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DIFFERENTIAL EFFECTS OF DIGITONIN ON SOME ENZYME ACTIVITIES OF THE SODIUM PUMP

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SUMMARY

1. When bovine brain membrane preparations containing the Na^+ pump are treated with increasing amounts of digitonin, the several enzyme activities characteristic of the pump are progressively inhibited, but not to the same degree.

2. At a digitonin-protein ratio just sufficient to inhibit the (Na^+, K^+)-ATPase (the membrane adenosine 5′-triphosphate phosphohydrolase (EC 3.6.1.3) requiring Na^+, K^+ and Mg^{2+} for full activity) activity completely, about one-fourth of the K^+ phosphatase activity is still present. Corresponding amounts of the Na^+-dependent ADP-ATP exchange activity and the Na^+-dependent labeling by [γ-^{32}P]ATP also survive.

3. Treatment of the membranes with digitonin in the presence of ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetate (EGTA) results in somewhat greater survival of the K^+-phosphatase (the membrane acyl phosphate phosphohydrolase (EC 3.6.1.7) requiring K^+ and Mg^{2+} for full activity), while the Na^+-dependent ADP–ATP exchange activity is completely protected.

4. Sucrose gradient centrifugation of control and digitonin-treated preparations shows that the enzyme activities surviving loss of overall (Na^+, K^+)-ATPase function are released from the bulk of the membrane protein.

5. These studies show that under membrane-disrupting conditions the partial reactions catalyzed by the Na^+ pump have less stringent organizational requirements than the (Na^+, K^+)-ATPase with respect to maintenance of their catalytic functions.

INTRODUCTION

It is now generally accepted that the Na^+ pump of mammalian cell membranes manifests itself in broken cell preparations as a (Na^+, K^+)-ATPase (EC 3.6.1.3) activity. The biochemical basis of Na^+ and K^+ transport by this pump remains an intriguing biological problem, primarily because the pump is a small part of a complex membrane structure and is not easily released in active form for purification and further study.

Recently several laboratories have reported methods for obtaining (Na^+, K^+)-

Abbreviations: (Na^+, K^+)-ATPase, the membrane adenosine 5′-triphosphate phosphohydrolase (EC 3.6.1.3) requiring simultaneously Na^+, K^+ and Mg^{2+} for full activity; K^+-phosphatase, the membrane acyl phosphate phosphohydrolase (EC 3.6.1.7) requiring both K^+ and Mg^{2+} for full activity; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N*, *N*′-tetraacetate; TES, *N*-tris-(hydroxymethyl)methyl-2-aminoethane sulfonate.

ATPase (EC 3.6.1.3) preparations of greatly increased specific activity[1–6]. Each method employs detergents at some point in the procedure, and results in a final preparation that invariably contains several protein species separable on gel electrophoresis. Whether all of these species participate in pump function is not known, but seems unlikely. Skou[7] has estimated that the preparation reported to have the highest specific activity is about 40 % pure. This particular preparation consists of membrane particles, and Skou[7] suggests that other detergent-solubilized preparations consist of a complex between such particles and the detergent. While essentially complete breakdown of membrane structure can be achieved with high concentrations of certain detergents, such treatments result in complete loss of (Na^+, K^+)-ATPase activity. Therefore, detergent-based purification techniques usually seek conditions that balance degree of enzyme solubilization against enzyme inactivation. This situation suggests that the (Na^+, K^+)-ATPase is very sensitive to the state of organization of the membrane of which it is a part.

The (Na^+, K^+)-ATPase also catalyzes several partial reactions. The initial Na^+-dependent reaction with ATP to form phosphorylated enzyme can be detected in two ways: with the aid of [γ-^{32}P]ATP, measuring ^{32}P incorporation into protein[8]; and as an ADP-ATP exchange activity[9]. The terminal portion of the reaction sequence can be detected as a K^+-dependent acetyl phosphatase activity[10]. Both portions of the reaction sequence are associated with all (Na^+, K^+)-ATPase preparations in constant proportion[11], and are undiminished in the most highly purified preparations (ref. 2, 3)*.

There have been no studies to date of the effects of membrane disrupting agents on the partial reactions of the (Na^+, K^+)-ATPase. In particular, the question arises whether the partial activities are catalyzed only by completely functional (Na^+, K^+)-ATPase preparations or whether they can continue to function even when disruption causes loss of overall ATPase activity. Answering this question may provide insight into how the Na^+ pump is organized, in particular with regard to the communication between the two ends of the reaction sequence. To date, only one pump subunit of about 94000 molecular weight has been detected whereas the total ATPase system is estimated to have a molecular weight of 250000[16,1].

The results presented in this paper show that digitonin, previously used to solubilize (Na^+, K^+)-ATPase activity[17–19], completely inhibits that activity when used at higher concentrations. This complete inhibition of overall (Na^+, K^+)-ATPase activity can be carried out with complete retention of the Na^+-dependent ADP–ATP exchange activity, and partial retention of the K^+-phosphatase activity. Furthermore, these surviving partial reactions are released from the bulk of the membrane protein by digitonin, and may be separated from it on sucrose density gradients.

MATERIALS AND METHODS

Preparation of the membrane (Na^+,K^+)-ATPase

Fresh, bovine brains were obtained from a local slaughter-house and transported to the laboratory in ice. All subsequent operations were carried out in the cold.

* The K^+-phosphatase reaction sequence also apparently involves a phosphorylated intermediate, but there is controversy over the alkali metal ion effects on its formation and turnover[12–14]. Recent studies in this laboratory using acetyl[^{32}P]phosphate as substrate show that labeling of the enzyme requires only Mg^{2+} and is discharged by K^+ (ref. 15), a finding in accord with the known ion requirements of the enzyme.

The gray matter was collected, weighed and homogenized in 5 vol. of 0.25 M mannitol containing 0.03 M Tris, pH 7.4, and 0.001 M EDTA. The resulting suspension was centrifuged 15 min at 10000 × *g*, to collect the crude microsomal membrane fraction. Material sedimenting at 10000 × *g* was resuspended and homogenized twice more to obtain additional microsomal material. The resulting microsomal pellets were suspended in distilled water, assayed for protein content[20] and frozen until used.

To reduce the (Na^+, K^+)-independent ATPase activity, the microsomes were treated by a slight modification of the method of Nakao and coworkers[21]. This treatment removed 95 % of the contaminating ATPase with quantitative retention of the (Na^+,K^+)-ATPase. The enzyme prepared in this manner was stable in the refrigerator for several weeks.

Measurement of enzyme activities

(Na^+,K^+)-ATPase activity was routinely assayed at 37° in a medium containing 0.025 M Tris, pH 7.4, 0.005 M MgATP, 0.1 M NaCl, 0.015 M KCl, 0.005 M dithiothreitol, and approx. 0.5 mg membrane protein per ml. The reaction was stopped by adding an equal volume of 15 % trichloroacetic acid and the orthophosphate released was estimated by the method of Gomori[22]. The rate of hydrolysis of ATP was constant with time and increased linearly with enzyme concentration over the range used in these studies. All values reported are corrected for hydrolysis of ATP in the absence of Na^+ and K^+. Periodic checks showed that values obtained in this manner agreed with the ouabain-sensitive ATPase activity.

Because some of the digitonin concentrations used in this study interfere with the method of Gomori[22], an alternative measure of ATP hydrolysis was needed for such studies. In some experiments, the hydrolysis of [γ-^{32}P]ATP was followed, using the method of Nielson and Lehninger[23] but increasing to 4 the number of extractions with isobutanol–benzene to obtain quantitative extraction of [^{32}P]orthophosphate. The other procedure used was that of Berkowitz[24] as described by Uesugi *et al.*[25]. Because this procedure avoided the unwanted interference and was easier to use, later experiments with digitonin employed this method.

ADP–ATP exchange activity was measured by the method of Glaze and Wadkins[26]. The concentrations of ADP and ATP were 5 mM and 3 mM, respectively, and these concentrations did not change significantly during the course of the reaction. The Mg^{2+} concentration was 0.5 mM and all other conditions were as described for the (Na^+, K^+)-ATPase assay, except that K^+ was omitted. Samples were assayed both with and without Na^+ to estimate the Na^+-dependent exchange activity catalyzed by the pump.

K^+-phosphatase activity was measured by following the disappearance of acetyl phosphate from the medium. The incubation medium was as described for the (Na^+,K^+)-ATPase assay except that Na^+ was omitted, the Mg^{2+} concentration was 0.01 M, and the substrate was 0.01 M acetyl phosphate. Acetyl phosphate was measured by the method of Lipmann and Tuttle[27], which is not affected by the amounts of digitonin used here.

Na^+-dependent phosphorylation of the membranes by [γ-^{32}P]ATP

Enzyme preparations were phosphorylated by [γ-^{32}P]ATP at 37° in the same medium used to assay Na^+-dependent ADP–ATP exchange activity, except that

ADP was omitted and lower concentrations of ATP (usually about 50 μM) were used. Control experiments showed that the conditions were adequate to ensure phosphorylation without a significant drop in substrate concentration. The reaction was stopped after 10 sec by adding lauryl sulfate (Tris salt, pH 7.4) to a final concentration of 0.08 M. This concentration is ten-fold higher than needed to completely block (Na^+, K^+)-ATPase activity. The resulting clear solution was applied to a 1 cm $\times$ 90 cm column of Sephadex G-50 (20–80 μ) and the protein separated from low molecular weight radioactive material by eluting with water. This procedure adequately separates radioactive protein from [γ-^{32}P]ATP, with complete recovery of protein. Phospholipid is removed from the protein, and it is rendered enzymatically inactive. Assays were run with and without Na^+, to determine the amount of Na^+-dependent phosphorylation

Density gradient centrifugation

Control and digitonin-treated enzyme preparations were applied to the top of a linear sucrose gradient containing 25 mM *N*-tris-(hydroxymethyl)methyl-2-aminoethane sulfonate (TES) buffer, pH 7.4 at 0°. The samples were centrifuged in the Beckman SW40Ti swinging bucket rotor for 60 min at 100000 $\times$ *g*. The position of visible bands in the untreated control tubes was observed and the tubes sliced so that these visible bands constituted separate fractions. (Some fractions were collected that contained no visible material.) The digitonin-treated samples were then sliced at points on the tube identical to the controls, so that the fractions obtained corresponded to the same regions of the gradient.

Materials

[8-^{14}C]ADP was purchased from Schwarz Bioresearch as a 50% ethanolic solution. The ethanol was removed and the labeled ADP converted to the Tris salt by passage through Dowex 50–Tris. [γ-^{32}P]ATP was synthesized by the method of Glynn and Chappell[28] and converted to the Tris form. Digitonin was purchased from Sigma Chemical Company and Matheson, Coleman and Bell. No difference was noted between different preparations. Recrystallized digitonin gives results identical to those obtained using the commercial product, therefore the latter was used directly.

Mode of introduction of digitonin

Commercially supplied digitonin is slow to dissolve in water. Therefore it was first dissolved in absolute ethanol, the appropriate aliquot pipetted into the incubation container, and the solvent evaporated to dryness. This procedure resulted in a film of finely divided particles on the wall of the tube, which dissolved instantly in aqueous solution. All treatments with digitonin were carried out at ice temperature, with subsequent enzyme assays at the usual 37° after prewarming for 3 min. No time-dependent loss of any activity is seen over the assay periods used in this work; the change in activity observed appears to be essentially instantaneous. Storage of digitonin-treated enzyme preparations in the refrigerator results in slow but complete loss of K^+-phosphatase activity over several days.

RESULTS

When brain membranes are treated with increasing amounts of digitonin, the (Na^+, K^+)-ATPase activity is progressively inhibited until it disappears alto-

gether. A concomitant loss of the K^+-phosphatase activity proceeds to a somewhat smaller extent, so that at the point where ATPase activity disappears, about 30 % of the phosphatase activity survives (Fig. 1). The degree of inhibition of the (Na^+,K^+)-ATPase activity apparently relates more closely to the digitonin–protein ratio than to the digitonin concentration, so that this method of expressing the results is used. 16 mg digitonin per mg protein reproducibly inhibits the (Na^+,K^+)-ATPase by 95 % or more in different preparations, while allowing survival of 20–30 % of the K^+-phosphatase. Higher relative amounts of digitonin inhibit the phosphatase to a greater extent.

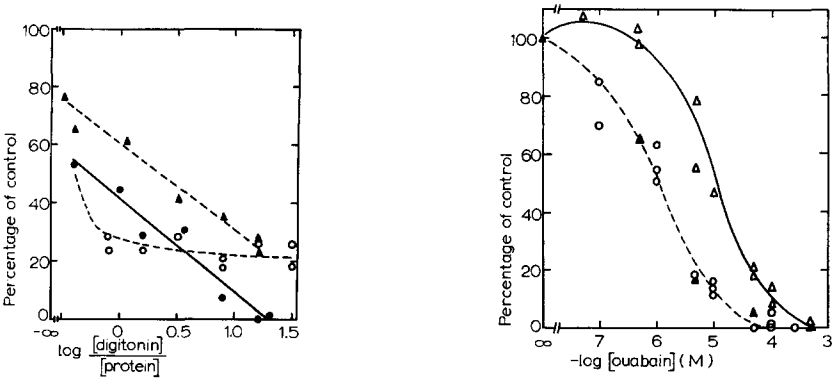

Fig. 1. Comparison of effects of digitonin on (Na^+, K^+)-ATPase, K^+-phosphatase and Na^+-dependent ADP–ATP exchange activities. ●, (Na^+, K^+)-ATPase; ▲, K^+-phosphatase; ○, Na^+-dependent ADP–ATP exchange activity.

Fig. 2. Effect of digitonin on ouabain-sensitivity of the surviving K^+-phosphatase activity. ○, control (Na^+, K^+)-ATPase; △, control K^+-phosphatase; ▲, K^+-phosphatase after treatment with 16.3 mg digitonin per mg protein. In the absence of ouabain the latter activity was 20.3 % of the untreated control.

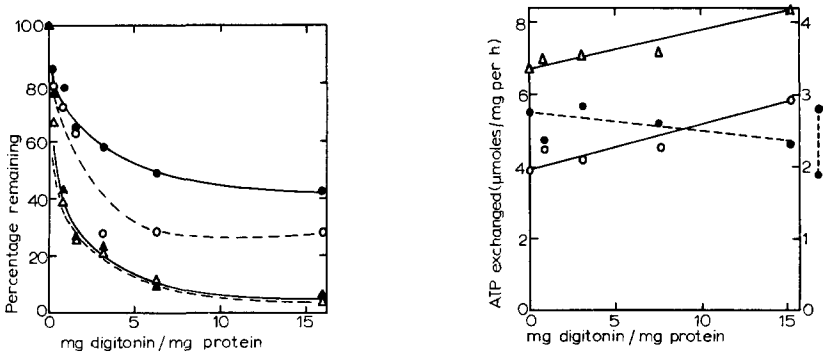

Fig. 3. Effect of EGTA on survival of K^+-phosphatase and (Na^+, K^+)-ATPase after digitonin treatment. Concentration of EGTA, 1 mM. Samples were incubated at ice temperature 30 min prior to assay. Dashed lines show samples without EGTA: (Na^+, K^+)-ATPase (△); K^+-phosphatase (○). Solid lines represent samples with EGTA: (Na^+, K^+)-ATPase (▲); K^+-phosphatase (●).

Fig. 4. Effect of digitonin on ADP–ATP exchange activity in the presence of EGTA. Concentration of EGTA (with equimolar Mg^{2+}), 3 mM. ○, Mg^{2+} present; △, Mg^{2+} *plus* Na^+ present; ●, Na^+-dependent activity (scale on right).

The surviving K^+-phosphatase must represent pump-associated activity, since it remains ouabain-sensitive, in fact more so than the untreated preparation (Fig. 2). The different sensitivities of untreated K^+-phosphatase and (Na^+, K^+)-ATPase to ouabain have been previously documented[29]. The ability of digitonin to render the phosphatase as sensitive as the (Na^+,K^+)-ATPase to ouabain may relate to digitonin's membrane-disrupting action, described below. The digitonin-treated preparation is inhibited by ATP and Na^+ to the same degree as the untreated control (results not shown). In accord with previous work[10] the inhibition by ATP does not require addition of Na^+.

The Na^+-dependent ADP–ATP exchange activity of the enzyme also partially survives digitonin treatment (Fig. 1). This finding is mirrored, as one would expect, in an associated survival of the Na^+-dependent phosphorylation of the enzyme by [γ-^{32}P]ATP (results not shown). Taken together, these findings suggest that the initial and final stages of the enzymatic reaction sequences are partially intact, but somehow uncoupled in the presence of digitonin.

At least part of the inhibition of K^+-phosphatase activity by digitonin can be reversed by the addition of ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*'-tetra-acetate (EGTA) (Fig. 3). The 1 mM level of EGTA used in this experiment is sufficient to produce a near-maximal effect. The K^+-phosphatase is not appreciably affected by the Mg^{2+} salt of EGTA when digitonin is not present. Furthermore, EGTA has no effect on the survival of overall (Na^+, K^+)-ATPase activity (lower curves, Fig. 3).

Addition of EGTA to digitonin-treated membrane preparations has an even more striking protective effect on the Na^+-dependent ADP–ATP exchange activity, as seen in Fig. 4 (compare Fig. 1). The Na^+-dependent ADP–ATP exchange catalyzed by untreated membrane preparations is relatively unaffected by EGTA, whereas the Na^+-independent exchange activity is stimulated somewhat by this agent (results not shown). This stimulation occurs in spite of the fact that both the microsome isolation and salt extraction procedures for enzyme preparation involve exposure to EDTA.

Table I shows two sample experiments from a series of 19 showing that the surviving "partial" activities become separated from the bulk of the membrane protein after digitonin treatment. Sucrose gradient patterns of the untreated enzyme show one major visible band, accompanied by a very faint additional band. Fraction 3 contains the major band; the minor band is in Fraction 1 in Expt. 1 and Fraction 2 in Expt. 2 (because the gradients differ). Digitonin treatment caused the major visible band to move to Fraction 4 near the bottom of the gradient, and the faint band to disappear. These band movements are reflected in the distribution of protein on the gradient.

The (Na^+, K^+)-ATPase activity of the untreated control (as well as its associated partial reactions) is found in both visible bands. In some experiments (such as Expt. 2), where the digitonin treatment did not completely inhibit the (Na^+, K^+)-ATPase activity, this surviving activity moves with the major protein band, and shows corresponding amounts of partial reaction activity. Even in this case, however, part of the K^+-phosphatase and Na^+-dependent ADP–ATP exchange activities remains on the top of the gradient, and shows little or no (Na^+, K^+)-ATPase activity. Furthermore, in most experiments the specific activity of each partial reaction at the top of the gradient is somewhat increased, despite the loss of a large fraction of

TABLE I

SEPARATION OF PARTIAL ACTIVITIES FROM BULK OF MEMBRANE PROTEINS

Enzyme was treated briefly in the cold with 16 mg digitonin per mg protein and submitted, along with an untreated control, to density gradient centrifugation as described under MATERIALS AND METHODS. Fraction 1 corresponds to the top of the tube. Expt. 1, 20–45 % sucrose; Expt. 2, 0–45 % sucrose. Values are percentages of total recovery in the control fractions. The relative specific activity is the ratio of the specific activity of the fraction indicated to that of Fraction 3 of the control.

Expt.	*Quantity measured*	*Sample*	*Percent recovery in fraction*			
			1	*2*	*3*	*4*
1*	Protein	Control	19.5	14.8	64.5	1.2
		Digitonin	5.5	10.5	5.3	78.7
	(Na^+, K^+)-ATPase	Control	34.7	4.3	61.0	0
		Digitonin	1.8	0.3	0.2	1.9
	Rel. spec. act.	Digitonin	0.42	0.04	0.04	0.03
	K^+-phosphatase	Control	24.0	5.6	68.5	1.9
		Digitonin	17.8	0	0	0
	Rel. spec. act.	Digitonin	1.77	0	0	0
2	Protein	Control	9.0	9.5	75.5	6.0
		Digitonin	20.5	12.3	15.9	55.3
	(Na^+, K^+)-ATPase	Control	0	14.5	85.5	0
		Digitonin	4.2	0	0	12.0
	Rel. spec. act.	Digitonin	0.18	0	0	0.19
	K^+-phosphatase	Control	0	11.0	89.0	0
		Digitonin	11.2**	0	0	13.2
	Rel. spec. act.	Digitonin	0.47	0	0	0.21
	Na^+-dependent	Control	0	11.2	88.8	0
	ADP–ATP exchange	Digitonin	34.8	0	0	27.7
	Rel. spec. act.	Digitonin	1.45	0	0	0.43

* The Na^+-dependent labeling of the enzyme by [γ-^{32}P]ATP in Fraction 3 of the control was 33 pmoles per mg protein. Fraction 4 of the digitonin-treated sample showed no detectable Na^+-dependent labeling. The other fractions were not analyzed.

** In most experiments, the recovery of K^+-phosphatase in this fraction was greater than twice this value, and the relative specific activity exceeded unity.

the corresponding total activity. These observations suggest that the partial activities, but not intact ATPase, are released from the bulk of the membrane.

DISCUSSION

The results presented show that the partial reactions of the Na^+,K^+-ATPase are less sensitive to digitonin than is the overall enzyme activity. At least part of the loss of the partial activities produced by digitonin can be prevented by EGTA, but this is not true for overall (Na^+,K^+)-ATPase activity. The protective effect of EGTA might be due to chelation of heavy metals released during membrane disruption by digitonin, or alternatively might be some sort of direct stimulatory effect on the partial reactions in the presence of digitonin (but not in its absence). One cannot distinguish between these two possibilities on the basis of the present results, but it seems unlikely in the case of the exchange reaction that a direct stimulation would exactly equal the digitonin inhibition, thus the term "protective" effect.

The Na^+-dependent ADP–ATP exchange activity and the K^+-phosphatase

differ in two aspects of their interaction with digitonin. The exchange is more sensitive to digitonin and can be completely protected by EGTA. The K^+-phosphatase, on the other hand, requires larger amounts of digitonin to produce the same degree of inactivation, and is also less susceptible to protection by EGTA. This difference can be rationalized with the aid of the reaction sequence proposed by Fahn *et al.*[30], and strongly supported by the results of Post and co-workers[31]:

$$\text{ATP} + E \overset{Mg^{2+},\,Na^+}{\rightleftharpoons} E_1\text{–}P + \text{ADP} \tag{1}$$

$$E_1\text{–}P \xrightarrow{Mg^{2+}} E_2\text{–}P \tag{2}$$

$$E_2\text{–}P \xrightarrow{K^+} E + P_i \tag{3}$$

Other evidence shows that the K^+-phosphatase enters this reaction sequence in the following manner[15]:

$$\text{Acetyl phosphate} + E \xrightarrow{Mg^{2+}} E_2\text{–}P + \text{acetate} \tag{4}$$

Digitonin cannot block Reactions 3 and 4 to an extent greater than 60 % in the presence of EGTA since this is the surviving level of K^+-phosphatase at the point where (Na^+,K^+)-ATPase is completely inhibited (Fig. 3). Furthermore, all of the Na^+-dependent exchange activity survives under these conditions (Fig. 4). Therefore, digitonin must block Reaction 2 completely to account for the complete loss of (Na^+,K^+)-ATPase activity. Since part of the K^+-phosphatase activity is inactivated by digitonin regardless of the presence of EGTA, the region or state of the pump catalyzing this activity may be the major site of interaction with digitonin.

It is clear that the partial activities surviving digitonin treatment of the membranes sediment differently on sucrose gradients than the bulk of the membrane protein. Since any surviving (Na^+,K^+)-ATPase activity remains with the bulk of the protein (Expt. 2, Table I), this suggests that in the process of loss of ATPase activity with digitonin, the physical state of the species catalyzing the surviving partial reactions is changed. The protective effect of EGTA must therefore occur only with these "solubilized" activities, since EGTA has no effect on the partial reactions or the (Na^+,K^+)-ATPase in untreated enzyme preparations, nor on the survival of the ATPase in the presence of digitonin. The increase in specific activity of both partial reactions usually seen on their release from the bulk of the membrane protein suggests that this process is somewhat selective.

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REFERENCES

1 A. Kahlenberg, N. C. Dulak, J. F. Dixon, P. R. Galsworthy and L. Hokin, *Arch. Biochem. Biophys*, 131 (1969) 253.
2 D. W. Towle and J. H. Copenhaver, Jr., *Biochim. Biophys. Acta*, 203 (1970) 124.
3 S. Uesugi, N. C. Dulak, J. F. Dixon, T. D. Hexum, J. L. Dahl, J. F. Perdue and L. E. Hokin, *J. Biol. Chem.*, 246 (1971) 531.
4 P. Leth Jorgensen and J. C. Skou, *Biochim. Biophys. Acta*, 233 (1971) 366.
5 P. Leth Jorgensen, J. C. Skou and L. P. Solomonson, *Biochim. Biophys. Acta*, 233 (1971) 381.
6 J. Kyte, *J. Biol. Chem.*, 246 (1971) 4157.
7 J. C. Skou, in D. R. Sanadi, *Current Topics in Bioenergetics*, Vol. 4, Academic Press, New York, 1971, p. 357.
8 R. L. Post, A. K. Sen and A. S. Rosenthal, *J. Biol. Chem.*, 240 (1965) 1437.
9 S. Fahn, G. J. Koval and R. W. Albers, *J. Biol. Chem.*, 241 (1966) 1882.
10 H. Bader and A. K. Sen, *Biochim. Biophys. Acta*, 118 (1966) 116.
11 H. Bader, R. L. Post and G. H. Bond, *Biochim. Biophys. Acta*, 150 (1968) 41.
12 G. H. Bond, H. Bader and R. L. Post, *Fed. Proc.*, 25 (1966) 567.
13 Y. Israel and E. Titus, *Biochim. Biophys. Acta*, 139 (1967) 450.
14 G. Sachs, J. D. Rose and B. I. Hirschowitz, *Arch. Biochem. Biophys.*, 119 (1967) 277.
15 W. F. Dudding and C. G. Winter, *Biochim. Biophys. Acta*, 241 (1971) 650.
16 G. R. Kepner and R. I. Macey, *Biochim. Biophys. Acta*, 183 (1969) 241.
17 A. Schwartz, H. S. Bachelard and H. McIlwain, *Biochem. J.*, 84 (1962) 626.
18 P. D. Swanson, H. F. Bradford and H. McIlwain, *Biochem. J.*, 92 (1964) 235.
19 H. F. Bradford, P. D. Swanson and D. B. Gammack, *Biochem. J.*, 92 (1964) 247.
20 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
21 T. Nakao, Y. Tashima, K. Nagano and M. Nakao, *Biochem. Biophys. Res. Comm.*, 19 (1965) 755.
22 G. Gomori, *J. Lab. Clin. Med.*, 27 (1941–42) 955.
23 S. O. Nielson and A. L. Lehninger, *J. Biol. Chem.*, 215 (1955) 555.
24 D. B. Berkowitz, Ph. D. Thesis, Univ. of Wisconsin (1965).
25 S. Uesugi, A. Kahlenberg, F. Medzihradsky and L. E. Hokin, *Arch. Biochem. Biophys.*, 130 (1969) 156.
26 R. P. Glaze and C. L. Wadkins, *J. Biol. Chem.*, 242 (1967) 2139.
27 F. Lipmann and L. C. Tuttle, *J. Biol. Chem.*, 159 (1945) 21.
28 I. M. Glynn and J. B. Chappell, *Biochem. J.*, 90 (1964) 147.
29 M. Fujita, T. Nakao, Y. Tashima, N. Mizuno, K. Nagano and M. Nakao, *Biochim. Biophys. Acta*, 117 (1966) 42.
30 S. Fahn, G. J. Koval and R. W. Albers *J. Biol. Chem.*, 241 (1966) 1882.
31 R. L. Post, S. Kume, T. Tobin, B. Orcutt and A. K. Sen, *J. Gen. Physiol.*, 54 (1969) 306 s.
32 C. G. Winter, *Fed. Proc.*, 29 (1970) 805 Abs.
33 C. G. Winter, *Symp. VIII, 8th Int. Congr. Biochem. Montreux, Switzerland, 1970.*