Photochemical Behavior of Folic Acid in Alkaline Aqueous Solutions and Evolution of Its Photoproducts

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The photolysis of folic acid (= *N*-(4-{[(2-amino-1,4-dihydro-4-oxopteridin-6-yl)methyl]amino}benzoyl)-L-glutamic acid) in alkaline aqueous solution (pH 10.0–11.0) was carried out at 350 nm at room temperature and monitored by UV/VIS spectrophotometry, anal. and prep. thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC, HPLC/MS). The folate species underwent at least two independent photo-oxidation pathways, which were not observed when the acid form was photolyzed at pH < 7. The presence of O_2 was essential in these oxidation pathways. Evidence for the role of singlet oxygen was established. In one of the pathways, the folate underwent cleavage, yielding 6-formylpterin (=2-amino-1,4-dihydro-4-oxopteridine-6-carboxaldehyde) and (4-aminobenzoyl)glutamic acid as photoproducts. The other pathway yielded a new photostable product **A** of molecular mass 455, which could be isolated and stored in acidic or neutral aqueous solution. However, **A** was rather unstable in alkaline media undergoing a thermal reaction to a product **B** of lower molecular mass (427). The kinetics of this thermal reaction was analyzed with a stopped-flow spectrophotometer. A linear dependence of the first-order rate constant with the OH^- concentration was observed. The corresponding bimolecular rate constant was 1.1 M^{-1} s^{-1}. The quantum yields of substrate consumption and of photoproduct formation were determined. The here-reported photochemical behavior of folate solutions departs from results in acid media, where phototransformation proceeded *via* the cleavage of the acid form into 6-formylpterin and (4-aminobenzoyl)glutamic acid as the first major photoproducts, and where no thermal reactions were observed.

1. Introduction. – Folic acid and related compounds play very important roles in biological processes. This compound, also known as pteroylglutamic acid (= *N*-(4-{[(2-amino-1,4-dihydro-4-oxopteridin-6-yl)methyl]amino}benzoyl)-L-glutamic acid), can be considered a conjugated pterin (=2-aminopteridin-4-(1*H*)-one), and is a vitamin that acts as a coenzyme in the metabolism of purine and pyrimidine bases [1]. Three subunits can be recognized in the structure of folic acid [2][3]: 6-methylpterin, 4-aminobenzoic acid, and glutamic acid (see *Fig. 1*). The folic acid derivative 5,10-methenyltetrahydrofolate is present as the light-harvesting antenna in DNA photolyases, which are involved in DNA-repair processes [4–6] after UV-light irradiation.

The pteridine moiety present in folic acid behaves as a weak acid in aqueous solutions. As reported by *Albert* [7] for several pterin derivatives, the dominant equilibrium at pH > 5 involves an amide group (acid form) and a phenolate group (base form). The pK_a of this equilibrium is *ca.* 8 for the different pterin derivatives studied [7–10], including folic acid itself. Other functional groups of the pterin moiety (*e.g.*, the

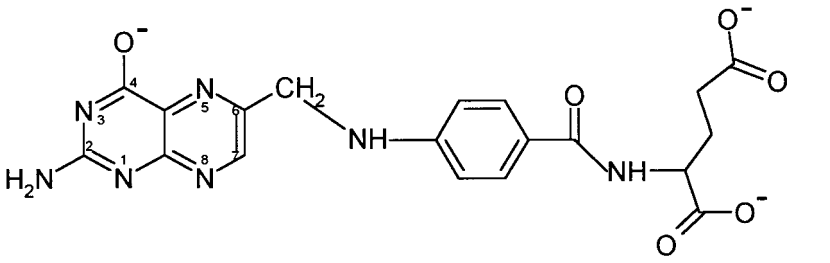


Fig. 1. *Molecular structure of folic acid in alkaline aqueous solutions* (pH > 8)

2-amino group or ring N-atoms) have pK_a values lower than 2 [7], and the pK_a of the carboxylic acid functions of the glutamic acid moiety of folic acid are lower than 5 [11].

The acid and base forms of the pterin moiety present differences in their electronic structures and charge densities that could also be reflected in its photochemical behavior. In fact, as reported in previous communications [12–18], the photochemistry as well as the photophysical behavior of the different acid–base forms of several pterin derivatives show significant differences.

There are few reports in the literature that deal with the photochemistry of folic acid. In 1949, *Lowry et al.* [17] employed indirect methods to identify products of photolysis of folic acid and proposed the formation of (4-aminobenzoyl)glutamic acid and 6-formylpterin (=2-amino-1,4-dihydro-4-oxopteridine-6-carboxaldehyde) as photoproducts. No information about the reaction pathway, the effect of O_2, or the quantum efficiency was reported. In a more-recent communication [10], we investigated the photochemistry of the acid form of this vitamin in aqueous solutions (pH 4.5–6.0). The photolysis quantum yields were reported, the photoproducts were identified (which were coincident with those proposed by *Lowry et al.* [17]), and some hints on its kinetic and mechanistic aspects were suggested. Indirect evidence for the role of singlet oxygen as a triggering agent for the photooxidation was shown, *i.e.*, the photodegradation of folic acid was inhibited by singlet-oxygen scavengers. The influence of 6-formylpterin, one of the photoproducts detected, on the rate of photodegradation was also described, *i.e.*, photodegradation was enhanced either when the concentration of 6-formylpterin increased during the photolysis or when this photoproduct was present in the initial solutions of folic acid. Therefore, excitation of 6-formylpterin led to more-efficient generation of singlet oxygen that induced the degradation of folic acid.

In the context of our investigations on photochemistry and photophysics of pterin derivatives [10][13][15][16][19] as well as potential biological implications [20], we now report the photolysis of folic acid in alkaline aqueous solutions, where only the base form of the molecule, the folate, is present (*Fig. 1*). Photoproducts were characterized, and the quantum efficiencies of the photodegradation of folate and of the formation of photoproducts as well as the effects of O_2 on these photochemical reactions were determined. The results were compared with those obtained for the photochemistry of folic acid in acid aqueous solution [10].

2. Experimental. – *Materials.* Folic acid (*Merck*) and the other pterin derivatives (*Schircks Laboratories*) were used without further purification. Solns. of folic acid stored in the absence of O_2 were stable (HPLC or TLC). Other chemicals from *Sigma-Aldrich* were used as received. The pH of aq. solns. was adjusted to 10.0–11.0 by adding drops of conc. HCl or NaOH solns. with a micropipette. The ionic strength was *ca.* 10^{-3} M in all the experiments.

Photolyses. The continuous photolysis of aq. folate solns. was carried out in the presence or in the absence of air. Deaerated solns. were obtained by bubbling with O_2-free N_2 for 20 min. *Rayonet RPR-3500*-Å lamps (*Southern N.E. Ultraviolet Co.*) were employed for irradiating. Photolysis products were identified or characterized by analyzing the absorbance changes during irradiation, by TLC, and by high performance liquid chromatography (HPLC).

UV/VIS Spectroscopy. UV/VIS Absorption spectra were collected with a *Cary 3* (*Varian*) spectrophotometer and refined with a program for smoothing and averaging signals. Quartz cells of 1-cm optical path length and double cells of 1 cm (for irradiation) and 0.2 cm (for absorbance measurements) were used. Absorption spectra of the solns. were recorded at regular intervals of irradiation time. From the absorbance changes recorded under these conditions, experimental difference (ED) and normalized experimental difference (NED) spectra were obtained. Reference difference (RD) and normalized reference difference (NRD) spectra were obtained from aq. solns. of commercial standards. The analysis based on these difference spectra is described elsewhere [10][13]. When necessary, further details are described in the Figure legends.

TLC Analyses. For anal. TLC, 100-μm *DEAE*-cellulose plates and aq. NH_4Cl solns. (0.3% (*w*/*v*) and 3% (*w*/*v*)) as eluent were employed. Irradiated folate solns. (450 μM) and standards of pterin derivatives were detected with 350-nm light. Under these conditions, most pterin derivatives show blue fluorescent spots. R_f Values obtained for several standard solns. under these conditions were reported elsewhere [10]. In some experiments, 250-μm cellulose plates with fluorescent indicator were employed to detect compounds that cannot be revealed by 350-nm light (*e.g.* (4-aminobenzoyl)glutamic and folic acid).

To separate some photolysis products, prep. TLC with 250-μm *DEAE*-cellulose plates were performed. To increase the amount of product, irradiated solns. of folate were concentrated at low pressure before carrying out the chromatographic runs. The same eluents as for anal. TLC were used. Products were extracted from the TLC plates with aq. solns. (0.5M HCl or 3% (*w*/*v*) NH_4Cl soln.). The solns. thus obtained were centrifuged and filtered before analysis.

HPLC Analyses. For monitoring the reaction and identifying photoproducts, a *Konic-Instruments 500-B* chromatograph, a *C18 Spherisorb-S5-ODS2* (250 × 46 mm) and a *Lambda-1000 Bischoff HPLC* spectrophotometer as detection system column were employed. The eluent flow rate was 1.0 ml/min. Eluent *1:* 7% MeCN/H_2O 6 : 4 (soln. *a*), 93% aq. soln. containing 20 mM potassium phosphate (pH 5.5) and 2.5 mM EDTA (soln. *b*). Eluent *2:* 5% MeOH, 95% aq. soln. containing 50 mM NaOAc and 5 mM citric acid (pH 5.2). Eluent *3:* 10% soln. *a*, 90% soln. *b*. Eluents *1* and *3* were employed for monitoring folic acid and other pterin derivatives at 340 nm, whereas eluent *2* was used for identification and quantification of (4-aminobenzoyl)glutamic acid at 280 nm.

Quantum Yields. The quantum yields for folate disappearance and 6-formylpterin formation were determined in experiments performed under different conditions. A chemical actinometer ($[Co^{III}Br(NH_3)_5]^{2+}$) was employed for the measurements of photon rates [21]. The initial concentration of reactant was adjusted so that more than 99.99% of the incident light at 350 nm was absorbed. The evolution of the concentrations of reactant and photoproducts during the irradiation time was followed by HPLC. Aq. solns. of commercial standards were employed for obtaining the corresponding calibration curves.

Oxygen Consumption. The O_2 consumption during the photolysis was measured with an O_2-selective electrode (*Orion*, model *37-08-99*). The experimental set-up for these measurements was described elsewhere [22].

Experiments in the Presence of Furfuryl Alcohol. Photolysis in the presence of 10 mM furfuryl alcohol (= furan-2-methanol; *Riedel-de Haën*) was carried out. This compound exhibits high reactivity towards singlet oxygen [23][24] reducing greatly its concentration when formed during the photolysis. In this group of experiments, folate and 6-formylpterin concentrations as a function of irradiation time were determined by HPLC. Results were compared with those obtained in the absence of the selective singlet-oxygen scavenger.

HPLC/MS Analyses. A *ThermoFinnigan-AS300* auto sampler connected to a pump *ThermoFinnigan*, model *TP 4000*, interfaced with a *LCQ-ThermoFinnigan* ion-trap mass spectrometer equipped with an ESI ion source was employed for MS and MS/MS in the negative-ion mode. Separations were obtained under gradient-elution conditions with a *RP-18-C18* column (*Lichrospher RP-18*, 250 × 4.6 mm, 5 μm particles (*Merck*, Darmstadt, Germany)) and a two-component mobile phase. Phase *A:* MeCN, phase *B:* aq. 0.05 mM NH_4OAc

(pH 6.8); flow rate 0.8 ml/min. Gradient conditions: *A*/*B* 10 : 90 at 0 min; 100% *A* at 30 min. High-purity N_2 was used as nebulizer and auxiliary gas. The heated capillary was set at 200° and the ion-optics parameters were optimized to the following values: spray voltage 4.5 kV, capillary voltage −8.0 V, tube-lens voltage 5.0 V, first-octapole voltage 2.25 V, inter-octapole-lens voltage 20.0 V, and second-octapole voltage 7.0 V.

Kinetic Studies. As a consequence of thermal chemical changes observed in irradiated solns. of folate, kinetic studies were carried out with a *SFA-20* stopped-flow accessory (*Hi-Tech*) and a *Cary-3* (*Varian*) spectrophotometer. Kinetic experiments were performed by mixing irradiated folate solns. or solns. of the photolysis products, separated previously by prep. TLC, with buffer solns. of K_2HPO_4/K_3PO_4 (0.025M) in the pH range 8–12. The pH was measured after mixing. Absorbance changes were recorded between 250 and 400 nm as a function of time, and rate constants were calculated as the average of at least 10 values obtained at different wavelengths.

3. Results and Discussion. – 3.1. *Preamble.* The behavior of the folate solutions under continuous irradiation in absence of O_2 is identical to that reported in the photolysis of the acid form of this vitamin, *i.e.*, folate does not undergo any chemical modification under irradiation at 350 nm in the absence of O_2.

3.2. *Evolution of the Spectrum of Folate Solutions.* As shown in *Fig. 2*,*a*, the spectrum of folate solutions changes significantly with irradiation time in the presence of O_2. However, most of the reported changes observed in the present study differ considerably from those observed in acid medium, where only the acid form of the pterin moiety is present [10]. The main differences are: *i*) In acid medium, NED and NRD spectra are almost indistinguishable. However, in alkaline medium, NED and NRD spectra are very different (*Fig. 2*,*b*). These results strongly suggest that, under these irradiation conditions, the folate anion does not yield the same photoproducts as found in acid media (6-formylpterin and (4-aminobenzoyl)glutamic acid), or that these compounds are not the only photoproducts.

ii) When the irradiated solutions of folate (pH 10–11) are stored in the dark, further chemical changes can be observed, as shown in *Fig. 3*. This reaction can be followed for a few hours *after interrupting the irradiation*. These results suggest that other photoproducts different from 6-formylpterin and (4-aminobenzoyl)glutamic acid are also present. These new photoproducts should be responsible for the observed thermal reactions. In addition, a strong dependence in the rate of spectral changes on the pH was detected; *i.e.*, the rate of spectral changes increases with the pH (see below, *Sect. 3.5*).

iii) Experiments were designed to investigate the role of O_2 in the thermal reaction. An air-saturated folate solution (450 μM) at pH 10.5 was irradiated during 50 min, then deareated by bubbling with N_2, and stored in the dark. The absorption spectra of the solution recorded at regular intervals of time after bubbling showed the same changes as observed in the presence of air, implying that O_2 is not involved in the observed thermal reaction.

3.3. *Characterization of the Photoproducts.* TLC Analyses showed the presence of four fluorescent products after irradiation. Two of the observed R_f values correspond to those of standard 6-carboxypterin (=2-amino-1,4-dihydro-4-oxopteridine-6-carboxylic acid) and 6-formylpterin, while the other two substances, **A** and **B**, could not be identified with any of the available standard pterin derivatives. As expected from previous studies on the photochemistry of pteridine compounds [19], 6-carboxypterin should arise in a later stage from the photolysis of 6-formylpterin. TLC Runs performed with plates containing fluorescent indicator showed the presence of a non-

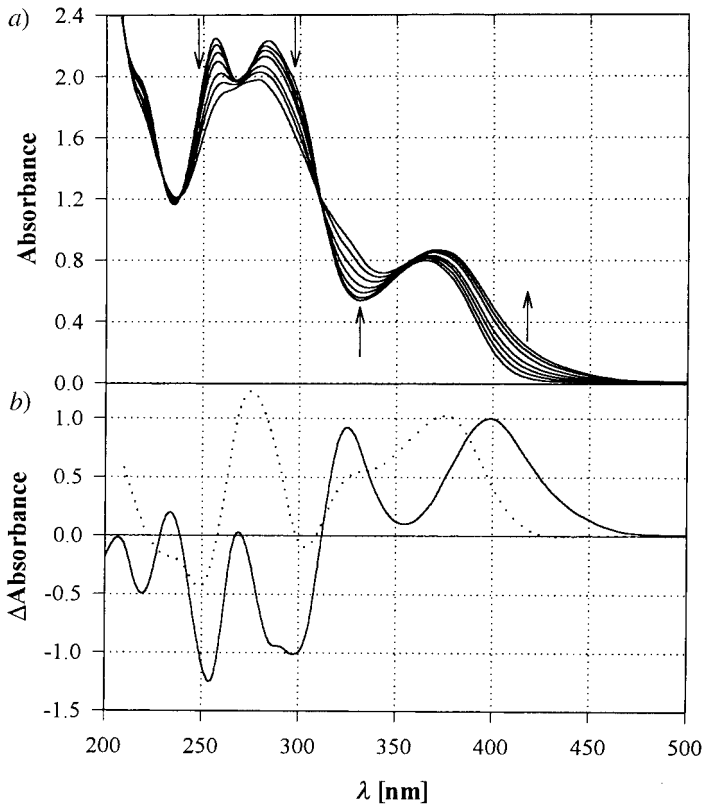


Fig. 2. a) *Evolution of the absorption spectrum of an irradiated solution of folate* (450 μM) *at pH 10.0 as a function of time* (air-saturated solutions, spectra recorded every 10 min; arrows indicate the observed changes). b) *NED Spectrum* (—) *obtained by subtracting the initial folate spectrum from the corresponding spectrum after 40 min of photolysis, and NRD spectrum* (·····) *obtained by subtracting the spectrum of a standard solution of folate from the spectrum of a standard solution of 6-formylpterin and (4-aminobenzoyl)glutamic acid, at the same concentration and pH.*

fluorescent product having a R_f value that corresponds to that of standard (4-aminobenzoyl)glutamic acid. Folate solutions, irradiated and then stored for several hours in the dark, contained no significant amounts of **A**, but increased amounts of **B** (by TLC). These results strongly suggest that the above-mentioned thermal reaction is the chemical transformation **A** → **B**.

HPLC Analyses revealed the presence of six substances in irradiated solutions of folate. Four of them were identified by comparing their retention times (t_R) with those of standard solutions, *i.e.*, 6-formylpterin, 6-carboxypterin, and folic acid (HPLC eluent *1*), as well as (4-aminobenzoyl)glutamic acid (HPLC eluent *2*). The other two substances could not be identified by comparison with the available standard solutions, which is in good agreement with the TLC results.

Solutions of substances **A** and **B** were obtained by prep. TLC (3% (*w*/*v*) NH_4Cl solution) from irradiated (50–60 min) solutions of folic acid. Substances **A** (R_f 0.63)

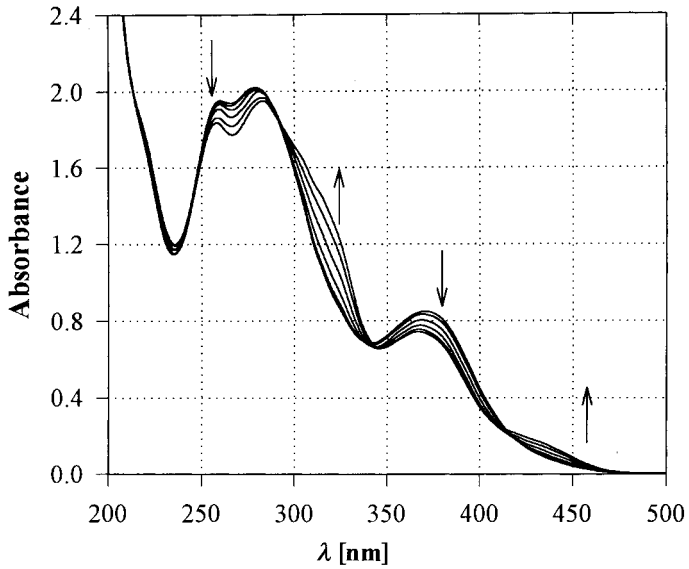


Fig. 3. *Evolution in the dark of the absorption spectrum of a folate solution* (450 μM) *at pH 11, irradiated for 50 min*. Experiment performed in the presence of O_2. After stopping the irradiation, the spectra were recorded at the following times: 0, 12, 30, 60, 105, and 150 min. Arrows indicate the observed changes.

and **B** (R_f *ca.* 0.02) were extracted from the plates with aqueous solutions (3% (*w*/*v*) NH_4Cl solution and 0.5M HCl, resp.), and normalized spectra of **A** and **B** in alkaline media were recorded (*Fig. 4,a*).

Substance **A** is stable in the absence of light at the pH of the NH_4Cl solution (pH 5.5), *e.g.*, no changes in the spectrum were detected after storing the solution for several days in the dark at room temperature. A similar spectral analysis of solutions of substance **B** revealed that this compound is stable in the dark in both acid and alkaline media.

However, substance **A** is not stable in the dark at pH > 8 since important changes in the spectrum were observed under these pH conditions. The rates of these changes showed strong dependence on the pH, being faster at higher pH (see *Sect. 3.5*). Alkaline solutions of **A** stored in the dark until completion of the thermal transformation furnished a product with a spectrum similar to that of substance **B** obtained by TLC. Moreover, also on TLC, the R_f value of this product was identical to that of **B**, and the NED spectrum obtained in this case (see *Fig. 4,b*) was similar to that obtained with irradiated solutions of folate (*e.g.*, experiment shown in *Fig. 3*). These results confirmed that **A** is transformed into **B** in alkaline media, and that this reaction is responsible for the spectral changes observed in irradiated solutions of folate after interrupting the irradiation.

Solutions of substances **A** and **B** were irradiated in alkaline media. No changes in the spectrum of **B** were detected after 2 h of irradiation. The changes in the spectrum of **A** were the same, irrespective of the irradiation. These results suggest that neither substance is photodegraded by UV-A light.

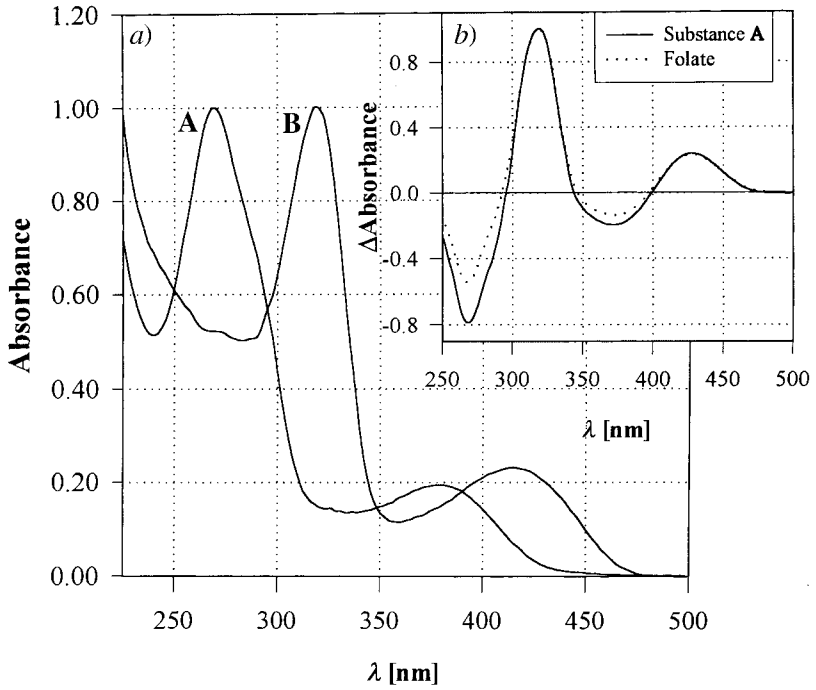


Fig. 4. a) *Normalized spectra of substances* **A** *and* **B** *in aqueous alkaline media.* (solutions obtained from prep. TLC). b) *NED Spectrum* (—) *obtained by subtracting the initial spectrum of an alkaline solution of* **A** (recorded immediately after alkalinizing a solution obtained by prep. TLC) *from the corresponding spectrum of the same solution, stored in the dark for several hours* (after completing the thermal transformation) *and NED spectrum* (···) *obtained by subtracting the initial spectrum of an irradiated solution of folate* (450 μM) *at pH 11* (recorded immediately after interrupting the irradiation) *from the corresponding spectrum of the same solution stored in the dark for several hours.*

Solutions of both products **A** and **B** and of folic acid were analyzed by HPLC/MS furnishing the molecular masses M_r 455 (*m*/*z* 454), 427 (*m*/*z* 426), and 441 (*m*/*z* 440), respectively. Oxidation of folic acid without cleavage could explain the increase in the molecular mass of **A**, which could be an epoxy derivative of folic acid. Substance **A** could suffer then a rearrangement with loss of the CO group of the pterin moiety (→ **B**). A more-detailed characterization of the chemical structure of substances **A** and **B** is the subject of future studies.

The MS/MS of **A** and **B** (*Fig. 5*) revealed that **A** loses H_2O (*m*/*z* 436) and, more importantly, a fragment of mass 28 (*m*/*z* 426) with a successive fragmentation pattern identical to that of **B**. There is agreement between this mass fragmentation and the chemical degradation pathway. Moreover, fragments at *m*/*z* 408 and 382 are formed, corresponding to losses of H_2O and CO_2 from *m*/*z* 426, the latter probably arising from one of the carboxylic groups of glutamic acid.

3.4. *Analysis of Concentration Profiles.* Foliate, 6-formylpterin, and 6-carboxypterin concentrations were measured as a function of irradiation time by means of two sets of HPLC analyses (eluent *1*): first, concentrated solutions of folate (> 500 μM) were

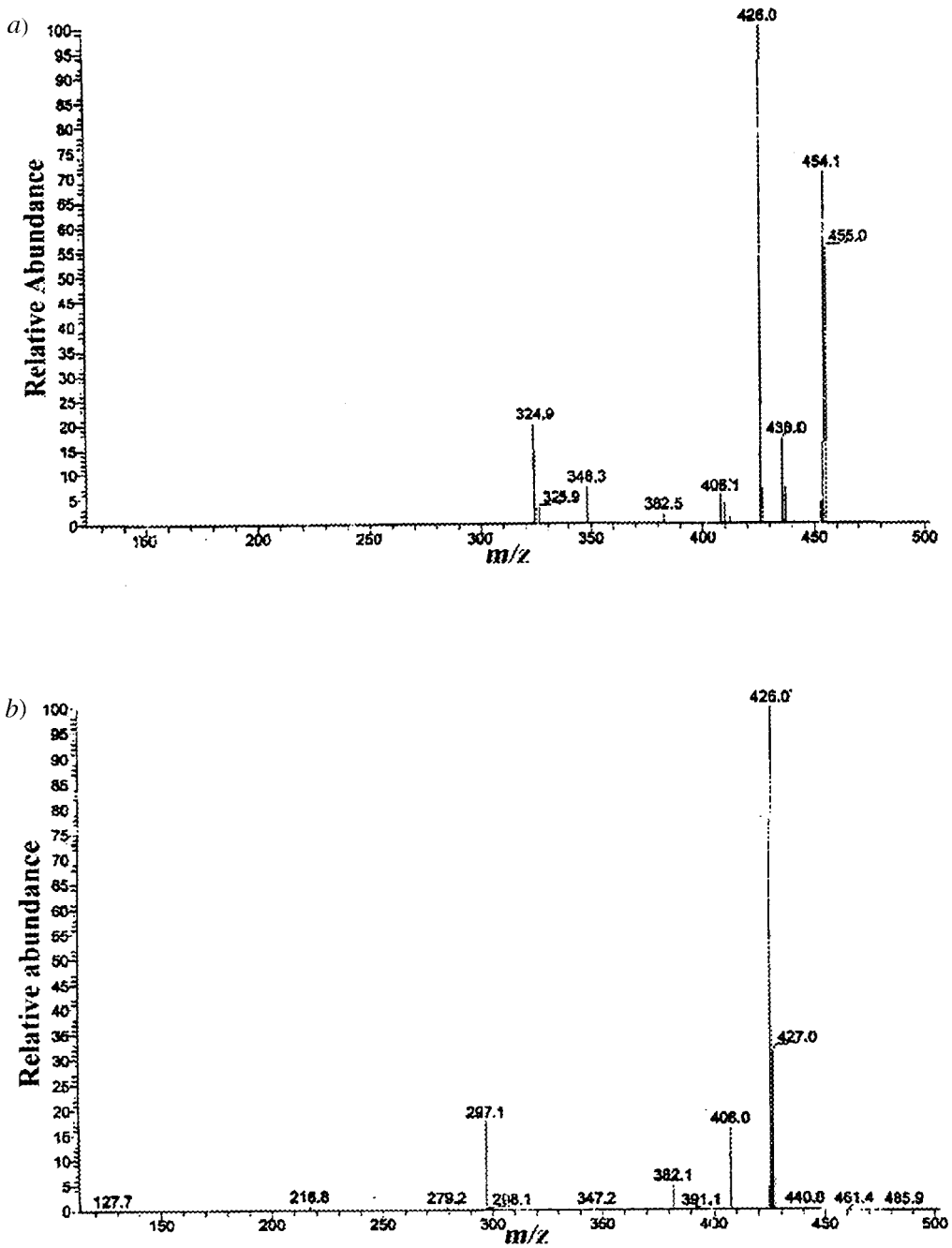


Fig. 5. *MS/MS of* a) *substance* **A** (*m*/*z* 454) *and* b) *substance* **B** (*m*/*z* 426). The corresponding solutions were obtained from prep. TLC.

irradiated and analyzed, and, second, folate/6-formylpterin mixtures were irradiated and analyzed.

The first group of analyses showed that, in the first 20 min of irradiation, the evolution of the concentrations of folate and 6-formylpterin follow a zero-order rate law (*Fig. 6*). In this first stage, no significant increase of the 6-carboxypterin concentration was observed, and it can be assumed that the whole incident radiation was absorbed by the folate. The quantum yield associated with the disappearance of folate was $5.1 \cdot 10^{-3}$, and that of 6-formylpterin formation was $2.9 \cdot 10^{-3}$. Therefore, consumption of folate to yield 6-formylpterin was not the only process taking place in the time range analyzed. After 20 min of irradiation, significant increases in the rate of photodegradation of folate and in the rate of formation of 6-formylpterin were observed (*Fig. 7*), coincident with a temporary increase in 6-carboxypterin concentration. As mentioned above, 6-carboxypterin is a photoproduct generated from 6-formylpterin [19]. Therefore, after *ca.* 20–30 min, a significant proportion of incident radiation was absorbed by 6-formylpterin, and the chemical transformation from folate to 6-formylpterin seems to be enhanced by the participation of excited 6-formylpterin. As can be seen in *Fig. 7*, during the first 60 min of irradiation under these conditions, the sum of folic acid, 6-formylpterin, and 6-carboxypterin concentrations was not constant, thus indicating that there is another pathway for folate photodegradation.

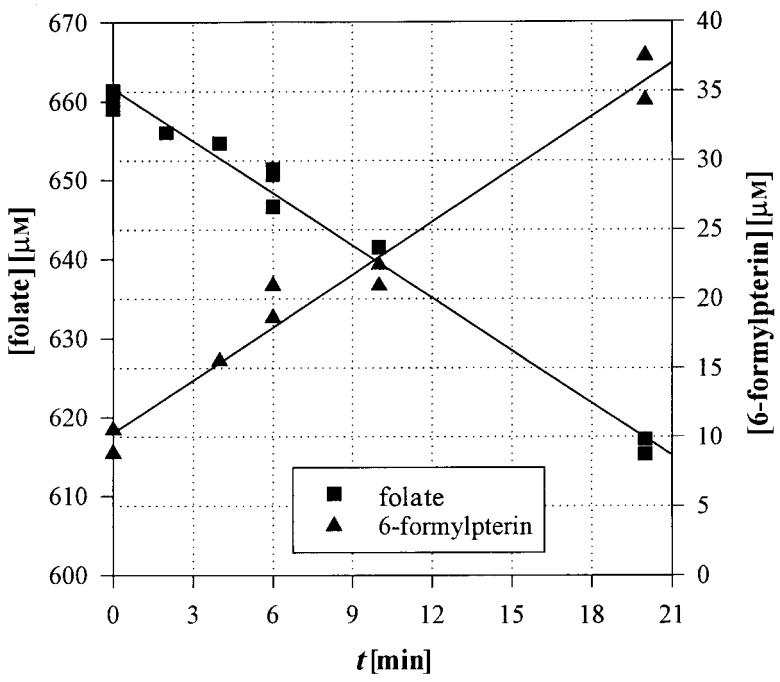


Fig. 6. *Changes in folate and 6-formylpterin concentrations in irradiated solution of folate* (660 μM) *at pH 11.0 as a function of time.* HPLC analysis of the first 20 min of photolysis.

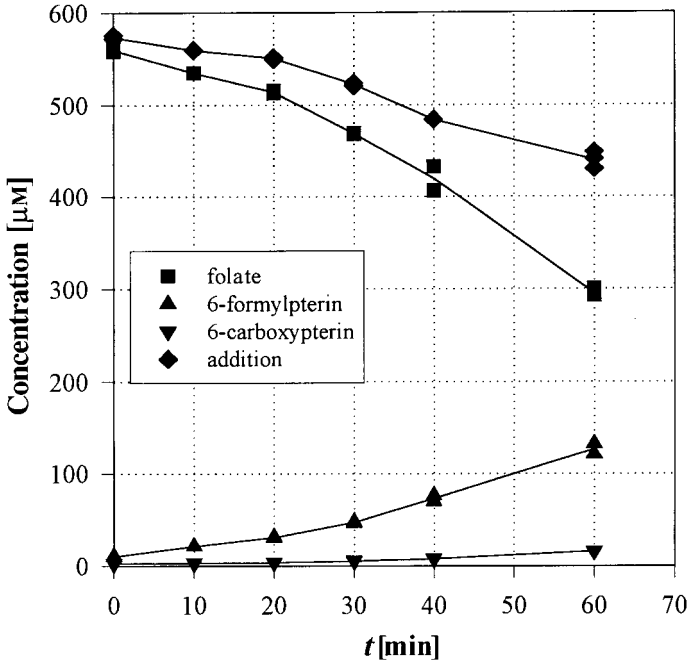


Fig. 7. *Changes in folate, 6-formylpterin, and 6-carboxypterin concentrations, determined by HPLC, in irradiated solution of 560* μM *folate at pH 11.0 as a function of time.* The algebraic sum of folate, 6-formylpterin, and 6-carboxypterin concentrations, for each time, is also shown (◆).

The second group of analyses were performed in such a way that only a low proportion of the incident radiation was absorbed by folate, whereas a higher proportion was absorbed by 6-formylpterin, *i.e.*, a mixture of folate (65 μM) and 6-formylpterin (150 μM) was irradiated (*Fig. 8*). An initial rate of consumption of 0.5 μM/min was estimated under these conditions, taking the rate of photons absorbed by folate as 23% of the incident photonic rate and the quantum yield of folate photodegradation reported above ($5.1 \cdot 10^{-3}$). However, a rate of 5.9 μM/min was obtained for the first 10 min of photolysis. These results confirm that absorption of radiation by 6-formylpterin induces the photodegradation of folate.

The concentration of (4-aminobenzoyl)glutamic acid was also measured by HPLC (eluent *2*) as a function of irradiation time. As expected, during the first 20 min of irradiation, the concentration follows a zero-order rate law, and, after 20 min, an increase of the rate of formation of (4-aminobenzoyl)glutamic acid was observed.

The relative concentration profiles of the two unidentified products **A** and **B** on irradiating solutions of folate (450 μM at pH 11.0) were determined by HPLC (eluent *3*) analyzing at a wavelength of 340 nm (t_R 3.2 (**A**) and 6.7 min (**B**)). The concentrations of both substances increased during the first 60 min of irradiation. Changes in the concentrations of **A** and **B** and folate were obtained as a function of time, after interrupting the irradiation (50 min) of a solution of folate (450 μM) at pH 11.0 (*Fig. 9*). In the analyzed time range, the concentration of **A** decreased while the concentration

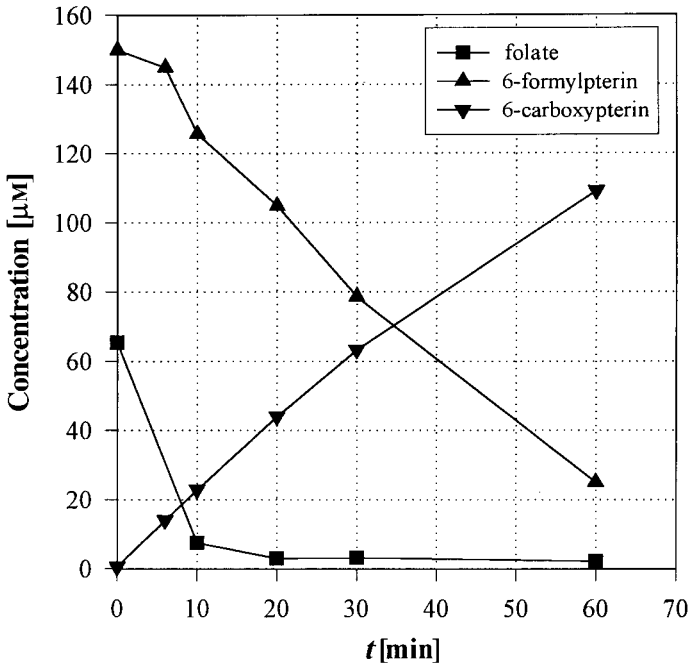


Fig. 8. *Changes in folate, 6-formylpterin, and 6-carboxypterin concentrations, determined by HPLC, in a solution of folate* (65 μM) *and 6-formylpterin* (150 μM) *at pH 11.0 as a function of the irradiation time*

of **B** increased, and no variation in folate concentration was observed. Other similar HPLC analyses (eluents *1* and *2*) showed that the concentrations of 6-formylpterin, 6-carboxypterin, and (4-aminobenzoyl)glutamic acid did not change after interrupting the irradiation. These results indicate that these substances, as well as folate, are not involved in the thermal reaction that occurs in irradiated solutions of folate.

3.5. *The Role of Oxygen and Kinetics.* A significant decrease in O_2 concentration during the photolysis was observed, indicating that photo-oxidation of folate is taking place. As shown in *Fig. 10*, after *ca.* 80 min of irradiation, an increase in the O_2 consumption rate was observed. The degradation of folate induced by 6-formylpterin or another product and the photo-oxidation of 6-formylpterin itself [19] may explain this observation.

In a previous work [10], we discussed the importance of singlet oxygen in the photochemistry of the acid form of folic acid. This reactive form of O_2 is formed in the presence of pterin derivatives, as previously reported [15][25]. Photolysis in the presence of furfuryl alcohol, a selective scavenger [23][24], was carried out to investigate whether singlet oxygen is involved in the mechanism of photodegradation of folate. The results obtained (not shown) indicate that both photodegradation pathways are strongly inhibited by furfuryl alcohol, suggesting that $^1O_2$ is involved as the active intermediate in photolysis of folate.

Kinetic experiments were performed by mixing irradiated solutions of folate (in most experiments, pH 9.5 – 10.0) or solutions of substance **A** (obtained by prep. TLC)

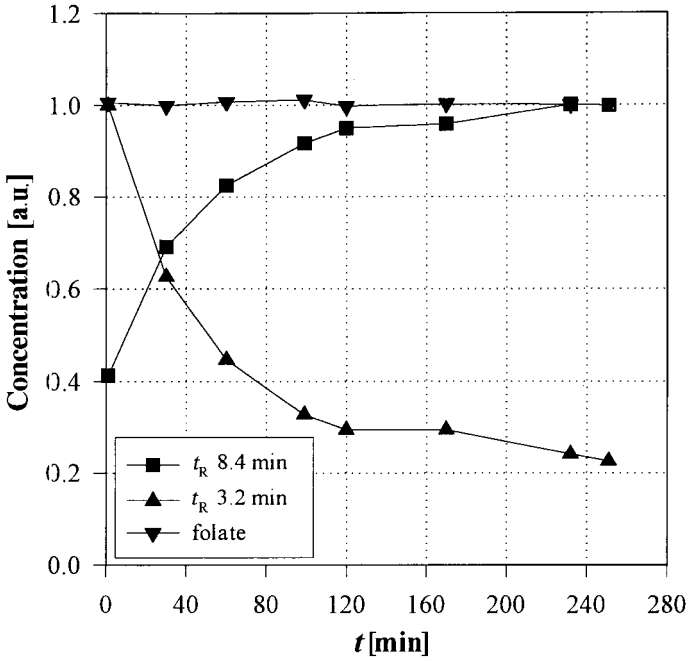


Fig. 9. *Changes in normalized concentrations of folate and substances* **A** (t_R 3.2 min) *and* **B** (t_R 8.4 min) *as a function of time, after interrupting the irradiation*. The folate solution (450 μM) at pH 11.0 was irradiated for 50 min and then analyzed by HPLC.

with buffer solutions of different pH values. Similar results were obtained in both groups of experiments. In all cases, only one process was observed in the dark. As shown in *Fig. 11*, this process follows a first-order rate law. In all experiments, the apparent rate constant (k_{app}) was found to be independent of wavelength. On the other hand, k_{app} showed a strong dependence on the pH, depending linearly on the OH^- concentration (*Fig. 12*).

Taking these results into account, the following rate law can be written: $-d[\mathbf{A}]/dt = k \cdot [OH^-] \cdot [\mathbf{A}]$, where **A** is the reactant of the thermal reaction and k is the second-order rate constant. A value of 1.1 M^{-1} s^{-1} was obtained for k from the data of *Fig. 12*.

4. Conclusions. – The results obtained are summarized in the *Scheme*. Some aspects of the photochemistry of the base form of folic acid, *i.e.*, of folate, are similar to those observed for its acid form, *i.e.*, in the absence of O_2, folate is photostable, whereas, in the presence of O_2, folate undergoes cleavage, yielding 6-formylpterin and (4-aminobenzoyl)glutamate as photoproducts [10]. However, the most important differences are associated with the presence of a new reaction pathway, leading to at least one new compound **A** having an M_r (455) higher than that of folic acid (441). This increase in the molecular mass may be due to the incorporation of an O-atom into the folate anion and the loss of two H-atoms. This reaction does not involve cleavage of the folate

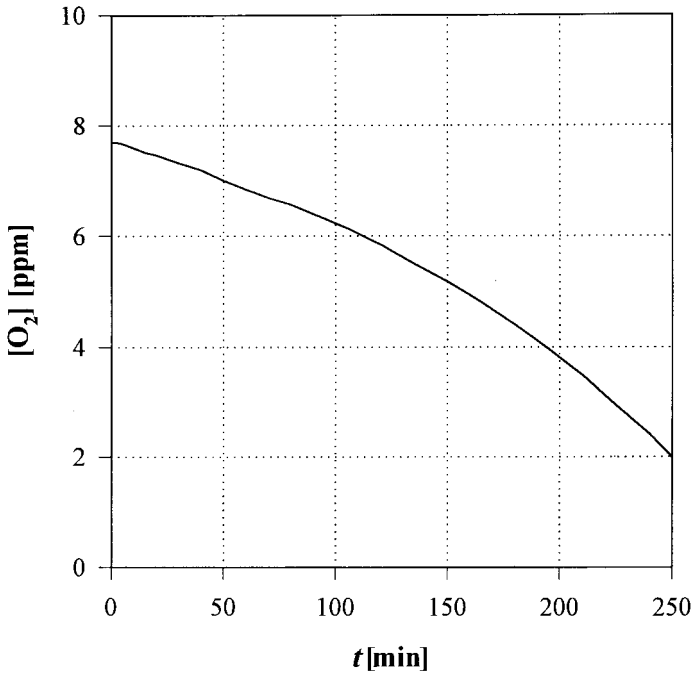


Fig. 10. *Changes in the O_2 concentration in an irradiated solution of folate* (450 μM) *at pH 11.0 as a function of time*

anion. Further studies aimed at complete identification of the structures of substances **A** and **B** are in progress. The folate behavior shows that this kind of molecule presents different reactivities in the base and acid forms as a consequence of the different charge distribution in the two forms.

Substance **A** is photostable, but, in alkaline media, it undergoes a dark thermal reaction yielding product **B**, which has lower molecular mass (427), whereby O_2 is not involved. The difference of M_r of **A** and **B** suggests a complex mechanism that may involve an internal rearrangement with loss of CO. This process follows a first-order rate law and the corresponding apparent rate constant (k_{app}) shows a linear dependence on the OH^- concentration.

In both acidic and basic media, singlet oxygen, very likely produced by energy-transfer from the electronically excited states of folic acid, is the oxidizing agent that starts the degradation reactions.

The presence of 6-formylpterin, either photochemically generated or added into the folic acid solution before irradiation, accelerates both photo-oxidation pathways. These processes require photoexcitation of 6-formylpterin, do not require the absorption of light by the folate anion, and are inhibited by a singlet oxygen scavenger. Therefore, excitation of 6-formylpterin leads to more efficient generation of singlet oxygen that induces the photolysis of folic acid by means of the two reactions described.

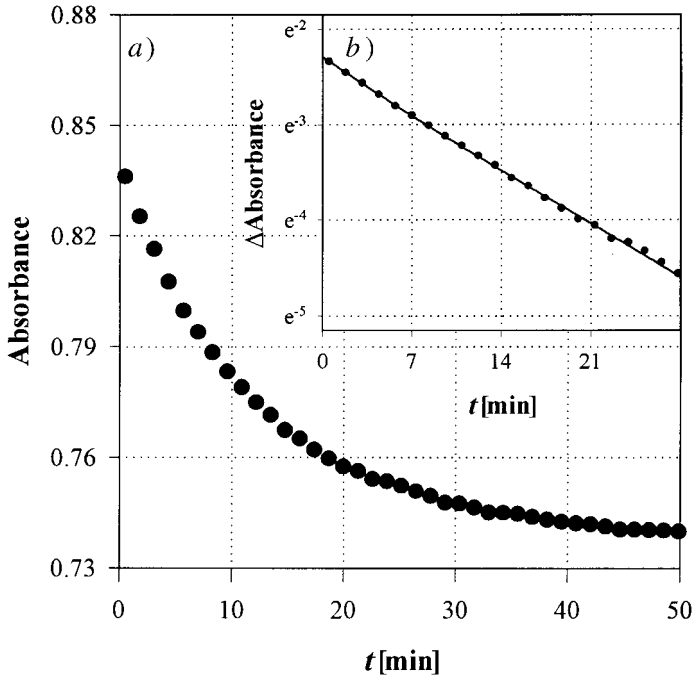


Fig. 11. *Experiment performed by mixing a folate solution* (pH 10.0) *irradiated for 50 min with phosphate buffer* (pH 10.5 after mixing): a) *Absorbance changes recorded at 374 nm as a function of time and* b) *first-order behavior of difference absorbance at 374 nm*

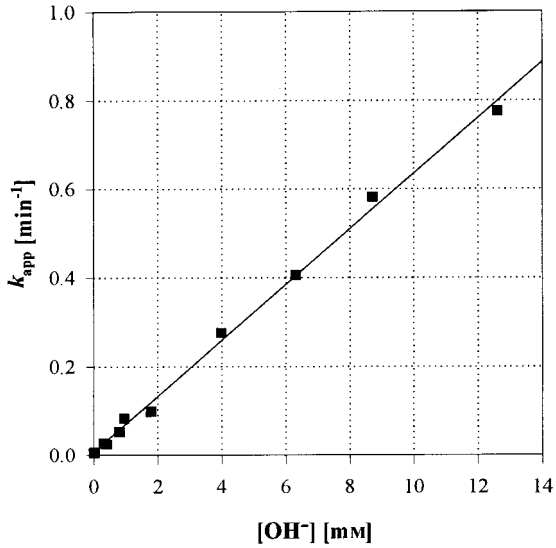
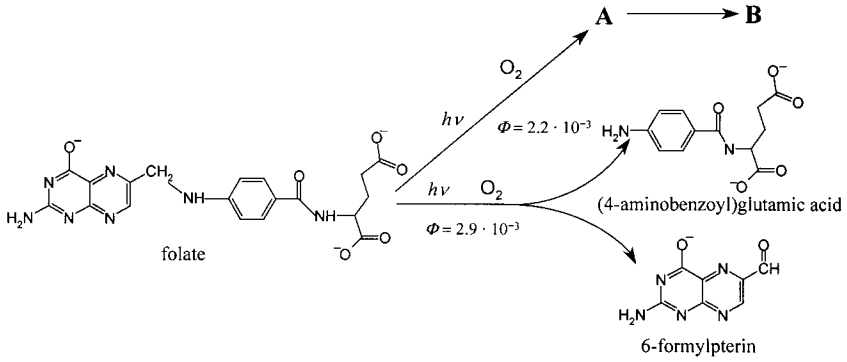


Fig. 12. *Linear dependence of* k_{app} *as a function of* OH^- *concentration*

Scheme 1



This work was supported in part by a project grant from the *Agencia de Promoción Científica y Tecnológica de Argentina* (*ANPCyT* Grant N° PICT 06-03531), *Consejo Nacional de Investigaciones Cientificas y Tecnológicas* (*CONICET*-PIP 4354), and Universidad Nacional de La Plata. *A. H. T.* wishes to thank the *Consejo Nacional de Investigaciones Cientificas y Tecnológicas de Argentina* (*CONICET*) for a research graduate grant. *C. B.* and *E. P.* wish to thank *MURST* (Rome).

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Received February 20, 2002