasma or urine, which indicated that absorption of the partially polymeric material (mol wt between 700 and 1500) may not have occurred by this time. The work of Bjarnason and Carpenter (1969) suggested that carbonylamine products, of at least a protein source, in urine would probably not be of dietary origin. They found that pure protein, in which the availability of lysine had been reduced by heat, resulted in increased fecal lysine, but little urinary lysine, indicating that absorption had not occurred. In a discussion of the nutritional significance of browning damage to foods, Clark and Tannenbaum (1974) reviewed their work and that of others that showed that a decrease in nutritional value of heated protein–glucose systems could not be accounted for by loss of lysine alone. Limit peptides that these workers prepared from insulin and glucose could not be completely digested to the bound carbohydrates; this type of undigestibility could account for unavailability of other amino acids. Recently, Tanaka et al. (1975) have shown that a simple Maillard reaction product, fructose-L-tryptophan, when placed in the colon can be passively absorbed, that some hydrolysis of this simple molecule occurs in vivo, and most is excreted in the urine without being metabolized.

Injected ^{14}C-labeled glucose–glycine browned products were very efficiently removed by the kidney and excreted in the urine; therefore, one may speculate that any naturally occurring low level browned products in animals and humans may also be removed efficiently if they are in the blood, but that their low level formation does not allow their detection by fluorescence techniques in mixtures of other highly fluorescent compounds.

LITERATURE CITED

Adhikari, H. R., Tappel, A. L., *J. Food Sci.* **38**, 486 (1973).
Anderson, E. E., *Biochemistry* **11**, 1224 (1972).
Bailey, A. J., *Biochim. Biophys. Acta* **160**, 477 (1968).
Bidlack, W. R., Tappel, A. L., *Lipids* **8**, 177 (1973a).
Bidlack, W. R., Tappel, A. L., *Lipids* **8**, 203 (1973b).
Bjarnason, J., Carpenter, K. J., *Br. J. Nutr.* **23**, 859 (1969).
Carpenter, K. J., *Nutr. Abstr. Rev.* **43**, 424 (1973).
Chio, K. S., Tappel, A. L., *Biochemistry* **8**, 2821 (1969a).
Chio, K. S., Tappel, A. L., *Biochemistry* **8**, 2827 (1969b).
Clark, A. V., Tannenbaum, S. R., *J. Agric. Food Chem.* **22**, 1089 (1974).
Dillard, C. J., Tappel, A. L., *Lipids* **6**, 715 (1971).
Dixon, H. B. F., *Biochem. J.* **129**, 203 (1972).
Feeney, R. E., Clary, J. J., Clark, J. R., *Nature (London)* **201**, 192 (1964).
Fink, L. M., Goto, T., Frankel, F., Weinstein, I. B., *Biochem. Biophys. Res. Commun.* **32**, 963 (1968).
Hannan, R. S., Lea, C. H., *Biochim. Biophys. Acta* **9**, 293 (1952).
Kiem, K., Lentner, C., Ed., "Documenta Geigy Scientific Tables", 7th ed, Ciba-Geigy Limited, Basle, Switzerland, 1970.
Klamerth, O. L., Levinsky, H., *FEBS Lett.* **3**, 205 (1967).
Lea, C. H., Hannan, R. S., *Biochim. Biophys. Acta* **3**, 313 (1949).
Nakanishi, K., Furutachi, N., Funamizu, M., Grunberger, D., Weinstein, I. B., *J. Am. Chem. Soc.* **92**, 7617 (1970).
Reiss, U. M., Ph.D. Thesis, University of California, Davis, Calif., 1972.
Reiss, U., Tappel, A. L., *Lipids* **8**, 199 (1973).
Reiss, U., Tappel, A. L., Chio, K. S., *Biochem. Biophys. Res. Commun.* **48**, 921 (1972).
Reynolds, T. M., *Adv. Food Res.* **14**, 167 (1965).
Roubal, W. T., Tappel, A. L., *Arch. Biochem. Biophys.* **113**, 5 (1966).
Steven, F. S., Coy, G. R., Jackson, D. S., *Biochim. Biophys. Acta* **271**, 114 (1972).
Steven, F. S., Jackson, D. S., Broady, K., *Biochim. Biophys. Acta* **188**, 334 (1968).
Tanaka, M., Lee, T.-C., Chichester, C. O., *J. Nutr.* **105**, 989 (1975).
Tappel, A. L., *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **32**, 1870 (1973).

Received for review June 9, 1975. Accepted October 8, 1975. This research was supported by Research Grant No. HD 05875 from the National Institute of Child Health and Human Development.

On the Local Isotherm Concept and Modes of Moisture Binding in Food Products

Héctor A. Iglesias and Jorge Chirife*

As mentioned in the literature it was found that application of Henderson's equation to moisture sorption data in foods and food components can be used to characterize three "local isotherms". However, the analysis of 174 sorption isotherms comprising 71 different food products indicates that, in disagreement with a published suggestion, local isotherms cannot be used to give a precise and unequivocal definition of the physical state of water in foods.

Numerous equations have been derived for describing water sorption isotherms of food materials, and Labuza (1968) and more recently Nellist and Hughes (1973) reviewed the applicability of most of them. One of the most widely used models relating water activity and amount of water sorbed is Henderson's equation (Henderson, 1952). Although in several cases a complete isotherm may be satisfactorily represented by a single pair of constants in Henderson's equation (Henderson, 1952; Agrawal et al., 1969; Iglesias and Chirife, 1976), it has been frequently observed that two or three "localized isotherms" may be distinguished (Rockland, 1957).

Rockland (1957, 1969) suggested that moisture sorption

Departamento de Industrias, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina.