Methylenediphosphonate, a Metabolic Poison in *Dictyostelium discoideum*. ^{31}P NMR Evidence for Accumulation of Adenosine 5′-(β,γ-Methylenetriphosphate) and Diadenosine 5′,5‴-P^1,P^4-(P^2,P^3-Methylenetetraphosphate)†

Gérard Klein,*,‡ Jean-Baptiste Martin,§ and Michel Satre‡

DRF/LBIO/Biochimie (UA 1130 du CNRS) and DRF/SPh/Résonance Magnétique en Biologie et en Médecine, Centre d'Etudes Nucléaires, 85X, 38041 Grenoble Cedex, France

Received September 14, 1987; Revised Manuscript Received November 3, 1987

ABSTRACT: The pyrophosphate analogue methylenediphosphonate efficiently inhibited the growth of the amoebae of the slime mold *Dictyostelium discoideum*. The mechanism of the toxicity of methylenediphosphonate has been investigated in vivo by ^{31}P NMR. The formation of nonhydrolyzable methylene analogues of ATP and diadenosine tetraphosphate, adenosine 5′-(β,γ-methylenetriphosphate) and diadenosine 5′,5‴-P^1,P^4-(P^2,P^3-methylenetetraphosphate), at the expense of cellular nucleoside triphosphates has been observed. These two compounds were identified from their ^{31}P NMR spectra in perchloric acid extracts from amoebae poisoned with methylenediphosphonate and may have been synthesized by reversible pyrophosphate exchange catalyzed by aminoacyl-tRNA synthetases.

The effects of compounds such as the P_i[1] analogue methylphosphonate (MP) and the PP_i analogues methylenediphosphonate (PCP) and imidodiphosphate (PNP) upon vegetative cells of *Dictyostelium discoideum* have been tested as part of a program to screen various phosphorylated substances for potential use in ^{31}P NMR as intracellular pH indicators (Gadian et al., 1982; Gillies et al., 1982). Although PCP could not be used to probe intracellular pH since its chemical shift scarcely varied with pH in the range pH 5–9 (δ = 16.1 ppm at pH 5 and δ = 16.4 ppm at pH 9), it surprisingly inhibited the growth of the amoebae of the slime mold *D. discoideum*. The ^{31}P NMR technique was used to investigate its mechanism of toxicity, and the formation of Ap_2Cp and Ap_2Cp_2A, the methylene analogues of ATP and Ap_4A, is demonstrated.

Experimental Procedures

Culture Conditions. D. discoideum, strain AX2 (ATCC 24397), was grown axenically at 22 ± 1 °C in peptone–yeast extract medium (Watts & Ashworth, 1970) supplemented with 18 g/L maltose. Amoebae were harvested in their exponential phase of growth (from 5×10^6 to 1×10^7 cells/mL) by centrifugation at 800*g* for 4 min in a Jouan GR 4.11 centrifuge at 4 °C. Cells were washed with ice-cold 20 mM Mes–Na buffer, pH 6.3, and the packed cell pellet (about 10^{10} cells) was stored on ice until NMR measurements.

Perchloric Acid Extracts. Perchloric acid extracts were performed with CDTA as chelating agent (Bass & Fromm, 1985). The extraction procedure involved the addition of 2.5 mL of ice-cold perchloric acid (70%) directly to the 20-mL cell suspension. The solution was kept on ice for 15 min and centrifuged for 10 min at 40 000 rpm at 4 °C in a Beckman R40 rotor. The supernatant was carefully decanted, adjusted to 7 mM CDTA, neutralized with KOH, and stored on ice for 15 min. Potassium perchlorate was eliminated by a centrifugation at 40 000 rpm for 15 min, and the supernatant was lyophilized. The freeze-dried sample was resuspended in 3 mL of 6% D_2O (v/v) and 40 mM Tris-HCl at a final pH of 8.5, and insoluble residues were eliminated by centrifugation.

^{31}P NMR Spectroscopy of Whole Cells and Perchloric Acid Extracts. In vivo NMR spectra of whole cells were obtained at 22 °C on a Bruker WM200 WB spectrometer at 81.01 MHz in 25-mm tubes in a final volume of 20 mL. General NMR conditions for living cells were described before (Satre & Martin, 1985; Satre et al., 1986; Martin et al., 1987). A capillary containing 50 mM MP, pH 6.3, was added as an internal chemical shift reference at 24.3 ppm. NMR spectra of perchloric acid extracts were recorded in 10-mm tubes at 22 °C on a Bruker WM250 spectrometer at 101.27 MHz with spinning of the tube at 12 Hz. Acquisition conditions used 10-μs pulses at 4-s intervals. Two levels of decoupling were used: 2.5 W for 0.48 s and 0.5 W for 3.52 s. The number of accumulated scans was 1800. Free induction decays were accumulated by using 8K data points and were zero-filled to 32K prior to Fourier transformation. A line broadening of 0.5 Hz was applied. Specific conditions are described in the legends of the figures. All chemical shifts are given relative to 85% orthophosphoric acid at 0 ppm.

Simulation of ^{31}P NMR Spectra. The ^{31}P NMR spectra were analyzed as an AKL system for Ap_2Cp and as an AA′XX′ system for Ap_2Cp_2A (Pople et al., 1959). Simulated NMR spectra were calculated on the Aspect 2000 computer (Bruker) operating the Parameter Adjustment in NMR by Iterative Calculation (PANIC) program and plotted with a line width of 1.7 Hz. Chemical shifts and coupling constants used initially for the simulations were obtained from the spectra

† This work was funded in part by grants from the Centre National de la Recherche Scientifique (UA 1130) and the Commissariat à l'Energie Atomique.

* Address correspondence to this author.

‡ DRF/LBIO/Biochimie (UA 1130 du CNRS), Centre d'Etudes Nucléaires.

§ DRF/SPh/Résonance Magnétique en Biologie et en Médecine, Centre d'Etudes Nucléaires.

[1] Abbreviations: ApCp, adenosine 5′-(α,β-methylenediphosphate); $ApCp_2$, adenosine 5′-(α,β-methylenetriphosphate); Ap_2Cp, adenosine 5′-(β,γ-methylenetriphosphate); Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; Ap_4N, adenosine nucleoside 5′,5‴-P^1,P^4-tetraphosphate; Ap_2Cp_2A, diadenosine 5′,5‴-P^1,P^4-(P^2,P^3-methylenetetraphosphate); CDTA, 1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid; MP, methylphosphonate; P_i, inorganic phosphate; PCP, methylenediphosphonate; PNP, imidodiphosphate; PP_i, pyrophosphate; Mes, 2-(*N*-morpholino)-ethanesulfonic acid.

0006-2960/88/0427-1897$01.50/0

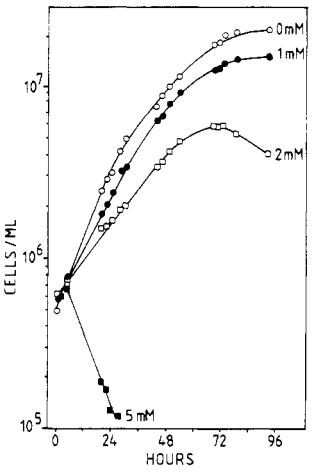

FIGURE 1: Effect of PCP on the growth of *D. discoideum* in axenic medium. The following PCP concentrations were used: (●) 1 mM; (□) 2 mM; (■) 5 mM; and (○) control without PCP.

of perchloric acid extracts of the PCP-poisoned cells for Ap_2Cp_2A and Ap_2Cp.

Origin of Chemicals. Adenosine 5′-(α,β-methylenediphosphate), adenosine 5′-(α,β-methylenetriphosphate), and adenosine 5′-(β,γ-methylenetriphosphate) were obtained from Pharmacia Biochemicals. Methylenediphosphonic acid and imidodiphosphate were purchased from Sigma. Methylphosphonic acid was from Alfa-Ventron.

RESULTS

Inhibition of the Growth of Dictyostelium discoideum Amoebae by PCP. Various concentrations of PCP in the range of 1–5 mM were tested for their effect on the growth of *D. discoideum* amoebae in axenic medium. Representative growth curves are shown in Figure 1. Control amoebae grew with a doubling time of 9 h at 21 °C and reached a plateau of 2×10^7 cells/mL. At a concentration of 1 mM, PCP only slightly inhibited the growth (doubling time = 12 h, plateau at 1.5×10^7 cells/mL), and at 2 mM PCP, inhibition was already significant with a doubling time increased to 19 h and a 3-fold lower cell yield (6×10^6 cells/mL). A slow lysis phase of the cells (half-time = 38 h) started once the plateau was reached. A strong inhibitory action of PCP was observed at a concentration of 5 mM as growth was stopped completely 4 h after the addition of PCP. Cells then started to lyse with a half-time of 10 h. The inhibitory effect of PCP was due to cell death and not simply to a growth stasis since, if the cells were removed from the axenic medium containing 5 mM PCP after 8 h of incubation and suspended in new medium without PCP, they did not resume their growth and lysis progressed with the same half-time as a PCP-containing sample.

For comparison purposes, imidodiphosphate (PNP), another pyrophosphate analogue, was tested for inhibitory effects. At a concentration of 5 mM it had no effect on growth. In addition, the phosphate analogue methylphosphonate (MP) at concentrations as high as 20 mM only caused a 10% decrease in cell yield.

In Vivo ^{31}P NMR Evidence for the Synthesis of New Phosphorylated Compounds during Poisoning with PCP. The time-dependent effect of 5 mM PCP on the synthesis of phosphorylated compounds by aerobic cells suspended in a nonnutritive buffer (20 mM Mes–Na, pH 6.3) was followed by ^{31}P NMR. The in vivo spectrum of cells incubated with PCP was compared to that of control cells. Besides the huge peak at 16.1 ppm corresponding to added PCP itself, two new peaks progressively appeared at about 12 and 9 ppm (Figure 2B, peaks 1 and 2; see arrows). They occurred in regions of the spectrum in which no natural phosphorylated compounds resonated (Jentoft & Town, 1985; Satre & Martin, 1985; Kay et al., 1986). At the same time, an increase in intensity of the peak at −11 ppm (peak 3) was observed.

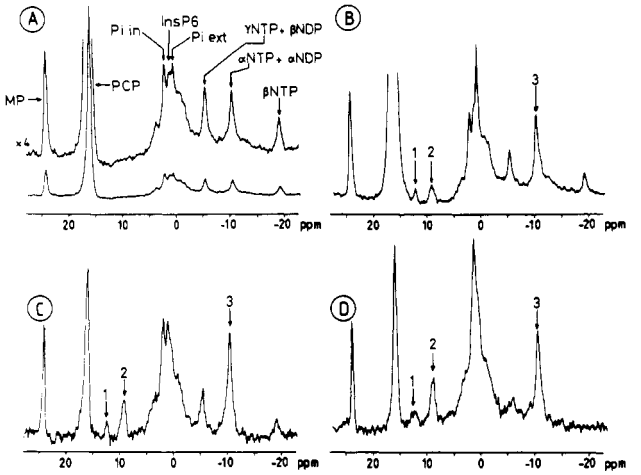

FIGURE 2: In vivo ^{31}P NMR spectra of *D. discoideum* amoebae incubated with PCP. Cells (4.5×10^8 cells/mL) were resuspended in 20 mL of 20 mM Mes–Na, pH 6.3, under oxygen. PCP was then added at a concentration of 5 mM, and ^{31}P NMR spectra were recorded every 15 min on a Bruker WM200 WB spectrometer (750 scans, 1.2-s interpulse interval). Representative spectra of the periods 0–15 and 135–150 min are shown in (A) and (B). Methylphosphonate (MP) was used as an external shift reference at 24.3 ppm. The position of inositol hexaphosphate (InsP6) is indicated. Attribution of the peaks was described elsewhere (Martin et al., 1987). Arrows 1–3 corresponded to newly formed compounds. PCP was then removed by centrifugation and washing of the cells. A 15-min ^{31}P NMR spectrum of the cells resuspended in fresh 20 mM Mes–Na buffer, pH 6.3, is represented in (C). Oxygen bubbling was finally replaced by nitrogen bubbling, and the spectrum of the cells in these anoxic conditions is shown in (D).

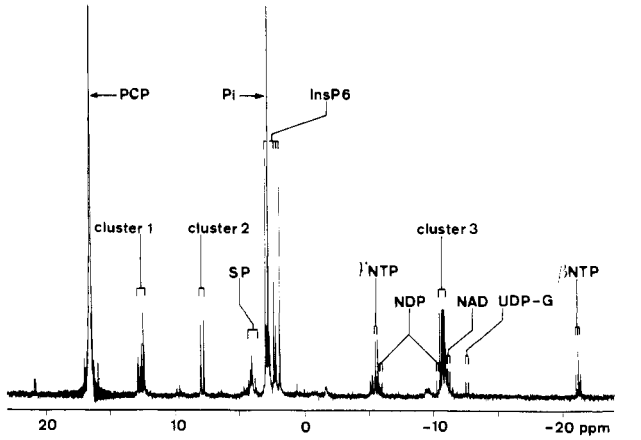

FIGURE 3: Proton-decoupled ^{31}P NMR spectrum of perchloric acid extract of *D. discoideum* cells incubated with PCP. A perchloric acid extract was prepared from aerobic cells that had been in contact with 5 mM PCP for 2 h (1.2×10^{10} cells, 20 mL). Its spectrum was then recorded on a Bruker WM250 spectrometer (1800 scans, 4-s repetition time). SP = sugar monophosphates (phosphomonoesters).

Identification of Ap_2Cp and Ap_2Cp_2A by ^{31}P NMR in Perchloric Acid Extracts of PCP-Poisoned Amoebae. The ^{31}P NMR spectrum of a perchloric acid extract of cells poisoned with PCP for 2 h is shown in Figure 3. As compared to an extract of control cells (Martin et al., 1987), and besides the peak corresponding to PCP itself, new peaks showed up in three clusters around 12, 8, and −11 ppm. Other minor peaks were also present in much smaller amounts, and their identification was not attempted in this work. The three major unknown clusters in the spectrum of the cellular extract

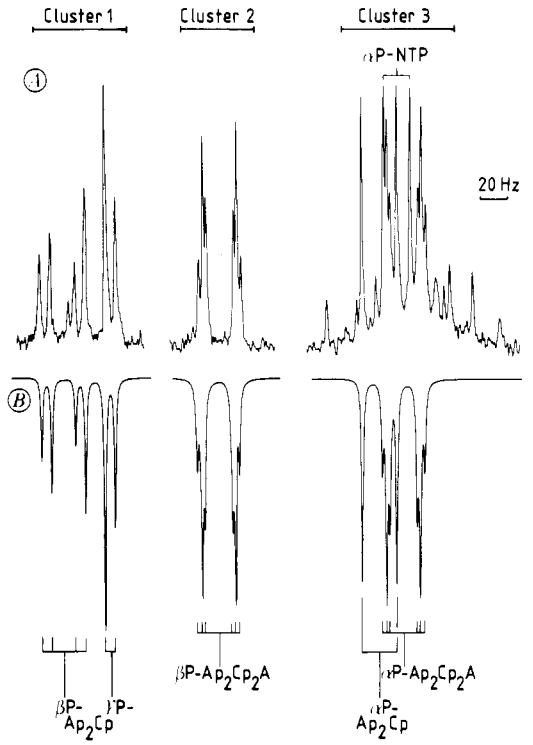

FIGURE 4: Identification of Ap_2Cp and Ap_2Cp_2A in the perchloric acid extract. In the upper part of the figure (A) are shown blowups of the ^{31}P NMR spectral regions of the perchloric acid extract corresponding to clusters 1–3 (see Figure 3). The resonance lines in cluster 3 corresponding to the α-phosphates of NTP are labeled. In the lower part (B) the PANIC-simulated spectra of Ap_2Cp and Ap_2Cp_2A are drawn at the same scale, but in an inverted position. At the level of cluster 3, the figure drawn corresponds to the sum of the two simulated spectra of Ap_2Cp and Ap_2Cp_2A. The β-phosphate (four resonance lines) and γ-phosphate (two resonance lines) of Ap_2Cp resonated in cluster 1 at 12 ppm whereas its α-phosphate had two resonances lines in cluster 3 at −11 ppm. The β-phosphates of Ap_2Cp_2A resonated as a complex doublet in cluster 2 at 8 ppm, and its α-phosphates had the symmetrical structure in cluster 3, characteristic of an AA′XX′ system.

corresponded well to the new peaks detected in the in vivo spectrum. Although the peak at −11 ppm in the in vivo spectrum from amoebae had exactly the same resonance position as the α-phosphate of NTP and NDP (Figure 2), in the perchloric acid extract it had a complex fine structure, showing that this cluster contained the resonance lines of the α-phosphate from nucleoside di- and triphosphates as well as that of new compounds (Figure 4).

The identification of the unknown compounds was aided by the following two facts: first, substitution of a P–O bond by a P–C bond in ADP or ATP, leading to methylene analogues of ADP or ATP, causes a downfield shift of 20–30 ppm for the resonances of the P atoms flanking the $-CH_2-$ group (Granot et al., 1980; Schliselfeld et al., 1982; Vogel & Bridger, 1982), and second, upon various metabolic stresses cells accumulate dinucleoside polyphosphates such as Ap_4A [see Zamecnik (1983) for a review].

One of the unknown compounds was thus found to be Ap_2Cp. When compared to a ^{31}P NMR spectrum of authentic Ap_2Cp, the resonance lines contained in cluster 1 around 12 ppm in the perchloric acid extracts of PCP-poisoned cells corresponded strictly, except for a single minor line, to the six resonance lines of the β-phosphate (four lines) and γ-phosphate (two lines) of Ap_2Cp. Furthermore, the α-phosphate of Ap_2Cp produced the expected doublet, identified in cluster 3 at −11 ppm. The definitive attribution was made by directly adding authentic Ap_2Cp to the perchloric acid extract. This led to

Table I: ^{31}P NMR Chemical Shifts (δ) and Coupling Constants (J) of Ap_2Cp_2A and Ap_2Cp[a]

	pH	δ(1) (ppm)	δ(2) (ppm)	δ(3) (ppm)	δ(4) (ppm)	ref
Ap_2Cp_2A	8.5	−10.85	7.79	7.79	−10.85	*c*
	–[b]	−11.0	7.42	7.42	−11.0	*d*
Ap_2Cp	8.5	−10.71	12.61	12.27		*c*
	8.0	−10.2	12.0	14.0		*e*
	8.2	−10.30	11.07	14.05		*f*
	8.5	−11	10.0	13.5		*g*

	pH	J(12) (Hz)	J(23) (Hz)	J(34) (Hz)	ref
Ap_2Cp_2A	8.5	25.3	5.5	25.3	*c*
	–	25.6	–	25.6	*d*
Ap_2Cp	8.5	26.3	7.7		*c*
	–	26.5	8.0		*e*
	8.2	26.1	8.0		*f*
	7.7	26.9	7.4		*g*

[a] The numbers attributed to the phosphates were as follows:

AA′XX′ system (Ap_2Cp_2A) A–O–P–O–P–C–P–O–P–O–A
1 2 3 4

AKL system (Ap_2Cp) A–O–P–O–P–C–P–OH
1 2 3

The iterated coupling constants J(13), J(14), and J(24) were always smaller than 0.4 Hz. [b] A dash (–) indicates value not reported. [c] This work. [d] Tarussova et al., 1983. [e] Vogel & Bridger, 1982. [f] Granot et al., 1980. [g] Schliselfeld et al., 1982.

the simultaneous increase of the eight resonance lines attributed to Ap_2Cp in the spectrum of the perchloric acid extract with no evidence of any extra lines in other positions of the spectrum. Confirmatory evidence for the chemical shift assignments and the multiplet structure was obtained by simulation of the ^{31}P NMR spectrum of Ap_2Cp as an AKL system with the α-phosphate (A), β-phosphate (K), and γ-phosphate (L) as illustrated in Figure 4B.

To the cluster at about 8 ppm, which consisted of a complex doublet of a main peak with two shoulders (Figure 4A), corresponded the symmetrical structure in the −11 ppm region in an uncoupled ^{31}P NMR spectrum. This was reminiscent of the ^{31}P NMR spectrum of Ap_2Cp_2A with its AA′XX′ structure in which the pattern in the end-group region is the mirror image of that in the middle-group region (Tarussova et al., 1983). Ap_2Cp_2A was therefore analyzed as an AA′XX′ system with the two α-phosphates A and A′ and the two β-phosphates X and X′. Its simulated ^{31}P NMR spectrum obtained with the PANIC program corresponded very accurately to the experimental spectrum with the symmetrical structures in the clusters at 8 and −11 ppm (Figure 4B). The β-phosphates of Ap_2Cp_2A resonated at the level of cluster 2 at 8 ppm, and the α-phosphates corresponded to the symmetrical structure at the level of cluster 3 at −11 ppm. The four other lines in cluster 3 were accounted for by the α-phosphates of ATP and of Ap_2Cp.

Table I summarizes the iterated chemical shifts (δ) and coupling constants (J) of Ap_2Cp_2A and of Ap_2Cp. Our results were in general agreement with previously published data (Granot et al., 1980; Schliselfeld et al., 1982; Tarussova et al., 1983; Vogel & Bridger, 1982). The small differences can be attributed to different pHs and ionic compositions of the solutions.

Intracellular Levels of PCP, Ap_2Cp, and Ap_2Cp_2A. Cells efficiently internalized PCP during the incubation. The resonance peak of PCP was clearly detected in cells that had been in contact with PCP and had been washed twice afterward in a medium without PCP. From the integrated intensity of that PCP resonance peak (Figure 2C), and by use of a cellular volume of 520 μm^3 (Klein & Satre, 1986), the intracellular

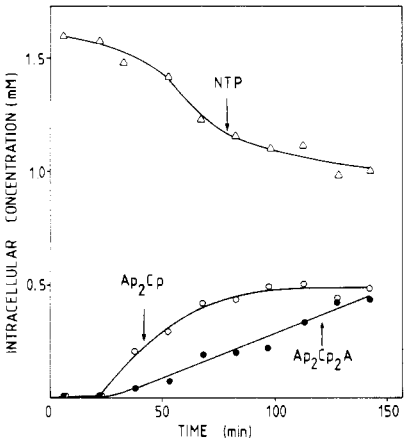

FIGURE 5: Kinetics of appearance of Ap_2Cp and Ap_2Cp_2A and of disappearance of NTP. The variations in the concentrations of Ap_2Cp, Ap_2Cp_2A, and nucleoside triphosphates are represented as a function of the incubation time with PCP. The concentrations of Ap_2Cp and Ap_2Cp_2A were calculated in the successive ^{31}P NMR spectra (see Figure 2) by comparison of the surface of their peaks with the surface of the β-phosphate of NTP which, at $t = 0$, represented 1.6 mM. For the calculations, it was taken into account that the peak at 12 ppm represented the γ-phosphate of Ap_2Cp (one phosphate group) and that the peak at 9 ppm corresponded to the β-phosphates of Ap_2Cp_2A (two phosphate groups).

level of PCP reached about 4 mM after 2.5 h of incubation, a value quite close to the concentration of external PCP. This level of intracellular PCP ensured that it was not a contaminating signal arising from residual external PCP. Also, if the wash step was performed immediately after the addition of PCP, no PCP was visible on a ^{31}P NMR spectrum of washed cells. The intensities of NMR peaks 1–3 corresponding to Ap_2Cp and Ap_2Cp_2A were not reduced by the washing step and a further incubation in a PCP-free medium, indicating that they were fully retained within the cell cytoplasm. In contrast, intracellular PCP was not retained inside the cell but was released into the medium.

After a lag time of about 30 min, intracellular concentrations of Ap_2Cp and Ap_2Cp_2A progressively increased as a function of incubation time with PCP (Figure 5). Ap_2Cp was synthesized with a higher initial rate than Ap_2Cp_2A but leveled off to 0.5 mM after about 90 min of incubation, while Ap_2Cp_2A level was still steadily increasing and reached about 0.5 mM after 2.5 h of incubation. A possible precursor–product relationship was further supported by an experiment in which cells poisoned with PCP were washed and incubated in a medium without PCP; as expected, it was found that Ap_2Cp was progressively converted into Ap_2Cp_2A. At the same time, the intracellular level of nucleoside triphosphates decreased, as followed by the intensity of their β-phosphate peak at −19.3 ppm. This suggested that Ap_2Cp and Ap_2Cp_2A were synthesized at the expense of the nucleoside triphosphates and/or that they perturbed the energetic machinery of the cell.

When oxygen was replaced by nitrogen, the nucleoside triphosphates completely disappeared whereas the level of Ap_2Cp and Ap_2Cp_2A remained unaffected (Figure 2D), indicating that these compounds were metabolically stable and were not destroyed by the cell in an anoxic situation.

Neither $ApCp_2$ nor ApCp was present in NMR-detectable amounts (detection limit about 0.05 mM) in the perchloric acid extracts of PCP-poisoned cells, as derived from the chemical shifts of the authentic compounds and directly by spiking these compounds into the perchloric extracts.

DISCUSSION

PCP, at moderate concentrations, completely inhibited

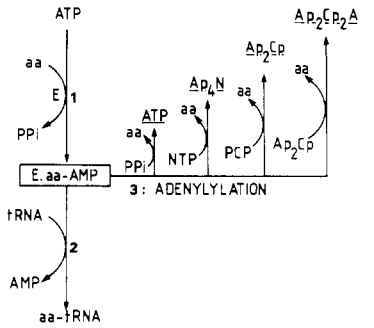

FIGURE 6: Adenylylation reactions catalyzed by aminoacyl-tRNA synthetases. In the main reaction path (left part of the figure), an amino acid reacts with ATP to yield an enzyme-bound aminoacyl adenylate and pyrophosphate (reaction 1). This mixed anhydride then normally produces an aminoacyl-tRNA (reaction 2) by reaction with tRNA. In the right part of the figure are depicted adenylylation side reactions (reactions 3) catalyzed by aminoacyl-tRNA synthetases. When a high concentration of PP_i is present, ATP can be regenerated (reversal of reaction 1). A nucleophilic attack of the aminoacyl adenylate by NTP leads to the synthesis of dinucleoside polyphosphates (Ap_4N). If PP_i is replaced by PCP, the methylene analogue of ATP, Ap_2Cp is synthesized. The reaction of Ap_2Cp with the aminoacyl adenylate leads in a second round to the synthesis of the methylene analogue of Ap_4A, Ap_2Cp_2A.

growth of *D. discoideum* amoebae in axenic medium. PCP was internalyzed by the amoebae, and its internal concentration reached a quasi-equilibrium with the concentration of PCP in the incubation medium.

In contrast to PCP, addition of PNP to *D. discoideum* at concentrations up to 5 mM had no effect on the growth of the amoebae. The reason for this might be that PNP itself is hydrolyzed by the cells as it is indeed a good substrate of inorganic pyrophosphatase (Smirnova et al., 1986) or of other nonspecific phosphatases, yielding phosphate and amidophosphate.

Incubation of *D. discoideum* with PCP was accompanied by the formation of two new intracellular compounds, identified by ^{31}P NMR to be the methylene analogues of ATP and Ap_4A, Ap_2Cp and Ap_2Cp_2A. The identification of Ap_2Cp in the perchloric acid extracts from PCP-poisoned amoebae was done unambiguously by addition of the authentic compound. Neither ApCp nor $ApCp_2$ was formed. Ap_2Cp_2A was identified by simulation of its ^{31}P NMR spectrum with the iterative PANIC program. The two compounds Ap_2Cp and Ap_2Cp_2A accounted for all the major unknown peaks in the clusters at 12, 8, and −11 ppm. Their intracellular concentrations reached about 0.5 mM. These PCP metabolites, accumulated in living amoebae, were likely to be involved in the mechanism of toxicity since cells first incubated with PCP and freed of the extra- and intracellular PCP remained unable to grow.

In vitro synthesis of Ap_2Cp has been described through the reversal of some aminoacyl-tRNA synthetases as discussed by Zamecnik (1983), and this suggested a possible in vivo mechanism for the synthesis of Ap_2Cp and Ap_2Cp_2A as shown in Figure 6. In this scheme, the nucleophilic attack on the phosphoryl group of the aminoacyl adenylate by PCP instead of PP_i led in a first step to the formation of the methylene analogue of ATP, Ap_2Cp, and in a second round to the methylene analogue of Ap_4A, Ap_2Cp_2A. PP_i, which should accumulate as ATP is utilized by the aminoacyl-tRNA synthetases, was most probably hydrolyzed by a pyrophosphatase and was not detectable in the NMR spectrum. Alternatively, Ap_2Cp_2A could be synthesized through the Ap_4A phosphorylase reaction, as recently described for Ap_4A (Brevet et al., 1987) in the yeast *Saccharomyces cerevisiae*. Ap_2Cp and Ap_2Cp_2A could possibly act as toxic compounds

by inhibiting cellular enzymes (Guranovski et al., 1987; Suzuki et al., 1987; Yount, 1975).

ACKNOWLEDGMENTS

We thank Professor Pierre V. Vignais for his constant support and advice throughout this work. We are grateful to Professor David Cotter for helpful discussions. We thank Jeannine Bournet for typing the manuscript and Marie-Françoise Foray and Robert Nardin for their help with the NMR simulations.

Registry No. PCP, 1984-15-2; Ap_2Cp, 3469-78-1; Ap_2Cp_2A, 88109-92-6.

REFERENCES

Bass, M. B., & Fromm, H. J. (1985) *Anal. Biochem. 145*, 292–303.

Brevet, A., Coste, H., Fromant, M., Plateau, P., & Blanquet, S. (1987) *Biochemistry 26*, 4763–4768.

Gadian, D. G., Radda, G. K., Dawson, M. J., & Wilkie, D. R. (1982) in *Intracellular pH: Its Measurement, Regulation, and Utilization in Cellular Functions* (Nuccitelli, R., & Deamer, D. W., Eds.) pp 61–77, Liss, New York.

Gillies, R. J., Alger, J. R., Den Hollander, J. A., & Shulman, R. G. (1982) in *Intracellular pH: Its Measurement, Regulation, and Utilization in Cellular Functions* (Nuccitelli, R., & Deamer, D. W., Eds.) pp 79–104, Liss, New York.

Granot, J., Mildvan, A. S., Bramson, H. N., & Kaiser, E. T. (1980) *Biochemistry 19*, 3537–3543.

Guranowski, A., Biryukov, A., Tarussova, N. B., Khomutov, R. M., & Jabukowski, H. (1987) *Biochemistry 26*, 3425–3429.

Jentoft, J. E., & Town, C. D. (1985) *J. Cell Biol. 101*, 778–784.

Kay, R. R., Gadian, D. G., & Williams, S. R. (1986) *J. Cell Sci. 83*, 163–179.

Klein, G., & Satre, M. (1986) *Biochem. Biophys. Res. Commun. 138*, 1146–1152.

Martin, J.-B., Foray, M.-F., Klein, G., & Satre, M. (1987) *Biochim. Biophys. Acta 931*, 16–25.

Pople, J. A., Schneider, W. G., & Bernstein, H. J. (1959) *High-Resolution Nuclear Magnetic Resonance*, pp 138–151, McGraw-Hill, New York.

Satre, M., & Martin, J.-B. (1985) *Biochem. Biophys. Res. Commun. 132*, 140–146.

Satre, M., Klein, G., & Martin, J.-B. (1986) *Biochimie 68*, 1253–1261.

Schliselfeld, L. H., Burt, C. T., & Labotka, R. J. (1982) *Biochemistry 21*, 317–320.

Smirnova, I. N., Baykov, A. A., & Avaeva, S. M. (1986) *FEBS Lett. 206*, 121–124.

Suzuki, H., Tanaka, Y., Buonamassa, D. T., Farina, B., & Leone, E. (1987) *Mol. Cell. Biochem. 74*, 17–20.

Tarussova, N. B., Shumiyanzeva, V. V., Krylov, A. C., Karpeisky, M. Ya., & Khomutov, R. M. (1983) *Bioorg. Khim. 9*, 838–843.

Vogel, H. J., & Bridger, W. A. (1982) *Biochemistry 21*, 394–401.

Watts, D. J., & Ashworth, J. M. (1970) *Biochem. J. 119*, 171–174.

Yount, R. G. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 1–56.

Zamecnik, P. (1983) *Anal. Biochem. 134*, 1–10.

Role of Fructose in Glycation and Cross-Linking of Proteins†

John D. McPherson, Brian H. Shilton, and Donald J. Walton*

Department of Biochemistry, Queen's University, Kingston, Ontario, Canada K7L 3N6

Received July 2, 1987; Revised Manuscript Received November 3, 1987

ABSTRACT: Incubation of carbohydrate-free human serum albumin (HSA) with fructose in an aqueous buffer at pH 7.4 resulted in glycation of ε-amino groups of lysyl residues. A recently developed procedure, involving analysis of hexitol amino acids by high-performance liquid chromatography of phenylthiocarbamyl derivatives, was used to show that 85% of the bound hexose was attached to protein via carbon 2 (C-2). The remainder was attached to protein via carbon 1 (C-1). When incubations were conducted with glucose under identical conditions, all the hexose was attached via C-1. Examination of human ocular lens proteins showed that the majority of the covalently bound hexose was connected to ε-amino groups of lysyl residues via C-1; this was attributed mainly to nonenzymatic glucosylation in vivo, which has already been documented. A significant proportion (10–20%) of the bound hexose was connected via C-2. In view of the HSA–hexose incubation results (above), this indicated that the lens proteins had reacted with endogenous fructose; i.e., they had undergone nonenzymatic fructosylation in vivo. The model protein bovine pancreatic ribonuclease A reacted with fructose and glucose at similar rates under physiological conditions. However, covalent, non-disulfide cross-linking, which could be inhibited by D-penicillamine, was induced 10 times more rapidly by fructose than by glucose. It is postulated that some of the protein cross-linking that occurs in vivo is fructose-induced. The possible significance of these processes in diabetic subjects is discussed.

The amino groups of some mammalian proteins react nonenzymatically with glucose, in vivo, to give Schiff bases such as I (Figure 1), which then undergo an Amadori rearrangement to form *N*-(1-deoxyfructos-1-yl) groups (II; Krantz et al., 1986). Subsequent reactions may result in the formation of cross-linked, fluorescent, protein derivatives (Elbe et al., 1983; Pongor et al., 1984), which may account for some of the complications of diabetes, such as cataract formation (Monnier et al., 1979), stiffening of collagen (Vishwanath et al., 1986), and vascular narrowing (Cerami et al., 1986).

In some organs, such as the ocular lens and peripheral nerves, fructose is biosynthesized by oxidation of sorbitol in

† This work was supported by the Canadian Diabetes Association.

0006-2960/88/0427-1901$01.50/0