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BINDING OF ATP TO AND RELEASE FROM MICROSOMAL ($Na^+ + K^+$)-ATPase

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SUMMARY

1. Turtle bladder microsomes, which possess ($Na^+ + K^+$)-ATPase activity, form Mg^{2+}-dependent acid-stable, radioactively labelled complexes during their incubation at pH 7.4 with 0.01 mM concentrations of [U-^{14}C]ATP, [γ-^{32}P]ATP, and [α-^{32}P]ATP.

2. The formation of a ^{14}C-labelled enzyme–ATP complex is demonstrated by the chromatographic isolation of intact [U-^{14}C]ATP which had been cleaved (by 3 different chemical methods) from its binding sites on the ^{14}C-labelled microsomal precipitates; and this is confirmed by an analogous isolation of intact [γ-^{32}P]ATP cleaved from γ-^{32}P-labelled precipitates.

3. The pH-dependent patterns of the formation of native complexes and of the breakdown of the acid-denatured complexes show that the ^{14}C-labelled microsomal protein is different from the Na^+-dependent γ-^{32}P-labelled protein. The pH dependency of ^{14}C labelling of native microsomes incubated with [U-^{14}C]ATP is consistent with the behavior expected of a phosphoramido bond; while that of γ-^{32}P labelling of native microsomes incubated with [γ-^{32}P]ATP is consistent with the behavior expected of an acyl phosphate bond.

4. The enzyme–ATP complex (or complexes) possesses at least two bonds between the enzyme and ATP: one between the γ-phosphate of ATP and the protein; and the other between the adenosine of ATP and the protein. The presence of an adenosinyl–protein bond is suggested by the fact that the same reagent removes all of the α-^{32}P label but only half of the U-^{14}C label from paired sets of microsomal complexes incubated under identical conditions except for the radioactive label (α-^{32}P *vs* U-^{14}C) on the substrate (ATP).

5. The pH-dependent pattern of ^{14}C labelling of microsomes by [U-^{14}C]ATP is nearly superimposable upon that of ($Na^+ + K^+$)-ATPase activity, while that of the Na^+-stimulated γ-^{32}P labelling of microsomes by [γ-^{32}P]ATP is nearly superimposible upon the mucosal pH dependence of Na^+ transport in the intact tissue.

6. The addition of 1.0 mM ATP or ADP chases half of the ^{14}C label from native microsomes which had become labelled during their incubation with 0.01 mM [U-^{14}C]ATP. The significance of these and other data with respect to the reversibility of the formation of an enzyme–ATP complex and its role in ADP:ATP exchange is discussed.

7. The assignment of parallel reaction paths to ($Na^+ + K^+$)-ATPase (E) and to the sodium-stimulatable phospho-enzyme (E') is discussed in relation to the nature of the intermediary complexes in the microsomal system; and in relation to the energy transduction and ion translocation required of active Na^+ transport in the intact system.

INTRODUCTION

Background

Previous work from this laboratory revealed the presence of a ^{14}C-labelled protein complex in acid-precipitated turtle bladder microsomes which had been pre-incubated in their native states with [8-^{14}C]ATP in earlier experiments[1] and with [U-^{14}C]ATP in more recent experiments[2,5]. Part of this ^{14}C-protein proved to be an E–ATP complex on the basis of recovering [^{14}C]ATP in the supernatants of acid-precipitated ^{14}C-labelled microsomes which had been partially degraded after treatment with hydroxylamine and molybdate[4].

The ^{14}C labelling of microsomes incubated with [U-^{14}C]ATP is rendered ouabain inhibitable only in the simultaneous presence of Mg^{2+}, Na^+, K^+[1,6,7], a property shared by the overall ($Na^+ + K^+$)-ATPase-catalyzed hydrolysis. This indicates that part of ^{14}C-labelled E–ATP is in series with the overall hydrolysis. It was expected that the formation of this ouabain-sensitive E-[U-^{14}C]ATP would also be in series with the formation of the Na^+-stimulated phospho-enzyme (Na^+-E–P) which forms only in the presence of Mg^{2+} and Na^+ and apparently breaks down only in the presence of Mg^{2+}, Na^+ and K^+. The apparent breakdown is blocked by ouabain only in the simultaneous presence of Mg^{2+}, Na^+, and K^+. Thus, the phospho-enzyme activity like ^{14}C labelling and ($Na^+ + K^+$)-ATPase is rendered ouabain sensitive only in the simultaneous presence of Mg^{2+}, Na^+, and K^+.

However, recent evidence suggests that neither the formation nor the lack of formation of Na^+-E–P is necessarily in series with the overall hydrolysis. For example, the increment of Na^+-E–P is formed in the presence of $Mg^{2+} + Na^+$ and disappears in the presence of $Mg^{2+} + Na^+ + K^+$ even when the ($Na^+ + K^+$)-induced increment of hydrolysis is completely inhibited by Ca^{2+} (ref. 5) or ITP[8,9].

Problems

The aforementioned findings raise questions concerning the properties of the ^{14}C- and ^{32}P-labelled proteins in acid-denatured microsomes as compared to those of the correspondingly labelled proteins in native microsomes.

One question is whether the acid-denatured states of E–ATP or E–P measured in microsomal precipitates are the same chemical entities as are the native states of these complexes formed at pH 7.0 during the incubation of the catalytically active protein. Though not rigorously proven, it is generally assumed that the bonds remaining in the acid-denatured protein complexes are the same as those formed in the native complexes. In this connection, covalent bonds have been demonstrated in the case of E–P[10–14] and have been suggested in the case of E–ATP[4].

A second question is whether the stability of the bonds in the acid-denatured complexes depends upon the previous presence of Na^+ and K^+ in the incubation

fluid during the reaction between the native microsomes and the substrate. This question provided the basis for that portion of the present experiments which deals with properties of the pre-formed acid-denatured microsomal complexes, *E*-ATP and *E*-*P*.

A third question is how the formation of a given native complex (*E*-ATP or *E*-*P*) depends upon the pH of the incubation fluid during the reaction between native microsomes and substrate. This question provided the basis for that portion of the present experiments which deals with some of the properties of bonds in the native microsomal complexes.

Purposes

Accordingly, experiments were designed to determine: (a) the stability of the acid-denatured, intermediary complexes, as well as the nature of the products released by the chemical breakdown of these denatured complexes; and to determine (b) the pH dependence of the formation of the native complexes, as well as the extent to which they can exchange their radioactive labels with ATP or ADP during the reaction between native microsomes and substrate.

The purposes of such experiments were to gain insight into the strength and qualitative nature of the bonds in the acid-denatured complexes, (*E*-ATP and *E*-*P*) and to determine whether they could be further identified by varying certain conditions required for their formation in their native states.

METHODS

A. Preparation

Mucosal epithelial cells were separated from the sub-mucosal layers of the isolated urinary bladder of fresh water turtle by incubation of bladder sacs in EDTA, containing Na^+ Ringer solutions according to the technique of Lipman *et al.*[15]. The suspension of mucosal cells was then homogenized and subjected to repeated differential ultracentrifugation at 0 °C in a Sorvall RC-2B refrigerated ultracentrifuge in order to separate the various sub-cellular fractions. As has been described[7], a supernatant fluid free of nuclei, mitochondria, and heavy microsomes was subjected to a centrifugal force of 35000 × *g* for 2 h in the Sorvall apparatus, and the resulting pellet, defined operationally as the microsomal fraction, was stored at −30 °C for 1 to 20 days prior to being used as the enzyme source in all of the present experiments.

B. Native microsomes

All experiments on native microsomal fractions were of the same basic design. In each flask 100 μl of a reaction mixture containing 50–100μg of microsomal protein was incubated with: 0.01 mM ATP (as the U-^{14}C-, α-^{32}P- or γ-^{32}P-labelled ATP); 3 mM $MgCl_2$; 85 mM NaCl when indicated; 5 mM KCl when indicated; 0.1 mM Tris–EDTA; 0.1 mM ouabain when indicated; 40 mM Tris–HCl or 50 mM of other buffers when indicated (see Methods C, below) final pH 7.3. When the final pH was other than 7.3, the buffer systems of Heppel[16] were used (see Methods C); and when the design required Na^+-free, K^+-free incubation fluids, Tris was substituted for Na^+ in these buffer systems.

Incubation reactions, initiated by the addition of ATP and lasting for 50 s at 0 °C, were terminated by the addition of 100 μl of 10 % (w/v) $HClO_4$, after which the mixture was centrifuged at 15000 × *g* for 15 min at 0 °C. The supernatant was discarded and the microsomal precipitate was washed with 200 μl of a cold (0 °C) solution containing 5 % $HClO_4$, 1 mM ATP, 1.0 mM ADP, 1.0 mM AMP and 1.0 mM P_i; then resuspended and recentrifuged at 15000 × *g* for 15 min at 0 °C. The washings with non-radioactive nucleotides and P_i and the centrifugations were repeated 4–6 times, or until the effluent wash fluid contained little or no detectable radioactivity.

The washed microsomal precipitates were then transferred to a vial containing the ethanol scintillation fluid[4]; and the radioactivity remaining on the microsomal precipitate was measured in a Beckman liquid scintillation counter (LS-230).

C. Acid-killed microsomes

The amount of radio label bound to the protein in acid-killed microsomal complexes (obtained as decribed under Methods B) was determined before and after reacting these complexes with buffer solutions of varying pH and with hydroxylamine and/or ammonium molybdate.

The amounts and kinds of radio-labelled nucleotides (ATP, ADP, and AMP) released into the supernatant fluids by the aforementioned reactions of the acid-killed microsomal complexes were also determined.

Buffer systems. In the experiments on acid-killed microsomes, the pH-dependent stability of the radio-labelled complexes was determined; and in the experiments on native microsomes, the pH dependence of the actively catalyzed process of radio labelling of microsomes was determined. In both sets of experiments, the final pH in each reaction flask was fixed at any desired level (between pH 2.0 and 10.2) by addition of the appropriate buffer[16] to a final concentration of 50 mM. The pH ranges and buffer systems were: pH 1.0–2.0, HCl–KCl; pH 2.0–2.5, glycine–HCl; pH 5.5–6.5, citric acid–sodium citrate; pH 6.5–7.0, citric acid–Na_2HPO_4; pH 7.0–8.0, NaH_2PO_4; pH 7.0–9.0, Tris–HCl; and 9.0–10.5, $NaHCO_3$–Na_2CO_3.

During exposure of the acid-denatured precipitates to the solution of pH 10.2 at 38 °C, there occurred a 20 % loss of protein from the microsomal precipitate; and this loss was accounted for in estimating the amount of residual labelling of such precipitates. Protein losses did not occur at any other level of pH to which the microsomes (native or acid denatured) were exposed. In fact, no such protein loss was detectable after exposure of the native microsomes to pH 10.2 at 0 °C.

Treatment fluids. The partial or complete splitting of bound radio label from the acid-killed microsomal precipitates was achieved by exposure of 100 μg of such precipitates for 10 min at 38 °C to 100 μl to any one of the following solutions: 5 % $HClO_4$; any one of the aforementioned buffer systems over the pH range of 2.0–10.2; 0.8 M hydroxylamine in HCl at pH 1.0–2.0 or hydrxylamine in Tris buffer at pH 7.0; and 0.2 % ammonium molybdate in 0.25M H_2SO_4.

The amount of splitting of the radio label from microsomal precipitate was estimated from measurements of ^{14}C or ^{32}P bound to the precipitates before and after their reaction with each of the above treatment fluids; and the percentage distribution of the ^{14}C- or ^{32}P-labelled nucleotides (ATP, ADP and AMP) released onto the supernatant fluid by such reactions was determined chromatographically as described in Methods D.

D. Materials and techniques

Reagents. The Tris and disodium salts of ATP, ADP, and AMP were obtained from Sigma Chemical Co., St. Louis, Mo. Isotopically labelled forms of ATP and ADP included: the ammonium salt of [U-^{14}C]ATP (580 Ci/mole); the ammonium salt of [8-^{14}C]ATP (60 Ci/mole); the ammonium salt of [8-^{14}C]ADP (45 Ci/mole); the sodium salt of [γ-^{32}P]ATP (1–10 Ci/mmole); and the acid form of [α-^{32}P]ATP (5 Ci/mmole). Cellulose MN 300-polyethyleneimine-impregnated chromatographic strips were obtained from Brinkman Instrument Inc., Westbury, Conn.

Chromatography. The aqueous solvent system used for the separation of nucleotides on the cellulose MN300-polyethyleneimine chromatographic strips was 0.25 M KH_2PO_4, pH 3.4. Aliquots of 40 μl of U-^{14}C-enriched or γ-^{32}P-enriched supernatant fluids (obtained from the various treatments of the labelled microsomal precipitates), together with "carrier amounts" (1.0 μl of 20 mM solutions) of non-radioactive ATP, ADP and AMP were applied to the base of the cellulose MN300-polyethyleneimine plates. Concomitantly, aliquots of standard solutions of [U-^{14}C]ATP or [γ-^{32}P]ATP together with carrier amounts of ATP were applied, and aliquots of [8-^{14}C]ADP with carrier amounts of non-radioactive ADP were similarly applied to the base of the cellulose MN300-polyethyleneimine plates. After 60 min of migration of the nucleotide-containing unknowns and standard solutions, the strips were dried in warm air and placed into a Wood ultraviolet lamp, whereby the differential migration of ATP, ADP and AMP was determined from the location of the fluorescent spots. The spots (unknowns and standards) were then excised under direct vision in the ultraviolet light and immersed in 10 ml of toluene scintillation fluid where the radioactivity was determined.

Techniques. The concentration of microsomal protein was determined by the method of Lowry *et al.*[17]. Counting of protein-bound or free radioactive labels (^{14}C and ^{32}P) was determined by the liquid scintillation technique described previously[4].

RESULTS

A. Acid-killed microsomal complexes

(1) Effect of pH on stability of complexes

Aliquots of acid-precipitated washed microsomes (previously labelled in the native state with [U-^{14}C]ATP at pH 7.4 as indicated under Methods B) were subsequently mixed with six different buffer solutions, the pH of which ranged from 2.0–10.2, and further handled as described under Methods C. The final pellets were washed several times with non-radioactive ATP, ADP and P_i (final concentrations 1.0 mM) at the same pH, and re-centrifuged prior to determining the residual ^{14}C or ^{32}P on the precipitate.

Fig. 1 is a plot of values of residual U-^{14}C bound to the microsomal precipitates as a function of the pH of the solution to which the precipitate had been exposed. The original native microsomes had been incubated with [U-^{14}C]ATP in the presence of Mg^{2+}. Not shown is an identical plot in two experiments on native microsomes incubated with [U-^{14}C]ATP in the presence Mg^{2+} and Na^+.

The stability of the ^{14}C-labelled complex (E–ATP) was maximal after exposure) to acid solutions (pH 1–3) and minimal after exposure to alkaline solutions (pH $>$8.5).

For example, the ^{14}C labelling of the precipitate decreased by 90 % after exposure to the solution of pH 10.2. This degree of alkali lability was also found after pre-incubation of the microsomes in the presence of Mg^{2+} and Na^+. The fact that addition of Na^+ to the original native incubation mixture had no discernible effect on the pH stability pattern of the ^{14}C-labelled microsomal precipitate is consistent with previously reported data showing that the amount of ^{14}C labelling of native microsomes incubated at pH 7.0 with [U-^{14}C]ATP and Mg^{2+} is not changed by addition of Na^+ and/or K^+ K^+ [4,5].

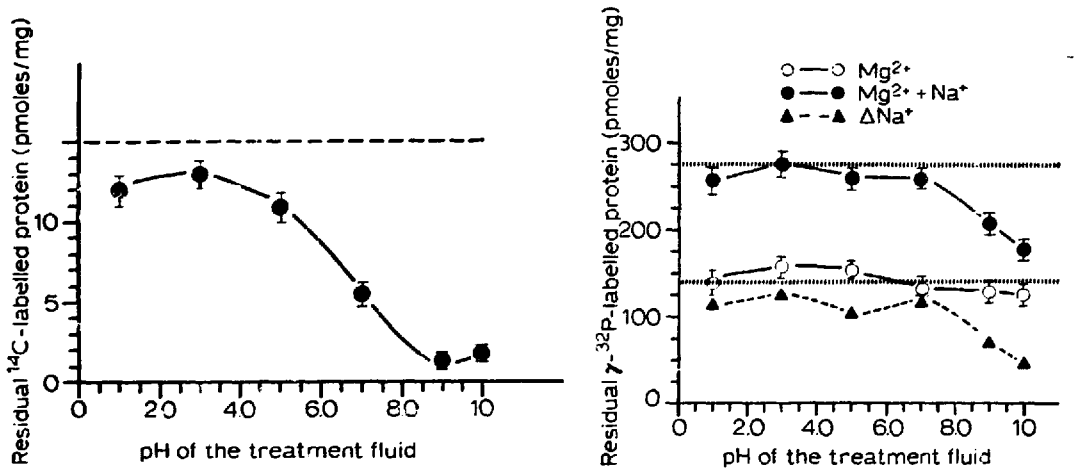

Fig. 1. Acid-denatured microsomes. Residual ^{14}C labelling of acid-precipitated microsomal complexes *versus* pH of the solution to which U-^{14}C-labelled microsomal precipitates had been exposed for 10 min at 38 °C. Units are expressed as the equivalent amount of bound nucleotide estimated from specific activity of [U-^{14}C]ATP, a parameter which cannot discriminate among the species: ATP, ADP, AMP, adenosine, adenine, or ribose. Each one of the solid circles and vertical bars denotes a mean value ± S.E. estimated from data of 8 experiments (n = 8). Dashed horizontal line denotes the mean amount of ^{14}C labelling of microsomes at 0 °C during incubation in the native state with [U-^{14}C]ATP in the presence of Mg^{2+} (n = 6) and Mg^{2+} + Na^+ (n = 2). Complete details on techniques and conditions of incubation, formation of complexes, acid denaturation, washing, and subsequent treatment of acid-denatured U-^{14}C-labelled microsomal precipitates are described in Methods.

Fig. 2. Acid-denatured microsomes. Residual ^{32}P labelling (expressed as the amount of γ-phosphoprotein) of acid-precipitated microsomal complexes *versus* pH of the solution to which γ-^{32}P-labelled microsomal precipitates were exposed for 10 min at 38 °C. Each one of the solid or open circles and vertical bars denotes a mean value ± S.E. estimated from paired data of 6 experiments. Dashed horizontal lines denote the mean amount of γ-^{32}P labelling of microsomes at 0 °C during incubation in the native state with [γ-^{32}P]ATP in the presence of Mg^{2+} (lower line) and of Mg^{2+} + Na^+ (upper line). Data denoted, ΔNa, were estimated by subtracting the Mg^{2+}-dependent from the (Mg^{2+} + Na^+)-dependent values. Complete details on techniques and conditions of incubation, acid precipitation, washing and treatment of acid-denatured γ-^{32}P-labelled microsomal precipitates are described in Methods.

The alkali lability of the ^{14}C-labelled protein complex shown in the figure does not disclose the exact site of cleavage of the [U-^{14}C]ATP–protein complex because none of the ATP-phosphate is labelled. Thus, it was pertinent to examine the pH stability of γ-^{32}P-labelled microsomal complexes obtained from native microsomes incubated with [γ-^{32}P]ATP under the same conditions as those used in generating the data of Fig. 1.

Fig. 2 is a plot of values of residual phosphate bound to the microsomal precipitate as a function of the pH of the solution to which the precipitate had been exposed.

The original native microsomes had been incubated with [γ-^{32}P]ATP at pH 7.3 in the presence of Mg^{2+} alone and in the presence of Mg^{2+} and Na^+. The dependence of the stability of the γ-^{32}P-labelled *E–P* complex on pH was related to the ionic conditions which had prevailed during the formation of this complex in its native state.

For example, the Mg^{2+}-dependent ^{32}P labelling of the microsomal precipitate was stable between pH 2.0 and 10.2. In contrast, the (Mg^{2+} + Na^+)-dependent ^{32}P labelling of the microsomal precipitates remained stable between pH 2.0 and 6.0 but was reduced at pH levels in excess of 6.0. The amount of the (Mg^{2+} + Na^+)-dependent *P* labelling, found after exposure of the precipitates to a pH of 10.2, was 75 % of that found after exposure to a pH of 1.0. The estimated Na^+-induced increment of phosphate binding after exposure of the precipitates to a pH 10.2 was less than half of that at pH 1.0.

(*2*) *Effect of hydroxylamine, molybdate and pH*

If the bond between the γ-phosphate and the enzyme in both the *E–P* and *E–*ATP complexes were an acyl phosphate, the addition of NH_2OH to either one or both of these phosphoproteins at pH 7.0 should cleave this bond to form the enzyme hydroxamate with the release of P_i from *E–P* and of ATP from *E–*ATP.

But the *E–*ATP complex is reduced by 60 % after exposure of the ^{14}C-labelled precipitate to a solution buffered at pH 7.0, even in the absence of NH_2OH (see Fig. 1). Consequently, the effect of the pH *per se* had to be included when testing for the effect of hydroxylamine on the stability of the ^{14}C binding to the microsomal precipitates.

Accordingly, radio-labelled microsomal precipitates (prepared as described in Methods B) were exposed to three levels of pH, 1.0, 7.0, and 10.2; to NH_2OH at pH 1.0 and 7.0; to molybdate at pH 1.0 and 7.0 and to NH_2OH *plus* MoO_4^{2-} at pH 1.0. The basis for the tests on the effect of molybdate was its reported catalytic effect on acyl phosphate breakdown at low levels of pH[18].

Table I shows mean values ± S.E. for the amounts of residual ^{14}C or ^{32}P labelling on microsomal precipitates before (Line 1) and after exposure to solutions of increasing alkalinity (Lines 2, 3, and 4); to NH_2OH in Tris buffer at pH 7.0 (Line 5); and after a sequential procedure consisting of a 10-min exposure to NH_2OH in 0.1 M HCl followed by centrifugation, decanting the supernatant and a 10-min exposure of the precipitate to ammonium molybdate in 0.25 M H_2SO_4 (Line 6). The microsomal precipitates had been produced from 3 sets of microsomes (6 experiments) similarly incubated with [γ-^{32}P]ATP.

Data in Line 1 ("none") show that the mean amount of ^{14}C labelling of the microsomes (15 pmoles/mg) was much less than the corresponding amounts of γ-^{32}P labelling (143 pmoles in the presence of Mg_2^+ alone, and 290 in the presence of Mg^{2+} + Na^+).

Data in Lines 2–4 show that increasing the pH of the solutions to which the precipitates were exposed resulted in a removal of 20–90 % of the ^{14}C label; of 0.7–8.0 % of the Mg^{2+}-dependent ^{32}P label; 11–38 % of the (Mg^{2+} + Na^+)-dependent γ-^{32}P label; and 21–67 % of the Na^+-dependent increment of the γ-^{32}P label. This is consistent with the data of Figs 1 and 2 indicating that the ^{14}C binding to microsomal precipitates is more alkali-labile than is the (Mg^{2+} + Na^+)-dependent γ-^{32}P binding; and that in contrast, the Mg^{2+}-dependent ^{32}P binding is relatively alkali stable under conditions of the 10-min exposure at 38 °C.

Data in Line 5 (NH_2OH, pH 7.0) show that the 10-min exposure of the labelled precipitates to NH_2OH at pH 7.0 resulted in the removal of practically all of the

TABLE I

CHEMICAL BREAKDOWN OF ^{14}C- AND γ-^{32}P-LABELLED MICROSOMAL PRECIPITATES

Mean values ± S.E. for the amount of labelling of acid-denatured microsomal complexes (expressed as the equivalent amount of bound nucleotide or bound γ-phosphate) after their formation at 0 °C from the incubation of native microsomes with [U-^{14}C]ATP or [γ-^{32}P]ATP (Row 1); and the residual amount of label on these precipitates after their exposure for 10 min at 38 °C to the various reagents listed in the first column (Rows 2–6). Complete details on conditions of preincubation and isotopic labelling of native microsomes and on the techniques of handling of acid-denatured microsomal precipitates are found in Methods.

Additions to pre-formed isotopically labelled microsomal precipitates for 10 min at 38 °C	*Residual binding to microsomal precipitates*			
	^{14}C *(pmoles/mg)*	γ-^{32}P *(pmoles/mg)*		
	Mg^{2+}, Na^+, K^+, *	Mg^{2+}	$Mg^{2+} + Na^+$	ΔNa^+, **
1. None	15 ± 1.1	143 ± 8.5	290 ± 18	147
2. $HClO_4$	12 ± 1.0	142 ± 9.0	258 ± 16	116
3. pH 7.0 (Tris)	5.5 ± 0.8	137 ± 11.0	265 ± 14	128
4. pH 10 (CO_3^{2-}–HCO_3^-)	1.5 ± 0.7	132 ± 8.5	180 ± 6.2	48
5. NH_2OH, pH 7.0	0.5 ± 0.3	135 ± 12.1	140 ± 6.6	±0
6. NH_2OH, MoO_4^{2-}, pH 1.0	8.0 ± 0.8	130 ± 8.8	191 ± 12.0	61

* With [U-^{14}C]ATP, 6 experiments on 3 sets of microsomes were performed involving incubation with Mg^{2+}; 2 with $Mg^{2+} + Na^+$, and 2 with $Mg^{2+} + Na^+ + K^+$.

** With [γ-^{32}P]ATP, 6 experiments on 3 sets of microsomes were performed with Mg^{2+} and 3 with $Mg^{2+} + Na^+$; and the values under the column designated ΔNa are the differences between the mean values designated $Mg^{2+} + Na^+$ less those designated Mg^{2+}.

^{14}C label and all of the Na^+-dependent increment of the γ-^{32}P label. However, hardly any of the Mg^{2+}-dependent γ-^{32}P labelling was affected by the exposure to NH_2OH at pH 7.0, as has been reported previously[19].

Data in Line 6 ("NH_2OH, MoO_4^{2-}, acid") show that the consecutive exposure of the labelled precipitates to NH_2OH and $(NH_4)_2MoO_4$ at pH 1.0 resulted in the loss of: 47 % (7 pmoles/mg) of the ^{14}C label; 58 % of the Na^+-stimulated increment of the γ-^{32}P label; and only 5 % of the Mg^{2+}-dependent γ-^{32}P label.

Not shown are the data from control experiments wherein the labelled precipitates were exposed to (a) acidic solutions of either NH_2OH alone or MoO_4^{2-} alone, or to (b) MoO_4^{2-} in H_2SO_4 followed by NH_2OH in HCl (*i.e.* reversing the order of the sequential exposure to NH_2OH and MoO_4^{2-}). The results of such exposures were not significantly different from those of exposure to $HClO_4$ alone (see Row 2 of Table I).

In short, the ^{14}C labelling did not occur after the sequence of exposure, MoO_4^{2-}–NH_2OH, but did occur after the sequence NH_2OH–MoO_4^{2-}. This suggests that NH_2OH alone at pH 1.0 must complex in some way with the ^{14}C-labelled precipitate, even though no ^{14}C is removed from the precipitate.

Data in Fig. 2 and Table I show that the Mg^{2+}-dependent γ-^{32}P-labelled complex is resistant to alkali and to NH_2OH. On the other hand, the Na^+-stimulated increment of the γ-^{32}P-labelled complex and the ^{14}C-labelled complex are alkali-labile, NH_2OH-reactive complexes. Although the proportion (53 %) of the ^{14}C label remaining on the precipitate after exposure to NH_2OH and MoO_4^{2-} was of a similar order as was that (42 %) of the Na^+-dependent γ-^{32}P remaining on the identically treated precipitate, the absolute amounts were quite different, (*e.g.* 8 pmoles/mg for the ^{14}C-labelled protein *vs* 61 for the Na^+–γ-^{32}P-labelled protein), which means that the resi-

dual ^{14}C labelling does not measure the same substance as does the residual γ-^{32}P labelling.

(3) *Experiments with* [α-^{32}P]*ATP*

During the reaction between ATP and the native microsomes, bonds may form between the adenosine moiety of ATP and the enzyme protein; and if so, these bonds, together with the γ-phosphoryl–protein bond, could remain intact in the acid-denatured microsomal precipitate. It was thought that the presence of such bonds could be elicited by the use of [α-^{32}P]ATP as the substrate for the formation of *E*-ATP by the microsomal enzyme system.

Under identical incubation conditions, it was expected that the estimated amount of α-^{32}P labelling of the microsomal protein during the incubation of microsomes with [α-^{32}P]ATP would be of the same order of magnitude as that of the [U-^{14}C]ATP, and that the amount of α-^{32}P, like the U-^{14}C labelling would be much less than the amount of the γ-^{32}P labelling of protein during the incubation of microsomes with [γ-^{32}P]ATP.

Accordingly, the following experiments involved the formation of α-^{32}P-labelled proteins by native microsomes and the degradation of α-^{32}P-labelled acid-denatured microsomal precipitates. The incubation conditions were identical to those employed for the corresponding experiments on [U-^{14}C]ATP.

The mean level $\pm$ S.E. of α-^{32}P labelling of 4 sets of microsomes incubated in the presence of Mg^{2+} (8.05 $\pm$ 0.6 pmoles/mg protein), was not significantly different (*P* 0.9) from that in 4 paired sets of microsomes incubated in the presence of Mg^{2+} + Na^+, 8.10 $\pm$ 0.7 pmoles/mg (see Table II). The magnitude of the α-^{32}P labelling, about 50–80% of that of U-^{14}C labelling of microsomes (10-15 pmoles/mg) incubated with [U-^{14}C]ATP in corresponding experiments (see Table II), was but 3–5% of that of γ-^{32}P labelling of microsomes (150–300 pmoles/mg) incubated with [γ-^{32}P]ATP.

Table II presents the effects of 5 methods of chemical degradation on mean values $\pm$ S.E. for the residual α-^{32}P labelling of 3 sets of acid-denatured, α-^{32}P-labelled microsomal precipitates. For comparative purposes, the analogous results on residual U-^{14}C labeling (taken from data in Table I) are also presented.

The fact that the amount of α-^{32}P labelling was less than that of U-^{14}C labelling suggests: (a) that the adenosine (or the adenine and/or ribose) moiety of ATP could have formed bonds with the enzyme protein, over and above those formed between the γ-phosphate of ATP and the protein; and (b) that an *E*–ATP complex makes up 50% of the U-^{14}C-labelled protein in the acid-precipitated protein. However, the comparative data on α-^{32}P and U-^{14}C labelling in two different sets of microsomes were not precise enough to make a firm decision on the presence or absence of non-phosphoryl bonds between adenosine and the protein. To approach such a problem, we studied the nature of the sequential breakdown products of pre-formed, acid-precipitated α-^{32}P-labelled microsomes.

A complete removal of the α-^{32}P or of the U-^{14}C label from the microsomal precipitates was found after their exposure to NH_2OH at pH 7.0 (Line 3) or to CO_3^{2-}–HCO_3^- buffer at pH 10 (Line 4). Consequently, these treatments provide no information on the possible bonds between ATP and the enzyme because only the residual binding was measured, which was essentially nil in both cases.

After exposure to acid alone (Treatment 2), the residual α-^{32}P labelling of the microsomal precipitates was reduced by 50% from 8.10 pmoles/mg to 3.74 pmoles/mg.

TABLE II

CHEMICAL BREAKDOWN OF α-^{32}P-LABELLED MICROSOMAL PRECIPITATES

Effects of pH, NH_2OH and MoO_4^{2-}. Mean values ± S.E. for the amount of α-^{32}P and U-^{14}C labelling on microsomal precipitates (expressed as the equivalent amount of bound nucleotide) formed from the incubation of native microsomes with [α-^{32}P]ATP or [U-^{14}C]ATP; and the residual amount of label on the precipitates after their exposure for 10 min at 38 °C to the reagents shown in the first column. The α-^{32}P labelling of microsomes (prior to acid precipitation) was performed in 4 paired experiments in 3 sets of microsomes, 2 in the presence of Mg^{2+}, and 2 in the presence of Mg^{2+} + Na^+; and the U-^{14}C labelling was performed in 6 paired experiments under the same conditions (see Table I). Complete details on techniques and conditions of incubation of native microsomes and subsequent treatment of labelled microsomal precipitates are given in Methods.

Additions to pre-formed isotopically labelled microsomal precipitates for 10 min at 38 °C	*Residual binding to microsomal precipitates of*	
	α-^{32}P *(pmoles/mg)*	U-^{14}C *(pmoles/mg)*
1. None	8.10 ± 0.7	15 ± 1.1
2. $HClO_4$	3.74 ± 0.2	12 ± 1.0
3. NH_2OH, pH 7.0	±0	0.5 ± 0.2
4. pH 10 (CO_3^{2-}–HCO_3^-)	±0	1.3 ± 0.3
5. NH_2OH, MoO_4^{2-}, pH 1.0	±0	8.1 ± 0.6

The same exposure to acid alone in [U-^{14}C]ATP-labelled precipitates lowered the ^{14}C labelling by 20% from 15 to 12 pmoles/mg. The fact that the percentage removal of α-^{32}P was greater than that of ^{14}C suggests that the bond between the C-4 of the ribose and the α-phosphate in the *E*–ATP complex is cleaved and that some adenosine remains bound to the complex after its exposure to $HClO_4$.

This hypothesis was clearly verified in the experiment involving exposure of the α-^{32}P- or the U-^{14}C-labelled microsomal precipitate successively to NH_2OH at pH 1.0 and $(NH_4)_2MoO_4$ at pH 1.0 (Treatment 5), after which 100% and 50% of the α-^{32}P and ^{14}C labels, respectively, were removed from the microsomal precipitate (Table II).

The presence of half of the ^{14}C label and none of the α-^{32}P label on the protein shows that: (a) all of the C-4 ribose–phosphate bonds in *E*–[α-^{32}P]ATP must have been split during the treatment with acid–NH_2OH and MoO_4^{2-}; that (b) ^{14}C-labelled adenosine (or adenine or ribose) remains bound in the microsomal protein, which suggests that at least one bond connecting the adenosine moiety of ATP to the native protein forms during the incubation.

At the same time there is a large amount (191 pmoles; see Table I) of residual γ-^{32}P labelling on the protein precipitate after the same treatment with NH_2OH and MoO_4^{2-}; and this labelling must be located at different sites than is the ^{14}C labelling. Hence, in addition to the bond between the γ-phosphate of ATP and the enzyme, there has to be at least one additional bond formed between the adenosine of the ATP and the enzyme during the formation of the primary enzyme–substrate complex by (Na^+ + K^+)-ATPase. On the other hand, these data do not necessarily indicate which moiety of the ^{14}C-labelled protein (the NH_2OH–MoO_4^{2-}-resistant or the NH_2OH–MoO_4^{2-}-sensitive part) can be assigned to the ouabain-sensitive. (Na^+ + K^+)-ATPase function.

(4) Nucleotide release

In order to determine the nature of the radio-labelled compounds released from the microsomal precipitates into the supernatant fluids following the aforementioned reactions, aliquots of supernatant fluids which had become enriched with ^{14}C or ^{32}P, were analyzed *via* cellulose MN300–polyethyleneimine chromatographic plates along with non-radioactive ATP, ADP, and AMP (as described under Methods D). The non-radioactive compounds provided the isotope carrier and nucleotide localizer for ^{14}C- and ^{32}P-labelled nucleotide standards and unknowns migrating in parallel along the chromatographic paper. The radioactivity (cpm) in each standard aliquot was approximately the same as that in each aliquot of unknown supernatant.

(a) Release of [γ-^{32}P]ATP.

Intact ATP, found in the supernatant fluid of acid–NH_2OH–MoO_4^{2-}-treated microsomal precipitates pre-incubated with [U-^{14}C]ATP[4], was also sought for in the supernatant of similarly treated microsomal precipitates pre-incubated with [γ-^{32}P]-ATP. The γ-^{32}P-labelled microsomes were perchloric acid treated and washed as described under Methods B. Residual γ-^{32}P labelling on the precipitate was determined before and after exposure to the appropriate reactive solutions side by side with determinations of [γ-^{32}P]ATP released into the supernatant.

Table III presents mean values for: γ-phosphate binding to microsomal precipitates pre-labelled in the native state under the specified ionic conditions with [γ-^{32}P]ATP both before (A) and after (B) exposure to acid–NH_2OH–MoO_4^{2-} solutions. Also shown are values for the estimated differences of γ-phosphate (C); and the amount of ATP released into the supernatant treatment solution (D) wherein it is identified as that moiety of released ^{32}P which migrates identically with the carrier ATP.

TABLE III

BREAKDOWN OF γ-^{32}P-LABELLED MICROSOMAL PRECIPITATES WITH RELEASE OF INTACT ATP INTO SUPERNATANTS

Mean values ± S.E. for: (A) the amount of labelling of acid-denatured microsomal complexes (in terms of an equimolar amount of bound nucleotide) after their formation from the incubation of native microsomes with [γ-^{32}P]ATP; for (B) the residual amount of γ-^{32}P on these precipitates after exposure for 10 min at 38 °C to the acid–NH_2OH–MoO_4^{2-} solution, for (C) the total amount of γ-^{32}P removed from the precipitates; and for (D) the amount of intact γ-^{32}P-labelled ATP recovered in the supernatant fluid. Six experiments performed on 3 sets of microsomes, involved incubations with Mg^{2+}, Mg^{2+} + Na^+ and Mg^{2+} + Na^+ + K^+. Complete details on techniques and conditions of incubation and subsequent treatment of labelled microsomal precipitates are given in Methods.

Parameter	*Ionic conditions (pmoles/mg protein)*			
	Mg^{2+}	$Mg^{2+} + Na^+$	$Mg^{2+} + Na^+ + K^+$	ΔNa^+
A. [γ-^{32}P]Phosphoprotein (initial)	93 ± 5.1	159 ± 10	74 ± 3.6	66 ± 5.1
B. [γ-^{32}P]Phosphoprotein (after treatment)	71 ± 6.0	111 ± 8.2	55 ± 3.1	40 ± 2.1
C Total *P* release (A-B)	22 ± 3.0	48 ± 4.8	19 ± 1.6	26 ± 3.3
D. Measured release of [γ-^{32}P]ATP	4 ± 1.0	4 ± 1.5	5 ± 2.0	~0

Data of Line A, similar to those reported here[1,5] and elsewhere[20], show that the Mg^{2+}-dependent formation of phosphoprotein was increased by the addition of Na^+; and decreased to less than Mg^{2+}-dependent levels by the addition of $Na^+ + K^+$.

Data of Lines A, B and C show that after exposure of the pre-labelled precipitates to NH_2OH and MoO_4^{2-}, the γ-phosphate binding decreased by 22 pmoles/mg or 24% in the Mg^{2+}-dependent phosphoprotein; by 48 pmoles or 30% in the ($Mg^{2+} + Na^+$)-dependent phosphoprotein; and by 19 pmoles or 26% in the ($Mg^{2+} + Na^+ + K^+$)-dependent phosphoprotein.

Data of Lines C and D show that the amount of [γ-^{32}P]ATP released from the acid-denatured γ-^{32}P-labelled phosphoproteins into the supernatant was independent of the ionic conditions previously used in the formation of the phosphoproteins. On the other hand, the amounts of non-ATP γ-^{32}P released were dependent on the previously used ionic conditions; and paralleled those of the previously reported γ-^{32}P labelling of these microsomes (compare Line C with A).

In short, Table III shows that: (a) most of the γ-^{32}P labelling of microsomes is due to non-ATP binding (presumably *E-P*); that (b) on the basis of reactivity with acid–NH_2OH–MoO_4^{2+}, the Mg^{2+}-dependent phosphoprotein is chemically similar to the ($Mg^{2+} - Na^+ + K^+$)-dependent phosphoprotein but clearly different from the ($Mg^{2+} + Na^+$)-dependent phosphoprotein; and that (c) the [γ-^{32}P]ATP released into the supernatant amounts to 25–35% of the U-^{14}C binding (see Table IV).

(*b*) *Release of* [*U-*^{14}*C*]*ATP.* Whereas the exposure of ^{14}C-labelled microsomal precipitates to acidic solutions of NH_2OH and MoO_4^{2-} removes half of the ^{14}C label, a similar exposure to NH_2OH at pH 7.0 or to CO_3^{2+}–HCO_3^- buffer at pH 10 removes virtually all of the ^{14}C label from the precipitates (Table I). The nature and distribution of ^{14}C-labelled nucleotides so released was determined chromatographically in the next set of experiments.

Table IV presents chromatographic data on the distribution of ^{14}C-labelled nucleotides released into the supernatant fluid after the designated treatment of ^{14}C-labelled microsomes (data in left panel).

Also included are derived data which show the distribution of nucleotides corrected for the breakdown of free ATP after it has been cleaved from the protein. The corrected values were calculated from the crude data in the panel denoted "ATP standard". The 100% levels of ^{14}C binding ranged from 10–20 pmoles/mg.

ATP recovered chromatographically, accounted for as much as 53% (5–10 pmoles/mg) of the bound and released ^{14}C of microsomal precipitates exposed to NH_2OH at pH 7.0; 24% (3–5 pmoles/mg) of the bound and released ^{14}C label of microsomal precipitates exposed to CO_3^{2-}–HCO_3^- solutions; and 40% (4–8 pmoles/mg) of the bound ^{14}C (or 79% of the released ^{14}C) of precipitates exposed to acid solutions of NH_2OH and MoO_4^{2-}.

AMP accounted for as much as 65% of the bound and released ^{14}C label of microsomal precipitates exposed to CO_3^{2-}–HCO_3^- solutions; 38% of the bound and released ^{14}C of precipitates exposed to NH_2OH at pH 7.0; but only for 6% of the bound ^{14}C (or 11% of the released ^{14}C) of precipitates exposed to acid solutions of NH_2OH and MoO_4^{2-}. When AMP appears in excess of that which can be explained by the breakdown of free ATP, it suggests that the bond between the α- and β-phosphates in protein-bound ATP is more susceptible to cleavage than it is in free ATP. The data on complete removal of the ^{14}C label with subsequent chromato-

TABLE IV

IDENTIFICATION OF ^{14}C-LABELLED NUCLEOTIDES RELEASED AFTER TREATMENT OF U-^{14}C-LABELLED MICROSOMES AND [U-^{14}C]ATP STANDARD

Identification and distribution of intact ^{14}C-labelled nucleotides released from three kinds of chemical breakdown of ^{14}C-labelled microsomal precipitates formed from the incubation of native microsomes with [U-^{14}C]ATP in the presence of Mg^{2+} in 2 experiments. Also shown is the pattern of ^{14}C-labelled nucleotide release from known amounts of pure [U-^{14}C]ATP subjected to the same three treatments. Raw values were estimated from the total cpm in 50 μl of supernatant fluid; and corrected values were estimated from the data on the simultaneous breakdown of pure unbound U-^{14}C-labelled ATP as the result of its exposure to the corresponding treatment solution designated in the first column. Complete details on techniques and conditions of incubation of native microsomes; and on treatment of microsomal precipitates with subsequent chromatographic recovery of intact nucleotides in supernatants are given in Methods.

Exposure of U-^{14}C-labelled microsomal precipitates and [U-^{14}C]ATP standards for 10 min at 38 °C to treatment designated below		*Percentages and identity of ^{14}C-labelled nucleotides appearing in supernatant fluid after designated treatment of:*					
		U-^{14}C-labelled microsomes			*[U-^{14}C]ATP standard*		
		ATP	*ADP*	*AMP*	*ATP*	*ADP*	*AMP*
1. NH_2OH, pH 7.0 (100% ^{14}C removal)	raw	21	26	53	40	32	28
	corrected	53	10	38			
2. NH_2OH, MoO_4^{2-}, pH 1.0 (50% ^{14}C removal)	raw	57	21	22	73	16	11
	corrected	79	12	12			
3. pH 10, CO_3^{2-}–HCO_3^- (100% ^{14}C removal)	raw	21	12	67	85	8	7
	corrected	24	9	65			

graphic recovery of ATP and AMP are consistent with those on complete removal of the α-^{32}P label after the same chemical treatments (see Section 3), although for these data, the bond between ribose and the γ-phosphate of protein bound ATP is also involved.

In summary, the data of Tables I–III indicate that incubation of microsomes with [U-^{14}C]ATP, with [α-^{32}P]ATP, or with [γ-^{32}P]ATP results in the formation of an acid-stable *E*–ATP complex. The acid-denatured form of this *E*–ATP complex, containing a non-exchangeable radio label, can be cleaved chemically with the release and recovery of free ATP after exposure of U-^{14}C- or γ-^{32}P-labelled precipitates to acid-NH_2OH-MoO_4^{2-}; to NH_2OH at pH 7.0; or to CO_3^{2-}–HCO_3^- solutions at pH 10.2.

Thus, at least 50% of the U-^{14}C label bound to the microsomal precipitate is a protein-bound ATP which can be cleaved from the insoluble complex (*e.g.* by the reaction with neutral NH_2OH) as an intact ATP molecule, which, in turn, appears in the supernatant where it is isolated, identified, and quantitatively recovered.

B. Properties of the native microsomal proteins

The hydroxylamine-induced breakdown of the U-^{14}C-labelled protein or of the Na^+ increment of the γ-^{32}P-labelled protein is consistent with the presence of a γ-phosphoryl–glutamyl bond in the acid-denatured microsomal complexes, as has been established in the case of the Na^+ phosphoenzyme by Hokin and his colleagues[10,11]. However, the acid–NH_2OH–MoO_4^{2-}-induced removal of all of the α-^{32}P label and only half of the U-^{14}C label is consistent with the presence of additional bonds between

the adenosine moiety of ATP and the acid-denatured microsomal protein in acid-denatured microsomes; and, by inference, in native microsomes.

Accordingly, experiments were designed to determine certain properties of the formation of the native microsomal complexes — in particular, the pH-dependent patterns and the reversibility of the reactions involved in their formation.

(*1*) *pH dependence of formation of native complexes.* The pH-dependent patterns of formation of the native enzyme–substrate complexes (*E*–ATP and *E*–*P*) were determined and compared with the pH-dependent stability of the acid-denatured enzyme–substrate complexes. It was expected that the half-maximal levels of formation of the native complexes would occur at pH levels consistent with the pK_a values of the amino acid residues to which the whole ATP molecule or its γ-phosphate is bonded; and that this "pK_a" of complex formation could be related to a class of substrate-binding amino acid residues on the enzyme protein.

Native microsomes were labelled at various pH levels with [U-^{14}C]ATP or [γ-^{32}P]ATP in the presence of Mg^{2+}, (Mg^{2+} + Na^+), or (Mg^{2+} + Na^+ + K^+), and acid killed.

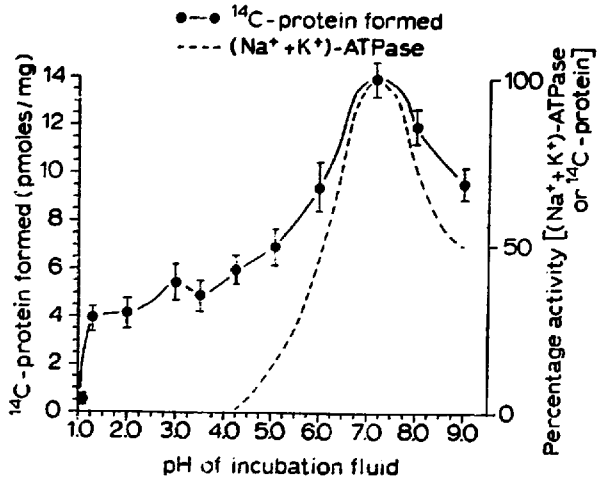

Fig. 3. Native microsomes. ^{14}C labelling of microsomes *versus* pH of incubation fluid. Each solid circle with the vertical bars denotes the mean value ± S.E. estimated from data of 10 experiments on microsomes incubated at 0 °C with [U-^{14}C]ATP in the presence of Mg^{2+} (n = 6), Mg^{2+} + Na^+ (n = 2), and Mg^{2+} + Na^+ + K^+ (n = 2). The dashed curve, taken from data of a previous report[21], denotes the pattern of (Na^+ + K^+)-ATPase expressed as percentage of the maximal activity at pH 7.4, (see scale of ordinate on right) *versus* pH of incubation fluid. Complete details on techniques, composition (substrate, ions, buffer, *etc.*), and conditions of incubation are described in Methods.

Fig. 3 is a plot of values of the ^{14}C labelling per mg of microsomal protein as a function of the pH of the incubation fluid. The amount of ^{14}C labelling of the native microsomes, near zero at pH 1.0, increased to reach a plateau of 4–5 pmoles/mg at pH levels of 2–3; increased further in a sigmoidal manner with a point of inflection (corresponding to 10 pmoles/mg) in the neighborhood of pH 6.0; and continued to increase to a maximum of 15 pmoles at pH 7.4. The half-maximal level of ^{14}C labelling occurs at a pH which is close to the pK_a of the imidazole group of a histidine residue on the protein; and is consistent with the possible presence of phosphoramido (–N–P) bonding in the *E*–ATP complex (see Discussion).

The dashed line in the plot represents a normalization of previously obtained

data[21] on the activity of $(Na^+ + K^+)$-ATPase as a function of the pH of the incubation fluid. There is a fairly close parallelism in the pH-dependent patterns of activity of both functions (*E*–ATP formation and $(Na^+ + K^+)$-ATPase.) This parallelism, together with the fact that both functions require the simultaneous presence of Mg^{2+}, Na^+ and K^+ in order to become inhibitable by ouabain[4], suggests that the reaction forming *E*–ATP is in a series sequence with the overall $(Na^+ + K^+)$-ATPase-catalyzed hydrolysis of ATP.

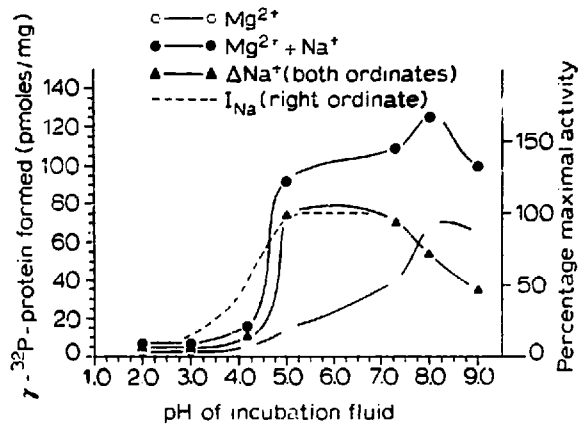

Fig. 4. Native microsomes. γ-^{32}P labelling (γ-phosphoprotein formation) of microsomes *versus* pH of incubation fluid. ○ and ● denote the mean values estimated from data of 6 paired experiments on microsomes which had been incubated with [γ-^{32}P]ATP in the presence of $Mg^{2+} + Na^+$. The Na^+-stimulated increment of γ-phosphoprotein was calculated by subtracting the amount formed with Mg^{2+} from that formed with $Mg^{2+} + Na^+$. The S.E. at the level of 100 pmoles/mg (not shown) was ± 4 pmoles/mg. The dashed curve, taken from previous data on the intact turtle bladder[22], indicates the pattern of net active transport of Na^+ (I_{Na}), expressed as percentage of the maximal transport rate of pH 7.4 (see scale of ordinate on right) so that it could be compared with the Na^+-stimulated increment of γ-^{32}P labelling of the microsomes. Complete details on techniques, composition (substrate, ions, buffer, *etc.*), and conditions of incubation are described in Methods.

Fig. 4, the format of which is similar to that of Fig. 3, is a plot of values of phosphoprotein formation (γ-^{32}P labelling) *versus* pH of the incubation fluid.

In the presence of Mg^{2+} alone, the γ-phosphate binding increased gradually from minimal levels at pH 4 to reach half-maximal levels at pH 7.4, and maximal levels at pH 8–9.

In the presence of $Mg^{2+} + Na^+$, the γ-phosphate binding, uniformly greater than that with Mg^{2+} alone, increased sharply from minimal levels at pH 4.0 to reach half-maximal levels at pH 4.3 and near-maximal levels at pH 5.0. The binding continued to increase gradually as the pH was increased to levels greater than pH 5.0 and reached a maximal level at pH 8.0.

The Na^+ increment of γ-phosphate binding estimated as the difference between the $Mg^{2+} + Na^+$ and Mg^{2+} phosphoprotein reached half-maximal levels at pH 4.3, and maximal levels at pH 5.0 to 7.0.

The half-maximal point of the Na^+-induced increment (as well as that of the $Mg^{2+} + Na^+$ moiety) of γ-phosphoprotein occurred at a pH close to the pK_a of the carboxyl group of a glutamyl or related acyl residue on the protein. This is consistent with the NH_2OH reactivity of the γ-^{32}P-labelled phosphoprotein (see Table I) and with the findings of Hokin *et al.*[10,11].

The dashed line in the plot represents a normalization of previously obtained data on net Na^+-transport rates (I_{Na}) *versus* the pH of the mucosal bathing fluid in the intact functioning turtle bladder[22]. The pH-dependent pattern of the Na^+-induced formation of phosphoprotein is similar to that of the net Na^+ transport, which suggests that the formation of the Na^+-induced *E–P* complex can become the rate-limiting step in the downhill, carrier-mediated translocation of Na^+ across the mucosal-facing membrane.

The pH associated with the half-maximal level of Na^+-phosphoprotein formation is almost two pH units lower than that associated with the half-maximal level of *E*–ATP formation. Moreover, previously reported data[5] show that the ion-dependent changes in phosphoprotein formation occur independently of the concomitant inhibition of ($Na^+ + K^+$)-ATPase under certain conditions (*e.g.* Ca^{2+} addition[5] or substitution of ITP for ATP[8,9]). Such differences suggest that the Na^+-phosphoprotein reaction path is not necessarily in series with the ($Na^+ + K^+$)-ATPase reaction pathway.

If the bonds in the acid-denatured complexes were the same as those which formed in the native complexes, the pH dependence of formation of the native microsomal complexes (Figs 3 and 4) might be related to that of the stability of the acid-denatured complexes (Figs 1 and 2). Whereas maximal levels of ^{14}C labelling of the native microsomes occur at pH 7.4 under the present conditions, the acid-denatured ^{14}C-labelled complex loses 60% of the ^{14}C label after exposure to the buffer solution at pH 7.4 (compare Fig. 3 with Fig. 1). Maximal levels of γ-^{32}P labelling of the native microsomes (in the presence of $Mg^{2+} + Na^+$) also occur at pH 7.4. (Fig. 4), but the acid-denatured γ-^{32}P-labelled complex remains unchanged after exposure to the buffer at pH 7.4 (Fig. 2). This suggests that the degree of dissociation and or the reactivity of the native *E*–ATP complex which forms at pH 7.4, is greater than that of the native Na^+-*E*–*P* which forms concomitantly at the same pH.

(*2*) *Interactions of native E–*[^{14}C]*ATP with ATP and ADP. (isotope chasing).* The reversibility of the formation of the phospho-enzyme, reflected in previously reported data on the ATP–ADP exchange reaction[6], suggests that $E \sim P$ donates its $\sim P$ group to ADP during the exchange reaction. In this connection, it was pertinent to determine the degree of exchangeability of the native, U-^{14}C-labelled complex with ATP and ADP. The exchange with ATP would be due in part to the reversibility of the primary complexation between *E* and ATP and in part to the breakdown of *E*–ATP into products; whereas that with ADP would be due in part to complexation between *E* and ADP and in part to the ATP–ADP exchange reaction *via* the *E*–ATP complex, *e.g.* $E\text{–}P + \text{ADP} \leftrightarrows E\text{–ATP} \rightleftharpoons E + \text{ATP}$ (ref. 1). It follows that the addition of either ATP or ADP should chase the ^{14}C label from native ^{14}C-labelled microsomes which had become labelled during their incubation with [U-^{14}C]ATP in the presence of Mg^{2+} or of $Mg^{2+} + Na^+ + K^+$. Therefore, non-radioactive ATP (1.0 mM) was added to native ^{14}C-labelled microsomes in order to determine the extent of the ^{14}C chasing.

Fig. 5 is a plot of mean values ± S.E. of ^{14}C binding to 14 batches of microsomes which had been incubated with 0.01 mM [U-^{14}C]ATP in the presence of Mg^{2+} in 7 experiments, and in the presence of $Mg^{2+} + Na^+ + K^+$ in 7 experiments. After the first 1.5–2.0 s of incubation, the amount of ^{14}C binding to microsomes reached maximal levels where it remained for 50–60 s.

Immediately before the addition of 1.0 mM ATP, the mean levels of ^{14}C labelling of the microsomal protein (100 μg in each incubation tube) were 1250 $\pm$ 65 cpm in the presence of Mg^{2+} and/or of $Mg^{2+} + Na^+ + K^+$. These crude values were taken individually as the 100 % level of ^{14}C labelling in each experiment.

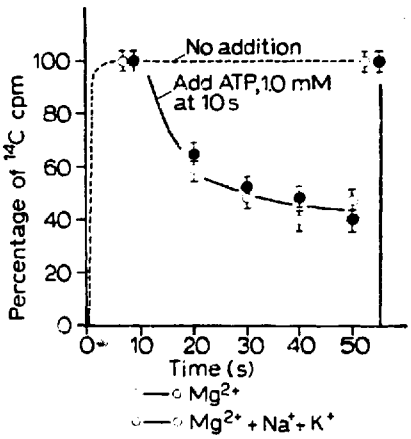

Fig. 5. Native microsomes (^{14}C chasing). ^{14}C labelling of microsomes *versus* time of incubation. Each circle with vertical bars denotes a mean value $\pm$ S.E. of the ^{14}C cpm bound to each aliquot of microsomal protein expressed as a percentage of the maximal level (1350 cpm) reached before the ^{14}C "chaser" (ATP) was added in 7 sets of paired experiments; and measured after terminating the incubation with $HClO_4$ and washing the resulting precipitate with cold ATP, ADP and P_i. Microsomes were incubated with [U-^{14}C]ATP in the presence of Mg^{2+} ($n = 7$) and $Mg^{2+} + K^+$ ($n = 7$).

Non-radioactive ATP was added to one aliquot and an equal volume of an ATP-free solution was added to the paired aliquot of each microsomal batch at the incubation time, $t = 10$ s. The addition of the "chaser" ATP resulted in a sequential reduction of the ^{14}C binding which reached levels of 50 % in 20 s and 40 % in 40 s. The levels of ^{14}C labelling in the presence of Mg^{2+} both before and after chasing were not significantly different from the corresponding levels in the presence of $Mg^{2+} + Na^+ + K^+$.

Not illustrated are data from three paired experiments on "^{14}C chasing" induced by the addition of 1.0 mM ADP to one aliquot and of 1.0 mM ATP to a paired aliquot of native, ^{14}C-labelled microsomes. The microsomes had been allowed to incubate with [U-^{14}C]ATP (0.01 mM) for 10 s at 0 °C in the presence of Mg^{2+} and $Mg^{2+} + Na^+ + K^+$ prior to addition of the non-radioactive nucleotide.

The rate of "chasing" of ^{14}C following the addition of unlabelled ADP was slower than that after ATP. The residual ^{14}C binding reached mean values of 80 % after 10 s and 58 % after 40 s in the presence of Mg^{2+} alone, or of Mg^{2+}, Na^+, and K^+ together. The corresponding levels of ^{14}C binding following the addition of unlabelled ATP to paired aliquots of the same microsomes reached mean values of 65 % after 10 s and 47 % after 40 s.

These data suggest the presence of exchangeable sites for ATP and ADP in the intermediate U-^{14}C-labelled enzyme complex. Although exchangeability of ADP with the *E*–ATP complex probably occurs *via* transphosphorylation of ADP by *E*–*P*, a portion of the reaction could be due to competition between ADP for binding sites on the enzyme. Further work is needed to determine whether the *E* component of $E \sim P$ is the same, is an isozyme of, or is independent of the *E* component of *E*–ATP.

DISCUSSION

A. Identity of E–ATP complexes

Inferential evidence for the formation of an *E*–ATP complex in native microsomes has been presented by Norby and Jensen[25,26] and by Hevgevary and Post[23]. Using rapid flow dialysis system, both groups calculated the amount of *E*–ATP formed by measuring the disappearance of [γ-^{32}P]ATP from a Mg^{2+}-free fluid in which the microsomal enzyme was incubated. The Mg^{2+}-free state of the undenatured enzyme was supposed to facilitate the formation of an *E*–ATP complex which would not degrade into its usual hydrolytic products (P_i, ADP, and *E*), but which would reach the required equilibrium state with its reactants ATP and *E*.*

Although the hydrolytic activity of (Na^+ + K^+)-ATPase is absolutely dependent upon the presence of Mg^{2+}, the investigators[23,25,26] inferred that the Mg^{2+}-free, non-hydrolyzing state of this enzyme forms the same bonds with the substrate as does the Mg^{2+}-rich, hydrolyzing state of the enzyme. Nevertheless, their microsomal preparation could have contained ATP-binding proteins other than (Na^+ + K^+)-ATPase, since its hydrolytic activity, when assayed in the presence of Mg^{2+}, was about 10% of the maximally attainable specific activity of (Na^+ + K^+)-ATPase[27].

The present data on nucleotide release from isotopically labelled microsomal precipitates provide a direct demonstration of the formation of an *E*–ATP complex (or complexes) during the incubation of microsomes with [U-^{14}C]ATP, [γ-^{32}P]ATP or [α-^{32}P]ATP. One may infer that part of this *E*–ATP complex is the primary enzyme–substrate complex of the (Na^+ + K^+)-ATPase reaction because of the following: (a) the ^{14}C labelling of microsomes incubated with [U-^{14}C]ATP is Mg^{2+} dependent; (b) the pH dependence pattern of this ^{14}C labelling is roughly superimposable upon that of (Na^+ + K^+)-ATPase (see Fig. 3 and ref. 21); (c) the simultaneous presence of Mg^{2+}, Na^+, and K^+ is required for the ouabain-induced inhibition of ^{14}C labelling as well as for the ouabain-induced inhibition of (Mg^{2+} + Na^+ + K^+)-ATPase activity; and (d) the ^{14}C label can be displaced from the *E*–[^{14}C]ATP complex by the addition of non-radioactive ATP or ADP during the incubation of native microsomes with 0.01 mM [U-^{14}C]ATP.

However, certain problems remain. For example: (a) the ATP binding related to (Na^+ + K^+)-ATPase has not yet been separated from ATP binding related to the other proteins in these and in other[23,25,26] microsomes; (b) the ouabain-sensitive moiety of the ^{14}C labelling is probably closely related to but not yet equated with the free [U-^{14}C]ATP released from its binding sites on the microsomal precipitates; and (c) the well-known techniques for estimating the binding of γ-^{32}P or ^{14}C to microsomes measure only the acid-stable non-exchangeable radio label on $HClO_4$-precipitated microsomes as they are isolated from the incubation mixture, so that some, but not all, of the bonds formed in the native state remain intact in the acid-denatured state. For example, native (Na^+ + K^+)-ATPase may well form acid-labile as well as acid-stable intermediary compounds during the hydrolytic reaction sequence[24]; and data on the ADP–ATP exchange reaction[6] and on the chasing of U-^{14}C by ATP and ADP

* The catalytically inert, Mg^{2+}-free preparation of *E*–[γ-^{32}P]ATP used by others would not be expected to exchange its label with ADP because the transphosphorylation step is presumably blocked under the Mg^{2+}-free conditions of these dialysis experiments. In this connection, Hegyvary and Post[23] have shown that the affinity of their protein complex for ATP was 10-fold greater than that for ADP.

(see Fig. 5) clearly show that a significant part of the radio label on native microsomes is exchangeable.

With these reservations in mind, we may proceed to compare some of the bond properties in the ^{14}C-labelled precipitates with those in γ-^{32}P and α-^{32}P-labelled precipitates.

B. Bonding in the microsomal complexes

A γ-phosphoryl–acyl bond accounts for most of the Na^+ increment of the γ-^{32}P labelling of microsomal protein[10,11] and for little or none of the Mg^{2+}-dependent γ-^{32}P labelling. But neither one of these γ-phosphate–protein bonds necessarily accounts for that moiety of the γ-^{32}P labelling which is released as free ATP after cleavage of the γ-^{32}P-labelled microsomal complex (see Tables I and III). This is because the present data do not provide unequivocal proof for the existence of an acid-stable γ-phosphoryl–protein bond in the *E*–ATP complex.

Quantitatively, the *E*–ATP complex, as isolated in the form of an acid-stable, U-^{14}C- or γ-^{32}P-labelled precipitate, amounts to only a small fraction (5–10%) of the correspondingly isolated *E–P* complexes. This may be ascribed to differences in the acid stability and nucleotide exchangeability of the complexes (E–[U-^{14}C]ATP and E–γ-$^{32}P_i$) during the $HClO_4$ precipitiation and washing procedure employed in their isolation.

Qualitatively, the patterns of pH-dependent stability of each of the acid-stable, non-exchangeable γ-^{32}P-labelled complexes is clearly distinct from that of the U-^{14}C-labelled complex (see Figs 1 and 2 and Table I). Thus, either a third type of γ-phosphoryl bond forms (*e.g.* a phosphoramido bond), or no acid-stable γ-phosphoryl bond forms during the formation of the *E*–ATP complex.

In all, the present data are consistent with the presence of at least two and possibly three qualitatively distinct types of bonds between functional groups on the microsomal enzyme and on the substrate molecule (ATP). First the presence of a γ-phosphoryl–acyl bond in ^{14}C- and γ-^{32}P-labelled complexes is consistent with the effects of NH_2OH (see Tables I and II) and with the pH-dependent pattern of γ-^{32}P labelling of native microsomes by [γ-^{32}P]ATP in the presence of Mg^{2+} and Na^+ (see Fig. 4). Secondly, the presence of an adenosinyl–protein bond is consistent with the effects of acid–NH_2OH–MoO_4^{2-} on ^{14}C- and α-^{32}P-labelled complexes (see Table II); and thirdly, the presence of a phosphoramido bond is consistent with the pH-dependent pattern of the ^{14}C labelling of native microsomes by [U-^{14}C]ATP (see Fig. 3). The phosphoramido-like pattern of the U-^{14}C labelling may reflect the behavior of an –N–P-link between the γ-phosphate of ATP and a nitrogen atom on the enzyme, or between a phosphate group on the enzyme and one of the N atoms on the adenine moiety of ATP.

The existence of two or three qualitatively different bonds in the intermediary complexes, together with other data on the independence of the formation and breakdown of Na^+-*E–P* enzyme from the overall ($Na^+ + K^+$) increment of hydrolysis (see Introduction and refs 5, 8 and 9), is consistent with two separate reaction sites operating in a parallel manner on the enzyme complex. One site, the hydrolytic one, is involved in the production of the ouabain-sensitive moiety of the *E*–ATP complex, and the other site, the transphosphorylating one, is involved in the production of the Na^+-*E–P* complex *via* the ouabain-resistant moiety of the *E*–ATP complex.

C. *Possible role of microsomal complexes in Na^+ transport*

The present data have physiological implications with respect to the delivery of energy and the translocation function of the Na^+ transport mechanism. The superimposition of the pattern of the pH dependence of formation of the U-^{14}C-labelled complex upon that of ($Na^+ + K^+$)-ATPase (see Fig. 3) suggests that the delivery of cellular energy for active Na^+ transport begins with the complexation of cytoplasmic ATP to ($Na^+ + K^+$)-ATPase (E) in the membrane. The superimposition of the pH dependence of the formation of the Na^+-phospho-enzyme (Na-E–P) upon that of the actual net sodium transport (I_{Na}) in the intact bladder (see Fig. 4) suggests that the translocation of Na^+ across the membrane begins with the complexation of Na^+ to an adjacent membrane enzyme, E' which may be a subunit of E.

The tentative model emerging from these data is as follows: Within the membrane there are two closely related, discrete functional units, (denoted E and E') which operate in a parallel manner to impel the active transport of Na^+ across the membrane. The E function (($Na^+ + K^+$)-ATPase), which serves as the energy transducer *via* complexation with ATP at the cytoplasmic interface of the membrane, requires and interacts with Mg^{2+}, Na^+, and K^+. The E' function (γ-phosphoenzyme), which serves as the Na^+ translocator, requires a cyclical change in its Na^+ affinity in order to impart directionality to the Na^+-transport process. It also requires and interacts with Mg^{2+}, Na^+, and K^+.

Problems remaining include: the exact manner of coupling or interaction of the two functions with each other; the nature of the interactions of ATP, Na^+, K^+ and ouabain with each of the two functions; and, in fact, the electrogenic or non-electrogenic nature of the membrane–ATPase–pump system.

Data herein suggest that additional experiments are necessary to test the validity of several recent concepts on Na^+–K^+ interactions with the membrane proteins[29,30]; and on Na^+–K^+ conductance channels[31,32]. Previous data on the electrical behavior of turtle bladder epithelia[28,33–35] *plus* those contained herein also suggest additional experiments to test the validity of recent concepts on the electrogenicity of cation pumping by epithelial cell membranes[30].

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