RELATIONSHIP BETWEEN PHOSPHORYLATION OF BLOOD PLATELET PROTEINS AND SECRETION OF PLATELET GRANULE CONSTITUENTS I. EFFECTS OF DIFFERENT AGGREGATING AGENTS

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SUMMARY: Exposure of human blood platelets containing [^{32}P]ATP to collagen fibres or to the divalent cation ionophore, A23187, selectively increased ^{32}P-labelling of polypeptides of mol. wt. 48,000 to 40,000 (by up to 200%) and 25,000 to 19,000 (by up to 50%). These phosphorylation reactions partly preceded aggregation of platelets by these agents, which is associated with secretion of platelet granule constituents. Phosphorylation reached a maximum before completion of aggregation and was followed by slow dephosphorylation. No increases in protein phosphorylation were observed when aggregation without secretion was induced by ADP. The results suggest that selective protein phosphorylations may be involved specifically in the secretory process.

Although platelets are known to contain cyclic AMP-dependent and independent protein kinases (1 - 4), phosphorylase kinase (5 - 7), myosin light chain kinase (8) and endogenous substrates for these enzymes, the roles of protein phosphorylation in platelet function have not yet been clearly established. This requires studies of the changes in phosphorylation of specific proteins in intact platelets. Using platelets that had been preincubated with [^{32}P]P_i, Lyons et al. (3) found that thrombin selectively increased ^{32}P incorporation into certain phosphopolypeptides. We have briefly reported that collagen and the divalent cation ionophore, A23187, exert similar effects (9). The present results provide evidence that these phosphorylations may play a role in the secretion of platelet granule constituents (the release action).

MATERIALS AND METHODS

Carrier-free [^{32}P]P_i was obtained from NEN Canada Ltd. and [G-^{3}H]5-hydroxytryptamine ([^{3}H]5-HT) from Amersham Corporation. ADP, human thrombin (3,000 NIH units/mg protein) and bovine tendon collagen were from Sigma Chemical Co. Suspensions of collagen fibres were prepared (10). Bovine serum albumin was supplied by ICN Canada Ltd. Human fibrinogen from Kabi AB was treated with diisopropylfluorophosphate before use (11). Potato apyrase (activity

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Abbreviation: SDS, sodium dodecyl sulfate.

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3 μmol/min/mg protein with 100 μM ADP at 37°C) was kindly supplied by Dr. J.F. Mustard. A23187, a gift from Eli Lilly and Co., was dissolved in dimethyl sulphoxide, which had no effects at the final concentration used (0.2%).

Suspensions of washed human platelets were prepared at 37°C by a modification of the method of Mustard et al. (12). The platelets were first washed with phosphate-free Tyrode's solution containing heparin (50 units/ml), apyrase (60 μg/ml) and bovine serum albumin (0.35%). After resuspension in the same medium without heparin at about 3 x 10^9 platelets/ml, they were then incubated for 60 min at 37°C with 0.8 mCi of carrier-free [^{32}P]P_i/ml and 1 μM [^{3}H]5-HT (18 Ci/mmol); 4-8% of the [^{32}P]P_i and almost all the [^{3}H]5-HT were taken up. The platelets were then washed in Tyrode's solution (with phosphate) containing both bovine serum albumin and apyrase and were finally suspended at a count of 4 x 10^8/ml in albumin-free Tyrode's solution containing 6 μg apyrase/ml.

Pairs of incubation mixtures, each comprising 0.9 ml of platelet suspension and 0.1 ml of 0.154 M NaCl containing any additions, were stirred at 37°C in a two-channel aggregometer (Payton Associates Ltd.). Aggregating agents were added after stirring for 1 min and aggregation was recorded as the decrease in extinction or increase in transmission (ΔT(%)). Incubations were stopped after appropriate intervals, one of each pair by the addition of 0.1 ml of 0.077 M EDTA and centrifugation for 30 s at 12,000 g (for determination of release of platelet [^{3}H]5-HT) and the other by addition of 0.1 ml of 3 N $HClO_4$ (for determination of protein phosphorylation). In the first case, supernatant was counted for ^{3}H and ^{32}P by liquid scintillation spectrometry; ^{3}H counts were corrected for ^{32}P crossover and for any ^{3}H present in supernatant from unstimulated controls and were expressed as percentages of the platelet-bound ^{3}H. In the second case, the acid-precipitated protein was washed with 0.3 N $HClO_4$ and dissolved in 0.2 ml of 0.1 M Na phosphate buffer, pH 7.0, containing 3% Na dodecylsulphate (SDS) and either 0.1 M dithiothreitol or 5% mercaptoethanol. Dissolved protein (about 80 μg in 25 μl) was electrophoresed on cylindrical SDS - 5.6% polyacrylamide gels and stained with Coomassie brilliant blue (13). Gels were destained in 10% acetic acid and scanned at 550 nm using a Gilford spectrophotometer with a linear transport. They were then frozen and divided into 1 mm slices with a Bio-Rad gel slicer (Model 190). Slices were placed in polythene vials containing 2 ml of 0.01% 4-methylumbelliferone in water and counted for Čerenkov radiation in a Beckman LS 230 scintillation counter (efficiency > 40%). The results were stored on Teletype tape and were superimposed on the densitometric scans of the corresponding gels using a Hewlett-Packard tape reader (HP9863A), calculator (HP9810A) and plotter (HP9862A). Corrections for decay of ^{32}P and for any discrepancies between the recorded lengths of gels and the number of slices obtained were applied.

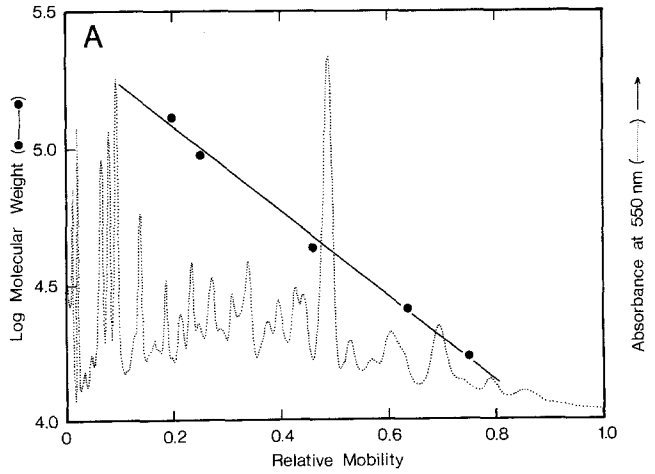
Acetone powders of platelet protein were prepared by addition of 9 vol. acetone at -20°C to 1 vol. platelet suspension or 1 vol. supernatant (48,000 g x 20 min) from suspensions of platelets lysed by dilution with 2 vol. water (0°C) followed by freezing and thawing. Precipitated protein was removed by centrifugation, washed with acetone and dried in vacuo. Actin was extracted from acetone powders and polymerized by established methods (14).

For determination of the specific activity of ATP in ^{32}P-labelled platelets, nucleotides were extracted with 0.5 N $HClO_4$, isolated by adsorption to and elution from charcoal (15) and separated by thin layer chromatography in two dimensions (16). ATP and AMP were eluted with water and their concentrations (from extinctions at 259 nm) and radioactivity determined. The specific activity of the γ-phosphate residue of the ATP in the platelet metabolic pool (60% of total platelet ATP (17)) was calculated assuming that the granule pool of ATP was unlabelled and, as little ^{32}P was found in AMP, that all the ^{32}P in [^{32}P]ATP was distributed equally between the β and γ-phosphate residues.

RESULTS

SDS-polyacrylamide gel electrophoresis of platelet protein (Fig.1A) resolved at least 28 polypeptides, including one identified as actin by its high concentration, apparent molecular weight of 43,000 and coelectrophoresis with partially purified platelet actin. Electrophoresis of material from ^{32}P-labelled platelets that had not been exposed to any aggregating agent demonstrated peaks of ^{32}P incorporation associated with at least ten of these polypeptides, though there was no relationship between the amounts of individual polypeptides and the labelling with ^{32}P (Fig. 1B). The largest amounts of ^{32}P were associated with two polypeptides of apparent mol. wt. 94,000 and 82,000. On stimulation of ^{32}P-labelled platelets by collagen, consistent changes were observed in only two regions of the ^{32}P profile (Fig. 1C). A marked (up to 200%) increase in ^{32}P incorporation occurred in polypeptides in the 48,000 to 40,000 mol. wt. range (P48-40), which included actin, and a moderate increase (up to 50%) occurred in the 25,000 to 19,000 range (P25-19). Exposure of platelets to collagen at 50-100 µg/ml for 2 min was maximally effective both in inducing secretion (release of [^{3}H]5-HT) and in increasing the labelling of these polypeptides with ^{32}P. A half-maximal effect of collagen on release was observed with 10-20 µg/ml and on ^{32}P incorporation into P48-40 with 5-8 µg/ml.

Evidence was obtained that the ^{32}P associated with these polypeptides largely represented ^{32}P incorporation into protein as alkali-labile phosphoester. It was found that although extraction of phospholipid from the acid-washed protein pellets with chloroform/methanol (2 : 1) removed about 75% of the ^{32}P present, there was no change in the ^{32}P profiles seen on subsequent electrophoresis, except for the disappearance of the large ^{32}P peak close to the tracking dye (Fig. 1). Incubation of solvent-extracted pellets with 0.8 M



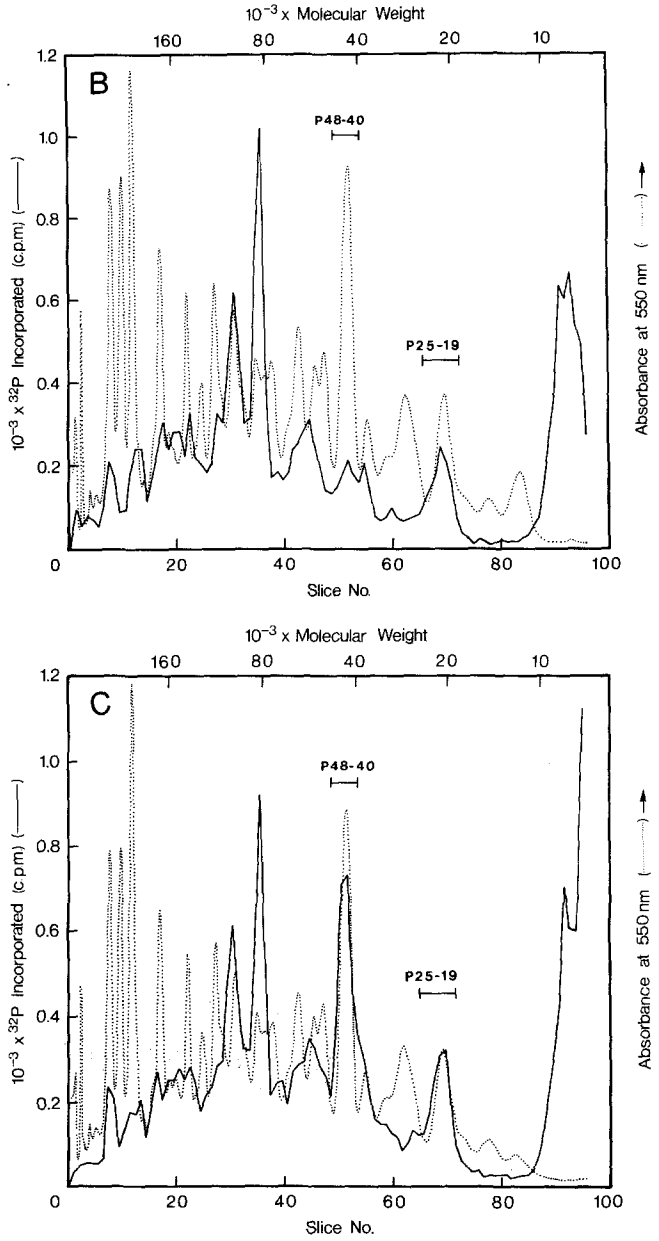


Fig. 1. Densitometric scans and ^{32}P-labelling of platelet polypeptides separated by SDS-polyacrylamide gel electrophoresis.

A. Calibration of gels with respect to mol. wt. Platelet protein was electrophoresed in parallel with the following proteins of known mol. wt.: *Escherichia coli* β-galactosidase, 130,000; muscle phosphorylase a, 94,000; ovalbumin, 43,000; chymotrypsinogen, 25,700; myoglobin, 17,200. Mobilities relative to that of the tracking dye are plotted against $\log_{10}$ mol. wt.

B. Control ^{32}P profile. Suspension of labelled platelets was stirred for 3 min with no addition other than 0.154 M NaCl.

C. Collagen-stimulated ^{32}P profile. Suspension of labelled platelets was stirred for 1 min before and for 2 min after addition of 0.154 M NaCl containing collagen (final concentration 50 μg/ml). Release of [^{3}H]5-HT was 54%.

Table 1. Platelet Aggregation, Release of [^{3}H]5-HT and Phosphorylation of Platelet Proteins with Different Aggregating Agents

Additions	Extent of aggregation ΔT(%)	Release of [^{3}H]5-HT (%)	^{32}P in P48-40		^{32}P in P25-19	
			c.p.m.	Δ(%)	c.p.m.	Δ(%)
None	0	-	1923	-	1864	-
ADP (20 μM)	4	0	2177	+ 13	2031	+ 9
ADP (50 μM)	5	0	1831	- 5	1921	+ 3
Collagen (10 μg/ml)	34	20	4673	+143	2611	+ 40
Collagen (50 μg/ml)	43	37	4820	+151	2698	+ 45
Thrombin (1 unit/ml)	54	59	5108	+166	2929	+ 57
Fibrinogen	0	-	1952	-	1900	-
ADP (20 μM) + Fibrinogen	43	1	1775	- 9	1735	- 9
ADP (50 μM) + Fibrinogen	47	0	1823	- 7	1830	- 4
DMSO	0	-	1933	-	1849	-
A23187(1.0 μM) + DMSO	61	35	4617	+139	2544	+ 38

Different aggregating agents (final concentrations indicated) were stirred for 1 min with samples of the same suspension of labelled platelets. Final concentrations of fibrinogen and DMSO were 0.4 mg/ml and 0.2% respectively, when present. Changes in ^{32}P in P48-40 (5 slices) and P25-19 (7 slices) were calculated as percentage increments over the values in controls without aggregating agents.

hydroxylamine in 0.05 M Na acetate buffer, pH 5.4, (10 min at 30°C) solubilized negligible amounts of ^{32}P, while incubation with 0.5 N NaOH (8 h at 37°C) released about 90% of the ^{32}P. Of this fraction, 80-85% was [^{32}P]P_i (precipitable as triethylamine [^{32}P]phosphomolybdate (18)). Collagen (50 μg/ml) increased the ^{32}P in extracted pellets that was recoverable as [^{32}P]P_i by this method by 40%.

The effects of other aggregating agents were compared with those of collagen (Table 1). A23187 and thrombin each had effects similar to those of collagen, causing both platelet aggregation and release of [^{3}H]5-HT that were associated with increased ^{32}P incorporation into P48-40 and P25-19. On the other hand, ADP, which in the presence of fibrinogen induced marked aggregation without release of [^{3}H]5-HT, did not cause any consistent changes in protein phosphorylation detectable by the methods used.

Almost half of the increased ^{32}P incorporation caused by collagen occurred before aggregation started (Fig. 2). With collagen at 50 μg/ml, the maximum ^{32}P incorporation into both P48-40 and P25-19 was seen after 1-2 min, before aggregation was complete. After 2 min, there was a gradual dephosphoryl-

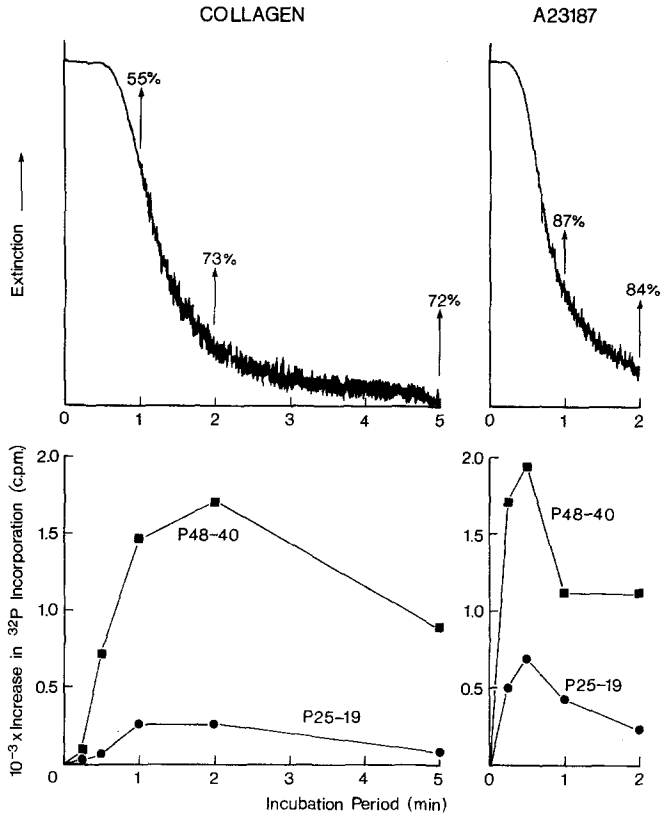


Fig. 2. Time course of ^{32}P incorporation into P48-40 and P25-19 in relation to platelet aggregation induced by collagen (50 µg/ml) and by A23187 (0.4 µM). The abscissae give incubation periods after addition of the aggregating agent. Values for ^{32}P in P48-40 (5 slices) and in P25-19 (7 slices) in control incubations without aggregating agents were subtracted from those found in stimulated platelets. The release of platelet [^{3}H]5-HT was measured at times indicated by arrows.

ation of P48-40 and P25-19 without any associated deaggregation of the platelets. A similar pattern was observed with 0.4 µM A23187, but aggregation started earlier and the time course of phosphorylation and dephosphorylation was accelerated. Maximum ^{32}P incorporation into both P48-40 and P25-19 occurred after 0.5 min, when aggregation had just begun, and 50% dephosphorylation was observed after about 2 min. The results show that phosphorylation (and dephosphorylation) of P48-40 and P25-19 proceed in parallel.

When platelet suspension was stirred for 1 min with collagen (100 µg/ml) in the presence of 5 mM EDTA, release of [^{3}H]5-HT and increased phosphorylation of P48-40 proceeded normally, but in the absence of platelet aggregation. If these platelets were then lysed by dilution of the suspension with 2 vol. 5 mM

EDTA ($0^{o}C$) followed by freezing and thawing and were then centrifuged at 48,000 g for 20 min at $0^{o}C$, marked ^{32}P incorporation into P48-40 was observed exclusively in the supernatant, which contained only about 50% of platelet actin. Acetone powders were prepared from control and collagen-stimulated platelet suspensions and from corresponding lysate supernatants without using EDTA. Actin was extracted and ^{32}P-labelled P48-40 was found in the actin-depleted insoluble fraction of acetone powders from collagen-stimulated platelets. None was found in the actin-enriched extracts.

From the specific activity of the γ-phosphate residue of the [^{32}P]ATP in the platelet metabolic pool (see methods), it was calculated that P48-40 contained 0.23 nmol [^{32}P]phosphate/10^{9} platelets in unstimulated platelets and 0.68 nmol [^{32}P]phosphate/10^{9} platelets after 2 min exposure to collagen (100 µg/ml). If 44,000 g of P48-40 phosphopolypeptide contain 1 mol. of phosphate and 10^{9} platelets contain 2 mg protein, this increase in ^{32}P incorporation caused by collagen must represent labelling of 1% of the platelet protein. Actin was estimated by integration of the densitometric scans to account for about 10% of platelet protein.

DISCUSSION

A previous study (3) has shown that thrombin, which induces both platelet aggregation and the release reaction, increases phosphorylation of specific platelet proteins. Our work confirms this and shows that collagen and A23187, which also cause both aggregation and release, exert identical effects. However, we have also found that ADP, which in the presence of physiological divalent cation concentrations and fibrinogen causes aggregation without inducing the release reaction (11), does not under these conditions induce protein phosphorylation. Moreover, EDTA blocked collagen-induced platelet aggregation but not the release action or increased phosphorylation. These findings indicate that increased protein phosphorylation is specifically associated with the release reaction.

Increased phosphorylation of P48-40 and P25-19 proceeded in parallel, suggesting a common activating mechanism. As A23187 (and other release-inducing agents) probably act by promoting movement of Ca^{2+} ions from intracellular stores into the platelet cytosol (19, 20), these phosphorylation reactions may result from direct activation of Ca^{2+}-dependent protein kinases or possibly indirectly, via a Ca^{2+}-dependent increase in cyclic GMP. The eventual dephosphorylation of both P48-40 and P25-19 suggests that ^{32}P incorporation into these polypeptides is due to increases in the amounts of protein-bound phosphate, rather than to replacement of unlabelled by labelled phosphate. The increased phosphorylation

of platelet proteins induced by collagen or A23187, which partly preceded aggregation, may also partly precede the release reaction, as appreciable release of [^{3}H]5-HT does not occur prior to detectable aggregation (unpublished results with a rapid fixation technique (21)). Protein phosphorylation could therefore play a role in the secretory mechanism.

The values obtained for the percentage stimulation of ^{32}P incorporation into P48-40 and P25-19 by release-inducers are minimum estimates, as these segments of the polypeptide profile may contain several ^{32}P-labelled phosphopolypeptides, only one or two of which may show increased incorporation. The identity of the P48-40 phosphopolypeptide formed during the release reaction has not yet been determined but it is not extracted from acetone powders of platelet fractions with actin and, on the basis of specific activity measurements, amounts to not more than 1% of the platelet protein, much less than actin. Preliminary studies indicate that this phosphopolypeptide can be separated from actin by discontinuous SDS-polyacrylamide gel electrophoresis (22). The P25-19 phosphopolypeptide that also shows increased labelling may be the phosphorylated 20,000 mol. wt. light chain of platelet myosin (8, 23).

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REFERENCES

1. Kaulen, H.D. and Gross, R. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 471-480.
2. Steiner, M. (1975) Arch. Biochem. Biophys. 171, 245-254.
3. Lyons, R.M., Stanford, N. and Majerus, P.W. (1975) J. Clin. Invest. 56, 924-936.
4. Booyse, F.M., Marr, J., Yang, D-C., Guiliani, D. and Rafelson, M.E. (1976) Biochim. Biophys. Acta 422, 60-72.
5. Karpatkin, S. and Langer, R.M. (1969) J. Biol. Chem. 244, 1953-1955.
6. Gear, A.R.L. and Schneider, W. (1975) Biochim. Biophys. Acta 392, 111-120.
7. Chaiken, R., Pagano, D. and Detwiler, T.C. (1975) Biochim. Biophys. Acta 403, 315-325.
8. Daniel, J.L. and Adelstein, R.S. (1976) Biochemistry 15, 2370-2377.
9. Haslam, R.J. and Lynham, J.A. (1976) Biochem. Soc. Trans. 4, 694-697.
10. Haslam, R.J., Davidson, M.M.L. and McClenaghan, M.D. (1975) Nature 253, 455-457.
11. Mustard, J.F., Perry, D.W., Kinlough-Rathbone, R.L. and Packham, M.A. (1975) Am. J. Physiol. 228, 1757-1765.
12. Mustard, J.F., Perry, D.W., Ardlie, N.G. and Packham, M.A. (1972) Br. J. Haematol. 22, 193-204.
13. Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.
14. Spudich, J.A. and Watt, S. (1971) J. Biol. Chem. 246, 4866-4871.
15. Ireland, D.M. and Mills, D.C.B. (1966) Biochem. J. 99, 283-296.
16. Haslam, R.J. and McClenaghan, M.D. (1974) Biochem. J. 138, 317-320.

17. Agarwal, K.C. and Parks, R.E. (1975) Biochem. Pharmacol. 24, 2239-2248.
18. Sugino, Y. and Miyoshi, Y. (1964) J. Biol. Chem. 239, 2360-2364.
19. Feinman, R.D. and Detwiler, T.C. (1974) Nature 249, 172-173.
20. Charo, I.F., Feinman, R.D. and Detwiler, T.C. (1976) Biochem. Biophys. Res. Comm. 72, 1462-1467.
21. Costa, J.L. and Murphy, D.L. (1976) Nature 255, 407-408.
22. Haslam, R.J., Lynham, J.A. and Fox, J. (1977) Fed. Proc. 36, 566a.
23. Daniel, J.L., Holmsen, H. and Adelstein, R.S. (1976) Fed. Proc. 35, 806a.