BBA 75 151

(Na^+-K^+)-ACTIVATED ATPase IN KIDNEY CELL MEMBRANES OF NORMAL AND METHYLPREDNISOLONE-TREATED RATS

ANDRZEJ MANITIUS, KLAUS BENSCH AND FRANKLIN H. EPSTEIN

Departments of Internal Medicine and Pathology, Yale University School of Medicine, New Haven, Conn. (U.S.A.)

(Received February 12th, 1968)

SUMMARY

A method is described for the isolation of a purified preparation of plasma membranes from rat kidney tissue, with a high specific activity of (Na^+-K^+)-activated ATPase.

Treatment of rats for 4–5 days with methylprednisolone produced an increase in the specific activity of (Na^+-K^+)-ATPase in kidney homogenates, but not of Mg^{2+}-dependent ATPase or succinate dehydrogenase (EC 1.3.99.1). Methylprednisolone did not, however, increase the (Na^+-K^+)-ATPase per mg of protein in kidney plasma membranes.

The data are interpreted to support the hypothesis that glucocorticoids increase (Na^+-K^+)-ATPase activity in the kidney by increasing the quantity of plasma membrane per cell, rather than by increasing the activity of enzyme per unit of plasma membrane.

INTRODUCTION

It has recently been demonstrated that the specific activity of (Na^+-K^+)-activated ATP phosphohydrolase in light fractions of rat kidney homogenates can be greatly increased by physiological manoeuvers which increase net reabsorption of Na^+ by the kidney. These include the administration of methylprednisolone, and the production of compensatory renal hypertrophy by uninephrectomy[1]. The increase in the activity of (Na^+-K^+)-ATPase is consistent with the view that this enzyme is involved in the active transport of Na^+ from the glomerular filtrate[1,2]. "Microsomal" fractions of homogenized cells, as conventionally prepared, contain fragments of plasma cell membranes[3–4] as well as ribosomes and other material located in the interior of the intact cell. Since the active transport of Na^+ is thought to take place primarily at the surface of the renal tubular cell, it would be of great interest to determine whether the increase in (Na^+-K^+)-ATPase activity per mg of protein induced, for example, by glucocorticoids, would be apparent in a purified preparation of plasma membranes.

The present report describes a method for the isolation of highly purified plasma membranes with high (Na^+-K^+)-ATPase activity from rat kidney tissue. Treatment with methylprednisolone increased (Na^+-K^+)-ATPase in the whole homogenate of

the kidneys. There was, however, no difference between the (Na^+-K^+)-ATPase activity per mg of protein of plasma membranes obtained from normal rats and those treated with methylprednisolone. The data are interpreted to support the hypothesis that methylprednisolone increases (Na^+-K^+)-ATPase activity in the kidney by increasing the amount of plasma membrane per cell, rather than by enhancing the activity of enzyme per unit of plasma membrane.

MATERIALS AND METHODS

In all experiments male, Sprague–Dawley rats weighing 150–350 g were used.

1. Isolation of kidney cell plasma membranes

The procedure used was a modification of the method described by NEVILLE[6] for the isolation of liver cell membranes. For each preparation 6–10 rats were used. The animals were anaesthetized with ether and their kidneys removed. The capsule and the adjacent fat were discarded, the kidney placed in an ice-cold 0.001 M $NaHCO_3$ solution (pH 7.5) and sliced into small pieces. Four batches, each containing 30 ml of solution and 3–5 kidneys, were thus obtained. Subsequent procedures were carried out with the tissue cooled to 4° in an ice-cold water bath or a refrigerated centrifuge. Each batch was homogenized with 30 strokes of a teflon pestle in a Dounce homogenizer. Uniformly homogenized suspension obtained from all 4 batches was then transferred to a beaker and ice-cold 0.001 M $NaHCO_3$ solution was added to a total of 500–600 ml. The suspension was mixed for about 2 min and filtered through 8 layers of gauze. The filtrate (referred to subsequently as "whole homogenate") was poured into four 250-ml polyethylene bottles and centrifuged for 10 min at 1500 × *g* using a Sorvall centrifuge. Deceleration of the centrifuge at this as well at all further steps was slow, *i.e.*, no brake was used. The supernatant was discarded and the brown-reddish, jelly-like sediment retained. About 5 ml of bicarbonate solution were then added to each bottle and the sediment resuspended using a Vortex mixer. The resulting suspension was transferred out of all 4 bottles into a homogenizer. Each bottle was washed with 4 ml of the bicarbonate solution which was added to the homogenizing tube. Resuspension was completed with 5 strokes of the pestle, the fluid transferred into three 15-ml conical centrifuge tubes and centrifuged at 1220 × *g* for 10 min. After this centrifugation, clear supernatant fluid overlaid a two-layer sediment. The bottom layer formed a solid pellet; the upper one consisted of a pale-brown, fluffy material. The clear supernatant was drawn off, care being taken not to remove the fluffy precipitate. Thereafter 3 ml of the bicarbonate solution were gently added to the tube. This stirred and resuspended the fluffy layer of the sediment. The resulting suspension was removed with care being taken not to touch the solid pellet. Another 3 ml of the bicarbonate solution were then added gently to the tube. This suspended the remaining fluffy sediment which was then removed as before. Both suspensions obtained in the same way from all 3 tubes were poured into a homogenizing tube, 10 ml of bicarbonate solution were added and resuspension was completed using 3 gentle strokes of the pestle. This suspension was then poured into three 15-ml conical tubes and centrifuged again at 1200 × *g* for 10 min. After this centrifugation the sediment consisted mostly of the fluffy precipitate although a small solid pellet was always present at the bottom of the tube. The clear supernatant was drawn off and

the fluffy sediment removed as before except that 1.5 ml instead of 2 ml of the bicarbonate solution were added each time. The suspension obtained from all 3 centrifuge tubes was again combined and resuspended in a homogenizer. The resulting suspension was then placed in one 15-ml conical tube and centrifuged as before (1220 × *g*, 10 min). The supernatant was discarded and the fluffy sediment removed and placed in a 4.5-ml homogenizer. Bicarbonate solution was added to a total volume of 3.5 ml. Resuspension was completed with 3 gentle strokes of the pestle, 1.6 ml of the suspension were then transferred into each of two 13.5-ml lusteroid centrifuge tubes. To each tube 2 ml of a solution containing 69 % by weight of sucrose in water (*d* 1.34) were added slowly with constant stirring using a Vortex mixer. Over this, 5 ml of 41 % sucrose solution (*d* 1.18) were carefully layered. This, in turn, was overlaid with 36 % sucrose solution (*d* 1.16) until the tube was filled. The gradient tubes were capped and spun in a Spinco ultracentrifuge at 100 000 × *g* for 75 min. After centrifugation a white band composed of floating cell membranes was observed at the *d* 1.16/1.18 interface. The membranes were collected using a syringe with a No. 19 needle and resuspended in a homogenizer in a 20-fold larger volume of bicarbonate solution. The suspension was then centrifuged at 3000 × *g* for 30 min. The supernatant fluid was discarded and the sediment either used for examination by electron microscopy or resuspended in 3–4 ml of bicarbonate solution using a Vortex mixer. The final suspension was then placed in a freezer at −10° and used for enzymatic studies no later than 16 h after the isolation was completed.

II. Electron microscopy studies

Fixation for electron microscopy was carried out on minute pellets of membranes obtained by centrifugation; these were overlaid with an isotonic, buffered (pH 7.2) 2 % glutaraldehyde solution[7]. Ultrathin sections of Maraglas embedded[8] material were stained with lead salts[9] and examined with an Elmiskop 1.

*III. Studies of the effect of methylprednisolone**

106 rats were divided into 14 groups of 7 or 8 animals in each. 7 groups served as controls and 7 were given methylprednisolone. All animals had free access to water and food which consisted of Purina laboratory chow. For 4–5 days before being sacrified all experimental animals were injected with 2.5 mg of methylprednisolone daily. The control animals did not receive any treatment. In each experiment one control and one experimental group were sacrificed at the same time. In both groups, animals were of the same age and similar weight. Kidneys were removed under ether anaesthesia and whole homogenate and cell membranes were obtained as described above. During all steps of the procedure the same homogenizers and the same pestles were used for both control and experimental samples.

IV. Determinations of enzymatic activity

(Na^+-K^+)-ATPase and Mg^{2+}-ATPase were determined as described by KATZ AND EPSTEIN[1]. The incubation medium for measuring total ATPase activity contained 100 mM NaCl, 20 mM KCl, 5 mM MgATP and 10 mM imidazole buffer per l at pH 7.8. Mg^{2+}-ATPase was determined using a medium containing all components except KCl.

* In these experiments long-acting methylprednisolone acetate, Depo-Medrol, Upjohn, was used.

ATPase activity was measured by the amount of P_i liberated during incubation and determined by the method of FISKE AND SUBBAROW[10]. (Na^+-K^+)-ATPase activity was defined as the difference between total ATPase and Mg^{2+}-ATPase. Samples of whole homogenates were incubated for 15–30 min and cell membranes for 60 min. Results were expressed in μmoles of P_i released per mg of tissue protein per h. D-Glucose-6-phosphate phosphohydrolase (EC 3.1.3.9) was determined as described by HARPER[11] and the results expressed in μmoles of P_i released per mg of protein per h. Succinate dehydrogenase (EC 1.3.99.1) activity was measured by the method of BONNER[12] and expressed in arbitrary units as Δabsorbance reading per mg of tissue protein per h. Protein content of tissue samples was determined by the method of LOWRY *et al.*[13].

All results presented are averages of either 2 or 3 simultaneous determinations.

RESULTS

I. Morphological appraisal of cell membrane preparation (Fig. 1)

The membrane preparations, as revealed by electron microscopy, consisted of spherical or elongated vesicles with a content of extreme electron transparency; the

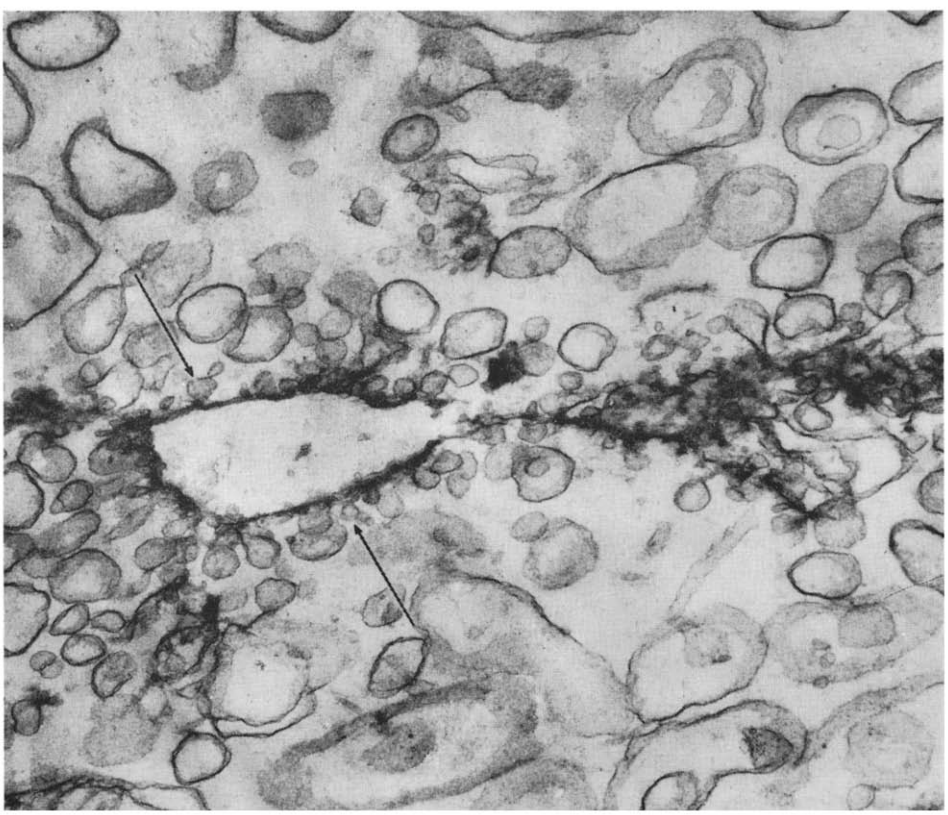

Fig. 1. Electron micrograph of pellet showing predominance of vesicular structures with very electron-transparent lumina; the hazy grey outline of parts of the limiting membrane of some of the vesicles is due to tangential sectioning of wrinkles of these structures. The arrows point to two of many small vesicles aligned along one side of a free-ending membrane. Magnification: 50 000 ×.

TABLE I

ENZYME ACTIVITY IN WHOLE HOMOGENATE AND CELL MEMBRANES OF KIDNEYS OF NORMAL AND METHYLPREDNISOLONE-TREATED RATS

Values for normal rats (C) are given in units (see text). Values for whole homogenate and cell membranes in methylprednisolone-treated rats (E) are expressed as percentage of the result found for the corresponding normal group. w.h. = whole homogenate; c.m. = cell membranes; enrichment = (activity in cell membranes)/(activity in whole homogenate).

Group No.	*$(Na^+$-$K^+)$-ATPase*			*Mg^{2+}-ATPase*			*Succinate dehydrogenase*			*Glucose-6-phosphatase*	
	w.h.	*c.m.*	*Enrichment*	*w.h.*	*c.m.*	*Enrichment*	*w.h.*	*c.m.*	*Enrichment*	*w.h.*	*c.m.*
1C	0.6	16.9	28.2	11.8	70.7	6.0	1.6	—	—	3.8	trace
1E	317%	108%	9.6	96%	119%	7.4	94%	—	—	211%	trace
2C	1.6	25.4	15.9	11.8	126.0	10.7	—	—	—	5.3	trace
2E	175%	83%	7.5	96%	61%	6.8	—	—	—	155%	trace
3C	1.5	17.3	11.5	13.9	46.8	3.4	1.2	1.4	1.2	5.7	trace
3E	100%	62%	7.2	95%	92%	3.3	100%	79%	0.9	146%	trace
4C	2.5	5.1	2.0	14.6	17.5	1.2	1.3	1.6	1.2	4.5	trace
4E	196%	100%	1.0	116%	113%	1.2	85%	94%	1.4	158%	trace
5C	1.3	20.3	15.6	18.6	223.5	12.0	1.1	2.0	1.8	5.0	trace
5E	169%	92%	8.5	103%	53%	6.2	100%	100%	1.8	152%	trace
6C	4.1	7.7	1.9	13.5	36.7	2.7	1.2	1.3	1.1	5.7	trace
6E	107%	119%	2.1	110%	116%	2.9	117%	108%	1.0	165%	trace
7C	2.1	8.1	3.9	15.8	134.2	8.5	1.0	2.8	2.8	4.0	trace
7E	162%	159%	3.8	97%	51%	4.5	100%	96%	2.7	193%	trace
Mean ± S.E.											
C	2.0 ± 0.47	14.4 ± 2.85	11.3 ± 3.58	14.3 ± 0.90	93.6 ± 27.2	6.4 ± 1.58	1.1 ± 0.22	1.8 ± 0.27	1.6 ± 0.32	4.9 ± 0.29	
E	175% ± 27	103% ± 12	5.7 ± 1.26	102% ± 3	86% ± 11	4.6 ± 0.87	99% ± 4	95% ± 5	1.6 ± 0.33	169% ± 9	

latter was identical with the surrounding of the vesicles (Fig. 1). These vesicles measured from 0.05 to 1 μ maximal dimension and occasionally one or more of the smaller vesicles were found to be present in the lumen of one of the larger vesicular structures. The walls of all of these vesicles were of identical and uniform thickness; wrinkling of the walls was common, with tangentially sectioned membranes frequently observable (Fig. 1). Among these vesicles were also free-ending membranes. Arrayed along the latter and usually confined to one side, were small vesicles of relatively uniform size (500–1000 Å). This arrangement was reminiscent of pinocytotic vesicles along a cell surface (Fig. 1). Structures probably depicting swollen mitochondria were rarely encountered; ribosomes attached to vesicles were extremely rare.

The method used in these studies produced an almost pure suspension of plasma membranes. The essential modification of this technique, when compared with the original method described by NEVILLE[6] for liver cell membranes, consists of introducing a supplementary layer of sucrose (*d* 1.18) in gradient centrifugation. A three-layer gradient, though of a different density, was also used by HAYS AND BARLAND[14] for the isolation of the membranes of the toad bladder, while a similar gradient was used by EMMELOT *et al.*[15] for liver cell membranes. If a supplementary layer is not used, the final preparation contains a large percentage of non-membranous material which has a density greater than 1.16 but lower than 1.18. There are two disadvantages of the method used in these studies. Firstly, there is a possibility that the final product obtained contains not only the plasma membranes of the cell but also some mitochondrial membranes. Secondly, the yield of the method is extremely small; from an initial 15–20 g of kidney tissue one finally obtains 2–4 mg of cell membrane protein.

II. Enzymatic activity of plasma membranes (Tables I and II, Fig. 2)

Plasma membranes of kidney cells are high in both (Na^+-K^+)-ATPase and

TABLE II

ACTIVITY OF (Na^+-K^+)-ATPase AND Mg^{2+}-ATPase IN WHOLE HOMOGENATE AND PLASMA MEMBRANES OF KIDNEYS OF NORMAL AND METHYLPREDNISOLONE-TREATED RATS

Values given are for mean and S.E. Enrichment = (activity in cell membranes)/(activity in whole homogenate).

	Whole homogenate (μmoles P_i per mg protein per h)	*Cell membranes (μmoles P_i per mg protein per h)*	*Enrichment*
(Na^+-K^+)-ATPase			
Control	2.0 ± 0.47	14.4 ± 2.85	11.3 ± 3.58
Experimental	3.0 ± 0.49	13.7 ± 2.19	5.7 ± 1.26
Mg^{2+}-ATPase			
Control	14.3 ± 0.90	93.6 ± 27.2	6.4 ± 1.58
Experimental	14.6 ± 1.09	64.8 ± 11.4	4.6 ± 0.87

Mg^{2+}-ATPase activity. In normal rats the specific activity of the plasma membrane fraction for (Na^+-K^+)-ATPase averaged 11.3 ± 3.6 (S.E.) times the activity per mg of protein in whole kidney homogenate. Mg^{+2}-ATPase was concentrated 6.4 ± 1.5 times. By contrast, there was scarcely any enrichment of succinate dehydrogenase activity, while glucose-6-phosphatase activity was virtually absent in plasma membranes. The low residual activity of succinate dehydrogenase, comparable to that in whole homogenate of kidneys, probably represents admixture of the membrane fraction with mitochondrial membranes. Similar low succinate dehydrogenase activities were present in the guinea-pig kidney plasma membrane preparations of COLEMAN AND FINEAN[16].

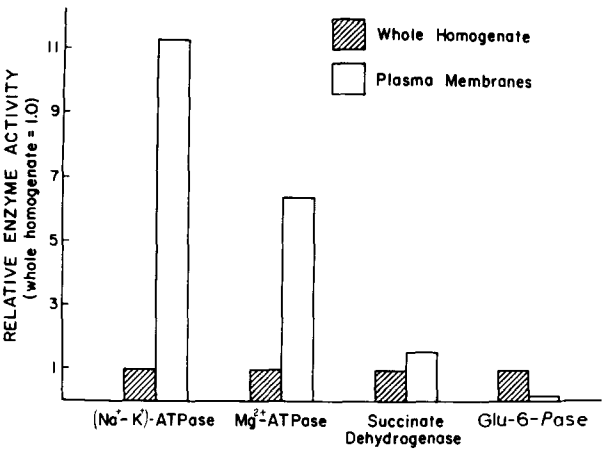

Fig. 2. Enrichment of enzymatic activity in plasma membranes of normal rats.

The degree of enrichment of ATPase activity in plasma membranes varied from day to day, but duplicate samples prepared in parallel at one time gave closely comparable results.

III. Effect of methylprednisolone on enzymatic activity of plasma membranes and whole homogenates (Tables I and II)

Treatment with methylprednisolone for 4–5 days increased (Na^+-K^+)-ATPase activity in whole kidney homogenates in 6 out of 7 experiments and by an average of 75%. Glucose-6-phosphatase also increased. As previously reported there was no change in Mg^{2+}-ATPase or succinate dehydrogenase activity[1].

In contrast to the findings in whole homogenate, the specific activity of (Na^+-K^+)-ATPase in purified plasma membranes was unaltered by methylprednisolone. The degree of enrichment of (Na^+-K^+)-ATPase in kidneys from control animals was therefore greater than that observed in rats treated with methylprednisolone.

It seems unlikely that treatment with hypertonic sucrose, necessary in the isolation procedure, obscured changes in (Na^+-K^+)-ATPase activity that were present. In additional experiments re-exposure of cell membranes to 69% sucrose solution did not alter the specific activity of this enzyme. Rat kidney microsomes, isolated as

described by KATZ AND EPSTEIN[1], showed no change in (Na^+-K^+)-ATPase activity when exposed to 36 % sucrose solution.

DISCUSSION

The method used for isolating plasma membranes of kidney cells is essentially a modification of that described by NEVILLE[6] for liver, involving homogenization in hypotonic solution and repeated washings with centrifugation on a sucrose density gradient. The final preparation appears quite homogeneous by electron microscopy. The considerable enrichment in (Na^+-K^+)-ATPase activity over the whole tissue homogenate, the absence of glucose-6-phosphatase activity (microsomal), and the low activity of succinate dehydrogenase (mitochondrial) support the histological evidence that the preparation consists chiefly of plasma membranes.

The mechanism by which (Na^+-K^+)-ATPase activity of the kidneys is influenced by corticosteroids is not clear. Adrenalectomy reduces (Na^+-K^+)-ATPase specific activity in the whole homogenate and microsomes of kidney tissue[1,17]. Treatment with glucocorticoids (but not with deoxycorticosterone or physiological doses of aldosterone) increases the activity of (Na^+-K^+)-ATPase, but not of Mg^{2+}-ATPase or of succinate dehydrogenase[1,17]. The activity of glucose-6-phosphatase, another microsomal enzyme, is also increased[1]. It has been proposed that the increase in (Na^+-K^+)-ATPase induced by glucocorticoids is related to an increase in glomerular filtration and hence sodium reabsorption per g of kidney tissue[1]. Since the site of active transport of Na^+ by kidney cells is thought to be the plasma membrane, a change in (Na^+-K^+)-ATPase related to changes in Na^+ transport might be reflected in a change in the specific activity of (Na^+-K^+)-ATPase in a purified preparation of plasma membranes.

In all but one of our experiments, methylprednisolone produced an increase in (Na^+-K^+)-ATPase in the whole homogenate of kidney. Nevertheless, the activity of (Na^+-K^+)-ATPase per mg of protein in plasma membranes was unchanged. Several explanations of this paradox are possible. First, it is conceivable that prednisone induces an increase in the (Na^+-K^+)-ATPase of endoplasmic reticulum, but not of the plasma membrane. (As an extension of this idea, it is possible that the endoplasmic reticulum is involved in transepithelial ion transport.) There is excellent evidence, however, that at least in the Ehrlich ascites cell, where this has been closely studied, most of the (Na^+-K^+)-ATPase of a broken cell homogenate derives from fragments immunologically identical to plasma membrane[3,4]. Second, it is possible that prednisolone induces an increase in (Na^+-K^+)-ATPase activity that is confined to a small portion, perhaps the basal or antiluminal border, of the limiting cell membrane. This might not be detected in the present studies because of dilution with membranes low in ATPase content. Finally, the simplest explanation of the data is that methylprednisolone stimulates an increase in the amount of plasma membrane per cell without inducing an increase in the specific activity of enzyme per unit of plasma membrane. This might be accomplished, for example, by proliferation of the basal infoldings of the limiting cell membrane at the antiluminal border, where Na^+ is pumped. The activity of the enzyme in the whole homogenate of kidney would therefore be increased without an increase in the enzymatic activity per mg of cell membrane. The observation that plasma membranes isolated from normal rat kidneys demonstrated a greater enrichment in (Na^+-K^+)-ATPase than those prepared from

rats treated with methylprednisolone is consistent with this hypothesis. Further evidence might be obtained from electron microphotographs of the kidneys of rats treated with steroids, or from assays of kidney tissue for other enzymes known to be constituents of the plasma membrane.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the technical assistance of Mrs. L. VALENTINE and Mrs. N. MYKETEY.

This work was aided by Grants (HE-00834, AM-5015 and GM-14834) from the National Institute of Health.

One of the authors (F.H.E.) is a recipient of a Research Career Award of the U.S. Public Health Service.

REFERENCES

1 A. I. KATZ AND F. H. EPSTEIN, *J. Clin. Invest.*, 46 (1967) 1999.
2 A. I. KATZ AND F. H. EPSTEIN, *Israel J. Med. Sci.*, 3 (1967) 155.
3 D. F. M. WALLACH AND V. B. KAMUT, *Proc. N. Dakota Acad. Sci.*, 52 (1964) 721.
4 V. B. KAMUT AND D. F. M. WALLACH, *Science*, 148 (1965) 1343.
5 D. F. M. WALLACH, V. B. KAMUT AND M. H. GAIL, *J. Cell Biol.*, 30 (1966) 601.
6 D. M. NEVILLE, JR., *J. Biophys. Biochem. Cytol.*, 8 (1960) 414.
7 D. D. SABATINI, K. G. BENSCH AND R. J. BARRNETT, *J. Cell Biol.*, 17 (1963) 19.
8 J. FREEMAN AND B. O. SPURLOCK, *J. Cell Biol.*, 13 (1962) 437.
9 M. J. KARNOVSKY, *J. Biophys. Biochem. Cytol.*, 11 (1961) 729.
10 C. M. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
11 A. E. HARPER, in H. V. BERGMEYER, *Methods of Enzymatic Analysis*, Academic Press, New York, 1965, p. 788.
12 W. D. BONNER, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Academic Press, New York, 1955, p. 722.
13 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
14 R. M. HAYS AND P. BARLAND, *J. Cell Biol.*, 31 (1966) 209.
15 P. EMMELOT, C. J. BOS, E. L. BENEDETTI AND PH. RÜMKE, *Biochim. Biophys. Acta*, 90 (1964) 126.
16 R. COLEMAN AND J. B. FINEAN, *Biochim. Biophys. Acta*, 125 (1966) 197.
17 E. J. LANDON, N. JEZAB AND L. FORTE, *Am. J. Physiol.*, 211 (1966) 1050.