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Solution Activity Product (K_{FAP}) and Simultaneous Demineralization–Remineralization in Bovine Tooth Enamel and Hydroxyapatite Pellets

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Abstract □ The effects of changing the ion activity product of the remineralization solution at pH 4.5 (pK_{FAP} 108–118) on the remineralization behavior of demineralized bovine tooth enamel and hydroxyapatite pellets have been studied. Solutions containing calcium-45, phosphate, and fluoride in acetate buffers were used. The ^{45}Ca/F molar ratios indicated the formation of fluoridated hydroxyapatite in the enamel or the pellet when the pK_{FAP} values for remineralizing solutions were <112. When the pK_{FAP} values were >112, the ^{45}Ca/F ratios were found to be ≪5. Also, when the pK_{FAP} values were large (>112), the remineralization patterns based on the fluoride distribution in the tooth (or pellet) were found to be different than when the pK_{FAP} values were small (<112). The hypothesis that a pK_{FAP} value of 112 is the demarcation between remineralization only and simultaneous dissolution–remineralization has been proposed based on these results.

Keyphrases □ Tooth enamel—bovine, demineralization and remineralization, effect of bulk solution activity product □ Hydroxyapatite—pellets, demineralization and remineralization, effect of bulk solution activity product □ Remineralization—demineralized bovine tooth enamel, hydroxyapatite pellets, effect of bulk solution activity product

Previous studies conducted in these laboratories (1) indicated that both bovine tooth enamel and hydroxyapatite pellets could be extensively remineralized in a fluoride-containing remineralizing solution after prior demineralization treatments for various lengths of time. After 3 or 6 hr of prior demineralization in a partially saturated acetate buffer at pH 4.5, fluoride uptake levels on the order of 1000 ppm were found after remineralization, at depths up to ~50 μm from the surface. The stoichiometry of the remineralized phase was shown to be fluoridated hydroxyapatite rather than calcium fluoride based on the ^{45}Ca/F ratio and determination by X-ray diffraction analysis. All of the remineralization studies (1) were conducted in solutions at an ionic product ($K_{FAP} = a^{10}_{Ca^{2+}} a^{6}_{PO_4^{3-}} a^{2}_{F^-}$) of $\sim 1 \times 10^{-108}$.

The purpose of the present study was to investigate the influence of varying the K_{FAP} value for the remineralizing solution. A case of special interest was where the K_{FAP} values are $>10^{-120}$, the solubility product for fluorapatite (2), but $<10^{-114}$, the ion activity product value below which dissolution takes place with hydroxyapatite pellets (3). A question of great interest was how the concomittant dissolution of hydroxyapatite, if it were to occur, would influence the remineralization behavior in this K_{FAP} region.

EXPERIMENTAL

Materials—*Bovine Teeth*—Teeth from 8-week-old strictly kosher calves were obtained from packing houses in the Chicago area. From these incisors, only those without any visible surface defects and with a reasonably flat surface were used for the experiments. The labial surfaces of these selected teeth were then ground with rotating sandpaper (No. 400 first, then No. 600) to remove the pellicle.

Hydroxyapatite Pellet—Synthetic hydroxyapatite crystals prepared using the procedure developed by Moreno (4) were used in the preparation of the pellets. Approximately 50 mg of hydroxyapatite, preequilibrated in a humidity chamber containing saturated potassium nitrate aqueous solution to maintain the humidity at ~67%, was compressed in a 0.62-cm diameter die with a force of 4540 kg using a laboratory press[1].

Preparation of Buffer Solutions—As in a previous study (1) a solution ~16% saturated (on a molar basis) with respect to the thermodynamic solubility of hydroxyapatite was used for demineralization. The solution was a 0.1 *M* acetate buffer containing 3.5 m*M* each of total calcium and phosphate. The pH was adjusted to 4.5 with sodium hydroxide and the ionic strength to 0.5 *M* by the addition of sodium chloride. For remineralization 0.1 *M* acetate buffers (pH 4.5) containing differing amounts of total calcium and phosphate depending on the desired bulk solution activity product ($K_{FAP} = a^{10}_{Ca^{2+}} a^{6}_{PO_4^{3-}} a^{2}_{F^-}$) as shown in Table I, were employed. These solutions also contained 10 ppm of fluoride and sodium chloride to adjust the ionic strength to 0.5 *M*. All the chemicals used for the preparation of buffer solutions were analytical grade. A predetermined amount of calcium-45 as $^{45}CaCl_2$ in water[2] was added to these solutions.

Demineralization—A bovine tooth was covered with dental inlay wax except for a 0.25-cm^2 area in the labial surface. In the pellet experiments, a hydroxyapatite pellet was completely covered with inlay wax except for one exposed surface (area, 0.25 cm^2). The tooth or pellet was then demineralized in the 16% partially saturated buffer solution for a period of 6 and 3 hr, respectively, after attaching a thin glass rod to each (length, ~14 cm). The glass rod was used to ensure careful handling during demineralization and remineralization. The use of 6 and 3 hr as the demineralization times was based on previous experimental results (1) which clearly indicated that the demineralizing solution was not saturated

[1] Carver Press.
[2] New England Nuclear, Boston, Mass.

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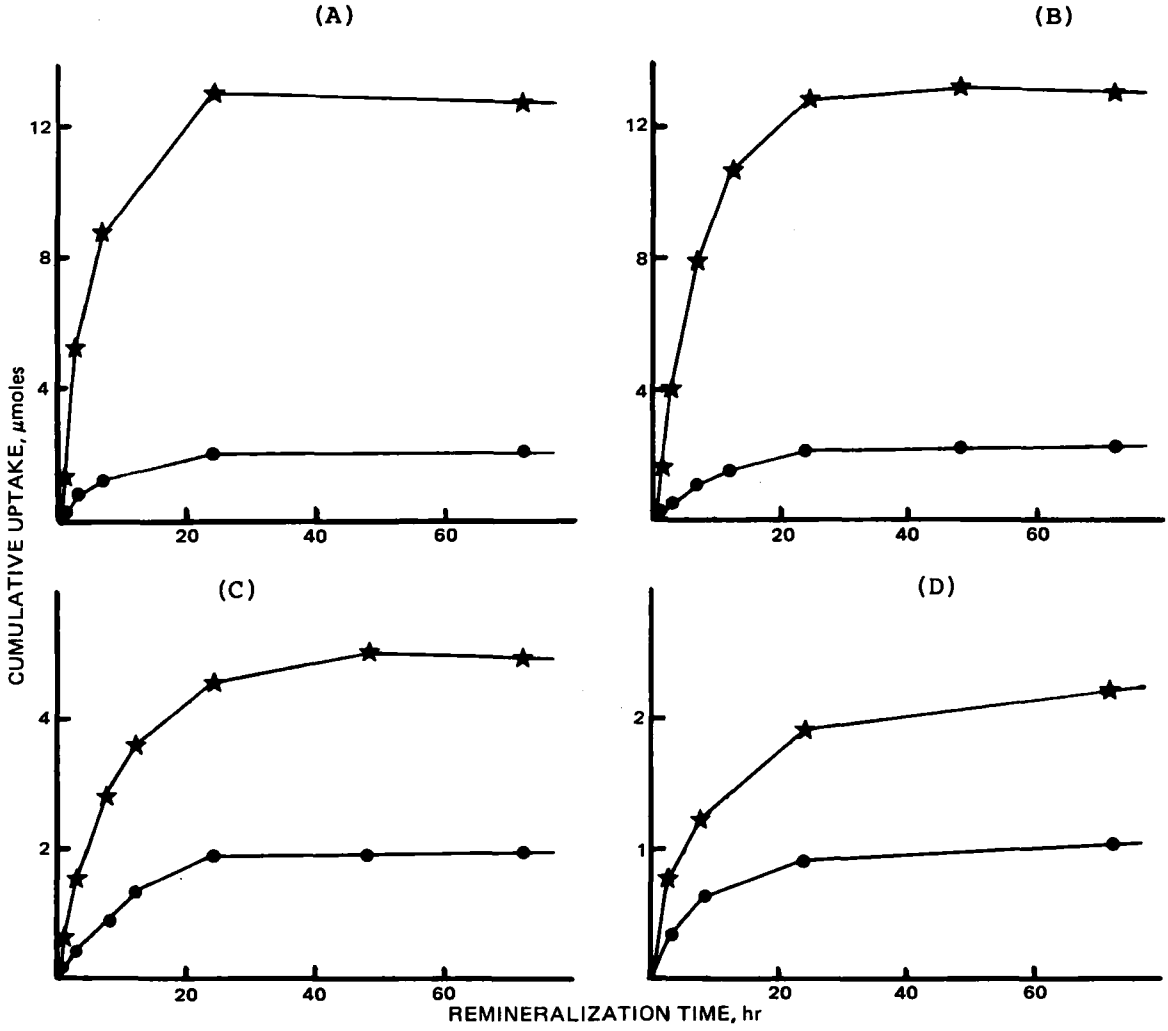

Figure 1—*Effect of remineralization time on the cumulative uptake of calcium-45 (★) and fluoride (●) ions in demineralized bovine tooth. Key: pK_{FAP} (A) 108; (B) 110; (C) 114; (D) 118.*

with respect to hydroxyapatite during that period. During demineralization the solution was shaken gently with a wrist action shaker[3] and kept at 30°. The volume of the buffer solution used was kept at 10 ml.

Remineralization—The demineralized tooth or pellet was washed completely with double-distilled water and dried. It was then subjected to the remineralization treatment for a period of 1–72 hr. During remineralization the solution was shaken gently in the wrist action shaker and kept at 30° as the remineralization proceeded.

Etching—The remineralized sample was thoroughly washed with double-distilled water and dried. The enamel was then etched in 1 ml of 0.5 *M* perchloric acid for successive periods of 30, 30, 120, 200, and 250 sec; the surface was washed with 1 ml of water after each etching and the washings were collected with the 0.5 *M* perchloric acid used for etching. In the case of pellets, the same procedure was repeated except the etchings were done for successive periods of 30, 30, 120, 200, 250, and 600 sec.

Analytical Techniques for the Estimation of Phosphate, Fluoride, and Calcium-45—Phosphate concentrations of the etching solutions were determined by the method of Gee *et al.* (5) in which the phospho-ammonium-molybdate complex formed was reduced by stannous chloride. The absorbance of the resulting blue solution was determined after 15 min at 720 nm[4]. Fluoride concentrations were determined by a fluoride ion electrode[5] using a low level total ionic strength-adjusting buffer. Concentrations of calcium-45 were determined using a scintillation counter[6].

Calculation of Layer Depth—The depth of the etched layers of the teeth were calculated from the amounts of phosphate removed from the etched layers, assuming the density of samples to be 2.95 g/ml. It was expected that this density value would be too large an estimate for the actual density near the surface; however, it is a convenient way to present the data and was adopted for this reason.

[3] Burrell Co., Pittsburgh, Pa.
[4] Model 25 Spectrophotometer; Beckman Instruments, Fullerton, Calif.
[5] Model 94-09; Orion Co., Cambridge, Mass.
[6] Model 9000 Liquid Scintillation System; Beckman Instruments, Fullerton, Calif.

RESULTS AND DISCUSSION

Effect of Remineralization Time—To determine the effect of remineralization time, bovine teeth demineralized for 6 hr were remin-

Table I—Calcium, Phosphate, and Fluoride Concentrations in Remineralizing Solutions Corresponding to Various pK_{FAP} Values [a]

pK_{FAP}	Ion Concentration Ca^{2+}, m*M*	PO_4^{3-}, m*M*	F^-, ppm	Ca/P Ratio
108	12.00	12.00	10	1.0
110	9.23	9.23	10	1.0
112	6.87	6.87	10	1.0
114	5.13	5.13	10	1.0
116	3.83	3.83	10	1.0
118	2.86	2.86	10	1.0
114	12.17	1.22	10	10.0
116	9.09	0.91	10	10.0
118	6.80	0.68	10	10.0
114	2.85	14.23	10	0.20
116	2.12	10.59	10	0.20
118	1.58	7.90	10	0.20
116	16.74	0.33	10	50.00
116	0.96	47.77	10	0.02

[a] pH 4.5, 0.5 μ, 0.1 *M* acetate buffer.

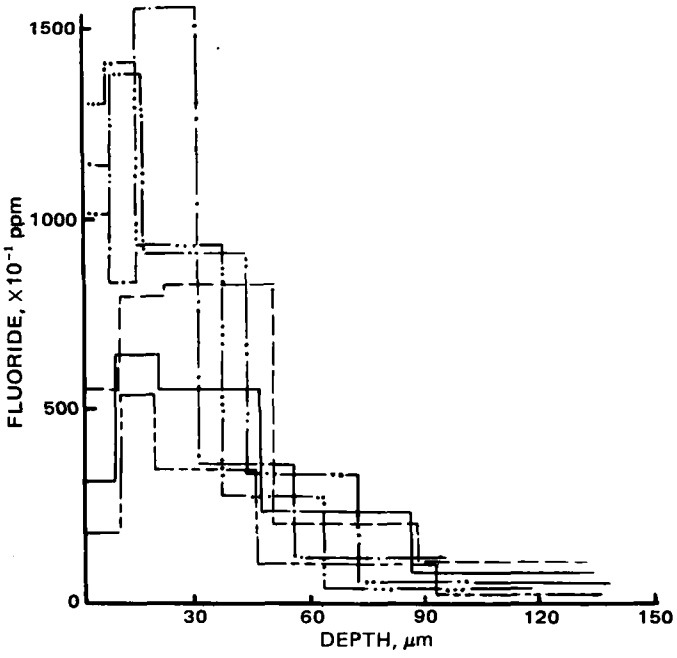

Figure 2—*The influence of driving force pK_{FAP} on the fluoride uptake of demineralized bovine teeth (pH 4.5, μ = 0.5, 0.1 M acetate buffer, Ca/P ratio = 1). Key: pK_{FAP} (— • • • —) 108; (— • • —) 110; (— • —) 112; (- - -) 114; (—) 116; (— - - —) 118.*

eralized for a period of 1–72 hr. For the purpose of remineralization, solutions with pK_{FAP} values of 108, 110, 114, and 118 were used. The effect of remineralization time on cumulative calcium-45 and fluoride uptake is shown in Fig. 1. These cumulative calcium-45 and fluoride uptake values were determined by summing the respective values obtained for each successive layer. Figure 1 clearly indicates that the remineralization is essentially complete in 24 hr except in the case where the remineralizing solution had a pK_{FAP} value of 118. In this case there was a slight increase in calcium-45 and fluoride concentrations from 24 to 72 hr of remineralization.

Effect of pK_{FAP} on Remineralization—To study the effect of the bulk solution activity product on remineralization, the remineralization studies were carried out by varying the pK_{FAP} values from 108 to 118. It was not possible to prepare a solution with a pK_{FAP} value <108 at pH 4.5 due to spontaneous precipitation during the pH adjustment. Calculations suggest that this may be due to the precipitation of dicalcium phosphate dihydrate.

All of the remineralization studies were carried out for a period of 24 hr. The cumulative uptake of calcium-45 and fluoride, and the ^{45}Ca/F molar ratio for each remineralized enamel layer and corresponding pK_{FAP} values, are shown in Table II. Calcium-45 uptake appeared to be constant at each pK_{FAP} value. Fluoride levels remained relatively constant until a pK_{FAP} of ~114 and seemed to drop somewhat at 116 and 118 (Table II). The ^{45}Ca/F molar ratios were near or somewhat above 5, which indicated the formation of fluoridated hydroxyapatite when the remineralizing solution had a pK_{FAP} of 108 or 110. At the larger pK_{FAP} values, however, the ratios decreased, reaching a value of 2.1 at pK_{FAP} 118.

A point of primary interest in this study was to determine the remineralization behavior when the pK_{FAP} value was >114. An attempt was made to determine whether the drop in the ^{45}Ca/F ratio at the higher pK_{FAP} values might be related to the system undergoing simultaneous dissolution of hydroxyapatite and remineralization (or recrystallization) of fluoroapatite[7]. The hypothesis that the deposited material in the enamel surface region actually had a Ca/F ratio of 10:2 was tested by varying the calcium-45 specific activity in the remineralizing solution. This was achieved by varying the bulk solution Ca/P ratios from 50 to 0.02 in the pK_{FAP} range of 114–118. As can be seen from Table III, there was an increase in the ^{45}Ca/F molar ratio with an increase in bulk solution Ca/P ratio and a decrease in the ^{45}Ca/F ratio with a decrease in Ca/P ratio in the remineralizing solution for the same pK_{FAP} value. This supported the idea that the deposited material in the enamel surface region actually had a Ca/F molar ratio close to 10:2 and that a simultaneous dissolution–remineralization process is occurring above a certain pK_{FAP} value. The results obtained were tested by correcting the results for the microenvironmental calcium-45 specific activity at the tooth surface as calculated by an approximate computer model. The details of the model parameters are as shown in the *Appendix*. The results obtained by assuming a surface pK_{FAP} of 110 at appropriate bulk solution concentration seemed to fit very well with the experimental values obtained. The theoretical values obtained from the model parameters are indicated in the last columns of Tables II and III.

Figure 2 gives the enamel fluoride concentration profiles for the bovine teeth experiments. While, as pointed out above, the cumulative uptakes are all of the same order of magnitude, it is seen that the remineralization pattern when the pK_{FAP} values are large is different from that when the pK_{FAP} values are small. At a pK_{FAP} of 108, there is significantly more remineralization near the tooth surface (~10–15 μm) than when the pK_{FAP} values of the solutions were large. These results are generally in agreement with a quantitative microradiographic study recently completed in these laboratories (7).

Hydroxyapatite pellets remineralized for 24 hr after 3 hr of demineralization were tested for calcium-45 and fluoride uptake as shown in Table IV. These results show that, as in the case of the bovine teeth experiments, calcium-45 uptake decreased with increasing pK_{FAP}, with no notable decrease in the fluoride uptake. The ^{45}Ca/F molar ratios also dropped below 5 with increasing pK_{FAP} of the remineralizing solutions, as was obtained in the case of bovine teeth.

Consideration of all the experimental results at pH 4.5 clearly indicates that the pK_{FAP} value of the remineralizing solution is the most important variable in determining the course of remineralization, with a pK_{FAP} of 112 being the demarcation between remineralization only and simulta-

Table II—Effect of pK_{FAP} on Bovine Tooth Remineralization [a]

pK_{FAP}	Calcium-45, μmoles	Fluoride, μmoles	^{45}Ca/F Ratio	
			Observed	Theoretical
108	13.1, 13.7[b]	1.92, 1.85	6.8, 7.4	—
110	12.5, 13.2	2.2, 2.6	5.7, 5.1	—
112	8.2, 8.0	2.0, 2.1	4.1, 3.9	3.87
114	4.5, 5.4	1.9, 2.0	2.4, 2.7	2.95
116	3.4, 2.8	1.6, 1.5	2.2, 1.9	2.24
118	1.9, 1.8	0.90, 0.85	2.1, 2.1	1.69

[a] At pH 4.5, Ca/P = 1.0, fluoride concentration = 10 ppm. [b] Duplicate values.

Table III—Effect of Ca/P Ratio on Bovine Tooth Remineralization [a]

pK_{FAP}	Ca/P Ratio	Calcium-45, μmoles	Fluoride, μmoles	^{45}Ca/F Ratio	
				Observed	Theoretical
114	10	6.2, 5.4[b]	1.7, 1.6	3.7, 3.4	4.01
114	0.2	2.5, 2.2	1.3, 1.2	1.94, 1.88	2.48
116	50	7.4, 6.4	1.72, 1.52	4.3, 4.2	4.3
116	10	4.0, 4.4	1.3, 1.2	3.0, 3.6	3.44
116	0.2	2.3, 2.0	1.2, 1.16	1.9, 1.7	1.74
116	0.02	1.0, 1.1	0.64, 0.66	1.6, 1.8	1.44
118	10	2.2, 2.5	0.9, 1.1	2.5, 2.3	2.86
118	0.2	1.2, 1.6	0.96, 0.95	1.2, 1.7	1.23

[a] At pH 4.5, fluoride concentration = 10 ppm, with 0.5 μ, 0.1 *M* acetate buffer. [b] Duplicate values.

Table IV—Hydroxyapatite Pellet Remineralization

pK_{FAP}	Calcium-45, μmoles	Fluoride, μmoles	^{45}Ca/F Ratio
108	7.7, 7.1[b]	1.2, 1.1	6.6, 6.1
110	7.0, 7.4	1.0, 1.1	6.8, 6.6
112	5.9, 5.4	0.9, 0.8	6.4, 6.5
114	5.0, 5.5	0.9, 1.0	5.6, 5.4
116	3.9, 2.6	1.0, 0.8	3.9, 3.5
118	3.0, 3.0	1.0, 1.1	3.1, 2.7

[a] At pH 4.5, fluoride concentration 10 ppm, Ca/P ratio = 1. [b] Duplicate values.

[7] Although the conditions and the nature of the experiments were different, Thiradilok and Feagin (6) observed that remineralization and dissolution–remineralization occur under different conditions in bovine enamel experiments. For example, when calcium, phosphate, and fluoride were all present in the remineralizing solutions (pH 7), remineralization was found to occur. However, if only calcium and fluoride or only phosphate and fluoride were present (and therefore the solution was initially unsaturated with respect to hydroxyapatite) these investigators found both dissolution and remineralization may take place. Their findings therefore are generally in good agreement with the present results.

neous dissolution–remineralization. Further experimental studies are in progress to determine whether the present conclusions regarding the "critical" pK_{FAP} value applies under other conditions (*e.g.*, at other pH values of the remineralizing solution). Also the similarities between the hydroxyapatite pellet experiments and bovine and human teeth studies are being evaluated further.

APPENDIX

Estimation of Calcium-45 Specific Activity at the Bovine Enamel Surface During Simultaneous Demineralization–Remineralization

It has been shown previously (3) that a hydroxyapatite pellet dissolves in the presence of partially saturated buffer containing fluoride when the solution is unsaturated with respect to a pK_{FAP} of ~114. In this dissolution situation, the solution concentration of calcium is higher in the pellet and at the hydroxyapatite surface than in the bulk solution, whereas the fluoride gradient goes the other way, favoring uptake of fluoride from the solution.

In this paper, bovine enamel was studied under similar conditions with the partially saturated bulk solution containing both calcium-45 and fluoride, and the uptake of both species was monitored. If it is assumed that bovine enamel behaves similarly to hydroxyapatite, then when the bulk solution has a pK_{FAP} of >112, the calcium concentration will be higher at the enamel surface since its apparent solubility under these conditions is governed by a pK_{FAP} of ~112. The result is that calcium-45 coming to the surface from the bulk solution will be diluted out by the higher "cold" calcium supplied by hydroxyapatite demineralization. Thus, at sites where calcium and fluoride uptake occurs, it would be expected that measuring uptake of calcium-45 would give too low a value for the calcium uptake as a result of the dilution of calcium-45 by "cold" calcium. The dilution of calcium-45 has been estimated and therefore the expected $^{45}Ca/F$ uptake ratio (assuming total Ca/F uptake is 10:2) was calculated by using a physical model incorporating the following assumptions:

1. The bovine enamel is assumed to dissolve stoichiometrically.
2. Fluoride and calcium-45 concentrations at the enamel surface are assumed equal to the bulk concentrations.
3. At the enamel surface a suitable expression for describing the surface solution ion activity product is:

$$K_{FAP} = 10^{-110} \text{ or } K_{HAP}(a^{10}_{Ca}a^{6}_{PO_4}a^{2}_{OH}) = 10^{-122}$$

4. The remineralization process in the enamel occurs with the ratio of deposited total Ca/F being 10:2.

Using this model, the expected $^{45}Ca/F$ uptake ratios have been calculated and seem to agree quite well with the experimental results over a range of bulk solution K_{FAP} values and for solution Ca/P ratios from 1:50 to 50:1.

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Analysis of Monobutyryl and Dibutyryl Derivatives of Adenosine 3′,5′-Monophosphate in Biological Samples Using Isocratic Ion Pair High-Performance Liquid Chromatography

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Received June 17, 1982, from the **Division of Toxicology, Department of Environmental Health Sciences* and ‡*Department of Anesthesiology/Critical Care Medicine, Johns Hopkins Medical Institutions, Baltimore, MD 21205.* Accepted for publication September 1, 1982.

Abstract □ Adenosine 3′,5′-monophosphate (cyclic AMP), its dibutyryl and monobutyryl derivatives, and a number of other naturally occurring adenine-containing compounds were separated by isocratic ion pair high-performance liquid chromatography. A mobile phase consisting of 30% methanol in 0.1 *M* KH_2PO_4 (pH 4.0) containing 1 m*M* tetramethylammonium hydroxide as the counterion was used to separate the butyryl derivatives. To sufficiently separate cyclic AMP from other adenine-containing compounds, a mobile phase containing 6% methanol in the same aqueous buffer plus counterion was used. Extraction of these cyclic nucleotides from deproteinized biological samples using disposable reverse-phase extraction columns is described. This not only eliminated lipophilic contaminants, but also served to concentrate the samples. The outlined procedures were used to determine the concentrations of the butyryl derivatives in lung tissue and perfusate following a 35-min lung perfusion with 100 μM N^6-$O^{2'}$-dibutyryl cyclic AMP. The role of this technique in the analysis of cyclic nucleotide derivatives as compared with conventional assay procedures is discussed.

Keyphrases □ Adenosine 3′,5′-monophosphate—dibutyryl and monobutyryl derivatives, lung tissue and perfusate, separation by isocratic ion pair high-performance liquid chromatography □ Analogues—dibutyryl and monobutyryl cyclic AMP, lung tissue and perfusate, separation by isocratic ion pair high-performance liquid chromatography □ High-performance liquid chromatography—ion pair, isocratic, cyclic AMP and its dibutyryl and monobutyryl derivatives, lung tissue and perfusate

Analogues of adenosine 3′,5′-monophosphate (cyclic AMP) were first synthesized to selectively mimic the effects of this cyclic nucleotide in various biological systems. The most extensively used analogue has been N^6-$O^{2'}$-dibutyryl cyclic AMP (1). In most instances the dibutyryl derivative has proved to be more biologically active when

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