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STUDIES ON PLASMA MEMBRANES

VI. DIFFERENCES IN THE EFFECT OF TEMPERATURE ON THE ATPase AND (Na^+-K^+)-ATPase ACTIVITIES OF PLASMA MEMBRANES ISOLATED FROM RAT LIVER AND HEPATOMA

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SUMMARY

1. Plasma membranes were isolated from homogenates of rat liver and rat hepatoma prepared in 1 mM $NaHCO_3$ containing 2 mM $CaCl_2$. 5'-Nucleotidase (EC 3.1.3.5), ATPase (EC 3.6.1.3) and (Na^+-K^+)-ATPase activities were assayed using (a) freshly isolated membranes incubated at temperatures from 10 to 50° and (b) membranes exposed to various temperatures prior to their assay.

2. The 5'-nucleotidase activity of both liver and hepatoma membranes increased over the entire temperature traject tested, the increase being exponential up to 30–35°.

3. The ATPase and (Na^+-K^+)-ATPase of liver membranes were characterized by a pronounced decrease of their temperature coefficient between 30 and 40°. At 50° the ATPase was impaired and the (Na^+-K^+)-ATPase activity markedly stimulated as compared with the corresponding activities at 40°. The temperature coefficient (20–50°) of the ATPase reaction catalyzed by the hepatoma membranes was very small, but no inhibition of the ATPase was noted at 50°. The (Na^+-K^+)-ATPase activity of the hepatoma membranes increased linearly from 20 to 50°.

4. Preincubation of the membranes at 50° followed by assay at 37° did not affect the ATPase but severely inhibited the (Na^+-K^+)-ATPase activity of the liver membranes, while causing the opposite effects on the hepatoma-membrane enzymes.

5. Thus, under the various experimental conditions examined, the temperature responses of the ATPase and (Na^+-K^+)-ATPase activities of the normal plasma membranes were distinct from those of the homologous neoplastic plasma membranes.

6. The results are discussed with reference to the possible role of the membrane structure in the temperature response of the enzymes.

INTRODUCTION

Plasma membranes isolated from rat liver exhibit appreciable ATPase (Mg^{2+}- or Ca^{2+}-dependent ATP phosphohydrolase), (Na^+-K^+)-ATPase (Na^+ *plus* K^+-activated Mg^{2+}-dependent ATP phosphohydrolase) and 5'-nucleotidase (5'-ribonucleotide phos-

phohydrolase; substrate: AMP) activities at 37° (refs. 1 and 2). The temperatures for the optimal reaction velocities of these enzymes, which are authentic components of the plasma membranes, are not known. In the experiments reported in this paper the effect of the temperature of incubation on the three enzymes of isolated rat liver and rat hepatoma plasma membranes was studied. Preincubation of the membranes at various temperatures prior to the assay of the ATPase and (Na^+-K^+)-ATPase was used as a test for the stability of these enzymes.

The effect of the temperature on the reaction velocity of an enzyme present in a biological membrane may be mediated by at least three groups of parameters (a) the intrinsic properties of the enzyme, including its stability, (b) inhibitors or activators which may be present or formed in the membrane and interact with the enzymes as a function of the temperature, and (c) the membrane structure in case the enzyme is critically dependent on a particular structural organization of the membrane (*e.g.*, the protein–lipid interaction) and the latter is affected either directly by the temperature or through a temperature-dependent interaction of compounds of the type mentioned under (b).

The present experiments were inspired by the previous findings that (i) the temperature coefficient of the ATPase reaction catalyzed by the liver plasma membranes was markedly decreased between 30 and 40° whereas that of the 5′-nucleotidase was not[2]; (ii) the two enzymes and the (Na^+-K^+)-ATPase differed markedly in their response to a number of conditions and agents capable of interfering with the membrane structure and/or membrane lipids[2,3]; (iii) the ultrastructure of the liver plasma membranes, but not that of the hepatoma plasma membranes was changed by increasing the temperature to 37° (refs. 4 and 5); (iv) differences between the morphological and chemical structure of the two types of membranes existed[5].

MATERIALS AND METHODS

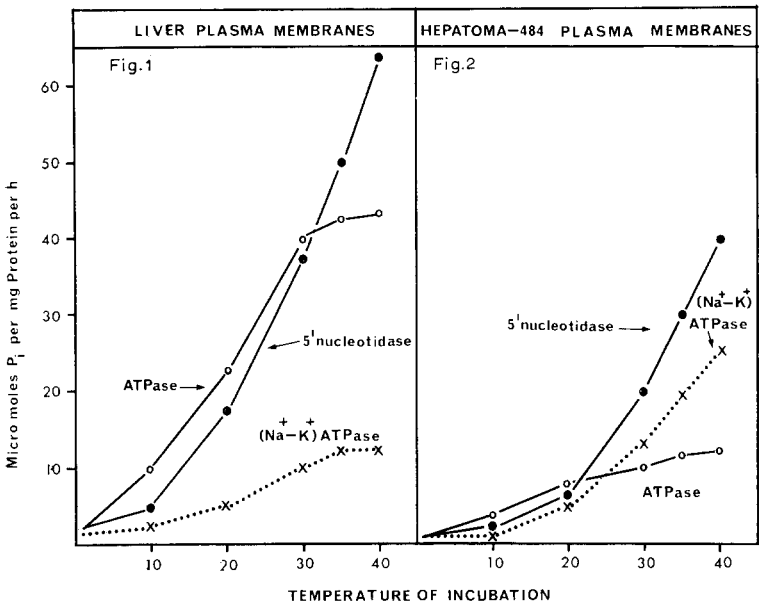
Plasma membranes were isolated from the livers of rats (inbred strain R-Amsterdam) and from the transplanted hepatocellular rat hepatoma-484, as previously described[1,6]. In both cases 1 mM $NaHCO_3$ (pH 7.5) containing 2 mM $CaCl_2$ was used as homogenization medium. All the low-speed centrifugations following the first centrifugation were carried out in 1 mM $NaHCO_3$. The 5′-nucleotidase, ATPase and (Na^+-K^+)-ATPase were assayed as mentioned in a previous paper[2]. The preincubation experiments were carried out by exposing plasma membranes, corresponding to about 2.0 mg protein per ml 25 mM Tris buffer (pH 7.4) to the various temperatures indicated in the text. After 15 min the suspensions were cooled to 0° for 5 or 10 min and 0.1-ml aliquots were subsequently used for enzyme assay.

RESULTS

5′-Nucleotidase, ATPase and (Na^+-K^+)-ATPase activities at 10–40°

The plasma membranes were isolated from homogenates prepared in 1 mM $NaHCO_3$ (pH 7.5) containing 2 mM $CaCl_2$. The presence of Ca^{2+} was required for the proper isolation of the hepatoma plasma membranes for the reason described previously[5,6]. Although Ca^{2+} was not needed for the isolation of the liver membranes, in the present experiments it was used for this purpose in order to make the comparison with the hepatoma membranes as close as possible.

Liver plasma membranes. The 5′-nucleotidase increased exponentially up to 30–35° as shown (not illustrated) by the linear function of the logarithm of the reaction velocities plotted against the reciprocal of the absolute temperature using the data of Fig. 1 which illustrate average values of 4 closely agreeing experiments. Although the nucleotidase activity deviated from the exponential course at 40°, the increase of the activity in the 30 (35) to 40° interval was still very pronounced.



Figs. 1 and 2. 5′-Mononucleotidase, ATPase and (Na^+-K^+)-ATPase activities of isolated rat liver and rat hepatoma plasma membranes as a function of the temperature of incubation (10–40°). Blanks without membranes were run at all temperatures to correct for spontaneous ATP hydrolysis.

At 10° the splitting of ATP catalyzed by the ATPase proceeded faster than did the phosphohydrolysis of AMP catalyzed by the 5′-nucleotidase. The two enzymes became about equally active at 30°. This was apparently due to the sudden and marked fall in the temperature coefficient of the ATPase reaction occurring between 30° and 40°, and the absence of such an effect in the case of the 5′-nucleotidase. The (Na^+-K^+)-ATPase activity, which was smaller than the ATPase activity, resembled the latter in its response to the temperature of incubation.

Hepatoma plasma membranes. The 5′-nucleotidase of the hepatoma membranes was about 40% as active as the corresponding liver-membrane enzyme. The 5′-nucleotidase activities of the hepatoma and liver membranes reacted in a similar manner to the rise of the temperature of incubation (Fig. 2, averages of 3 closely agreeing experiments). The ATPase activity of the hepatoma membranes was much smaller than that of the liver membranes. The relative increase of the hepatoma-membrane-ATPase activity with the temperature of incubation was less pronounced than in the case of the liver membranes, so that the curve relating the ATPase activity of the hepatoma membranes with temperature had a rather flat appearance (*cf.* also Fig. 4). As a result the ratio of the ATPase activity of the liver membranes to that of

the hepatoma membranes progressively increased from 2.5 at 10° to 3.9 at 40°. The ATPase and 5'-nucleotidase of the hepatoma membranes showed about the same activity at 20°, this temperature being 10° less than that observed for the corresponding situation of the two liver-membrane enzymes.

The most interesting finding was that the (Na^+-K^+)-ATPase activity of the hepatoma membranes, unlike that of the liver membranes, continued to increase linearly between 20 and 40°, and thus tended to resemble more the 5'-nucleotidase than the ATPase type of temperature response.

Effect of the temperature of preincubation on the ATPase and (Na^+-K^+)-ATPase activities

An effect of the temperature on the velocity of an enzyme reaction that stems from the heat instability of the enzyme itself can be demonstrated by exposing the enzyme (in the present case: membranes) to various temperatures prior to measuring its activity. The term preincubation refers to the latter treatment. Unless stated otherwise, the preincubated membranes were cooled and kept at 0° for 5–10 min in order that any reversible structural change due to the temperature might be expressed before the membranes were incubated in the enzyme assay medium. As shown in Table I, preincubation of the liver and hepatoma membranes at 37°, according to the procedure mentioned under MATERIALS AND METHODS, did not lead to a decrease of the ATPase activities of the membranes on subsequent incubation in the assay medium at 20 or 37°, as compared with the enzyme activities obtained after preincubation at 20° (or of the controls kept at 0°). By contrast, following preincubation and incubation at 37°, the (Na^+-K^+)-ATPase activity of the liver membranes was moderately decreased as compared with that of liver membranes preincubated at 20° (or kept at 0°) and incubated at 37°. An inhibition of the (Na^+-K^+)-ATPase resulting from preincubation and incubation at 20°, as compared with the enzyme activity of membranes kept at 0° and incubated at 20°, was also apparent. However, unlike the (Na^+-K^+)-ATPase of the liver membranes, the corresponding enzyme activity of the

TABLE I

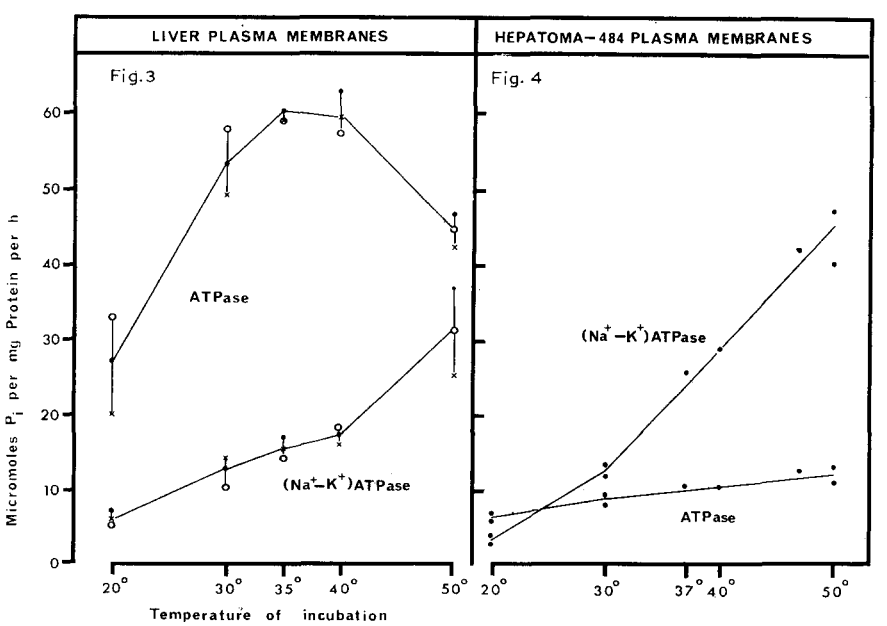
EFFECT OF PREINCUBATING LIVER AND HEPATOMA PLASMA MEMBRANES AT 20 AND 37° ON THE ATPase AND (Na^+-K^+)-ATPase ACTIVITIES AT 20 AND 37°

Membrane source	*Preincubation temperature*	*μmoles P_i/mg protein per h*							
		20°				*37°*			
		ATPase		*(Na^+-K^+)-ATPase*		*ATPase*		*(Na^+-K^+)-ATPase*	
		Expt. 1	*Expt. 2*	*Expt. 1*	*Expt. 2*	*Expt. 1*	*Expt. 2*	*Expt. 1*	*Expt. 2*
Liver	0°	22.6	22.9	5.4	4.8	44.9	37.7	13.4	16.2
	20°	22.3	22.4	3.7	2.9	48.2	40.2	14.7	16.0
	37°	24.1	22.2	2.9	2.4	45.1	37.7	9.1	11.9
		Expt. 3	*Expt. 4*	*Expt. 3*	*Expt. 4*	*Expt. 3*	*Expt. 4*	*Expt. 3*	*Expt. 4*
Hepatoma	0°	3.4	3.0	2.2	2.5	6.9	6.6	11.5	14.0
	20°	3.6	3.1	2.1	3.2	6.9	6.9	12.1	14.2
	37°	3.2	3.7	2.3	2.9	6.4	7.1	12.1	14.3

hepatoma membranes was not affected by preincubation. The present results suggest that the ATPase of liver and hepatoma membranes and the (Na^+-K^+)-ATPase of hepatoma membranes, in contrast to the (Na^+-K^+)-ATPase of liver membranes, are not inactivated by preincubation at 37°. Incidentally, the data on the control membranes of Table I confirm those illustrated in Figs. 1 and 2 by showing the great difference in the effect of temperature on the (Na^+-K^+)-ATPase activities of liver and hepatoma membranes which are, respectively, 2.5–3.5 and 5–7 times as active at 37 as at 20°. The present results following preincubation of the plasma membranes are completely different from those obtained by HOKIN AND REASA[7] using erythrocyte membranes. The ATPase of the two types of membranes is also differently affected[3] by phospholipase C (EC 3.1.4.3).

ATPase and (Na^+-K^+)-ATPase activities at 20–50°

Fig. 3, which illustrates 3 experiments with liver membranes, shows that when the temperature during incubation of the membranes in the assay medium was raised to 50°, the ATPase activity of the liver membranes markedly decreased whereas the (Na^+-K^+)-ATPase activity was stimulated. The highest specific (Na^+-K^+)-ATPase activity thus obtained amounted to 45.1 μmoles P_i released per mg protein per h. The increase of the (Na^+-K^+)-ATPase and the decrease of the ATPase activity in the 40–50° interval were in most cases stoichiometrically related (see also Tables III and IV). Fig. 4, illustrating 2 experiments with hepatoma membranes, apart from confirming the much smaller temperature coefficient (between 20 and 50°) of the ATPase reaction of the hepatoma membranes as compared with that of the liver membranes, shows that the hepatoma-membrane ATPase was not inhibited at 50°, in contrast to that of the liver membranes. Furthermore, it was found that the



Figs. 3 and 4. ATPase and (Na^+-K^+)-ATPase activities of liver and hepatoma plasma membranes at 20 to 50°.

(Na^+-K^+)-ATPase activity of the hepatoma membranes increased linearly up to 50°. At the latter temperature the ratio of the (Na^+-K^+)-ATPase to ATPase activities amounted to 0.7 for liver membranes and to 3.3 for hepatoma membranes. By including phosphoenolpyruvate and pyruvate kinase (EC 2.7.1.40) in the assay medium in order to trap the ADP that may inhibit the ATPase (ref. 2) it appeared (Fig. 5) that the particular temperature response of the liver membrane ATPase was not an artifact resulting from substrate exhaustion or product inhibition.

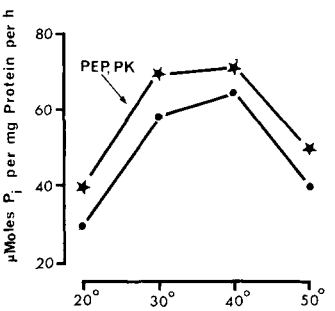


Fig. 5. ATPase activity of liver plasma membranes at 20 to 50° in the absence and presence of phosphoenolpyruvate (PEP, 8 µmoles) and pyruvate kinase (PK, 1.8 units). Phosphoenolpyruvate and pyruvate kinase were products of Boehringer u.S., Mannheim, Germany.

When the liver membranes were incubated at 60°, the ATPase was further decreased (as compared with incubation at 50°) and the (Na^+-K^+)-ATPase also became inhibited, *e.g.* at 37, 50 and 60° the ATPase activities amounted to 58.3, 42.8 and 30.9, and the (Na^+-K^+)-ATPase to 20.7, 35.8 and 11.7 µmoles P_i released, respectively. At 70° neither the ATPase nor the (Na^+-K^+)-ATPase of the hepatoma membranes showed activity, the membranes becoming markedly agglutinated.

Effect of preincubation at 50° on the ATPase and (Na^+-K^+)-ATPase activities

Preincubation of the liver membranes at 50° prior to incubation at 37° did not affect the ATPase and further decreased (as compared with preincubation at 37°) the (Na^+-K^+)-ATPase activity (Table II). The reverse effects were obtained with the corresponding enzymes of the hepatoma membranes, preincubation of the latter membranes at 50° now having no (significant) effect on the (Na^+-K^+)-ATPase but causing a substantial drop in the ATPase activity

Results similar to the aforementioned have been obtained with liver membranes after introduction of the following changes in the method of preincubation of the liver membranes. (a) The membrane suspension was not cooled after preincubation but 0.1-ml aliquots were directly transferred to the enzyme assay tubes (Table II, Expt. 3). (b) Preincubation of membranes equivalent to about 0.2 mg protein was carried out in 1.6 ml 25 mM Tris buffer (pH 7.4) followed by centrifugation for 10 min at 1500 × *g*, removal of the supernatant, addition of the enzyme assay media to the pellets, and resuspension of the membranes (Table II, Expt. 2 with liver membranes). (c) Preincubation as under (b) but using, instead of Tris, the ATPase assay medium with and without ATP and Na^+, centrifugation, washing with bidistilled water, centrifugation, addition of fresh assay media to the pellets and resuspension.

The opposite results obtained by incubating the liver membranes for enzyme

TABLE II

EFFECT OF PREINCUBATING LIVER AND HEPATOMA PLASMA MEMBRANES AT 20, 40 AND 50° ON THE ATPase AND (Na^+-K^+)-ATPase ACTIVITIES AT 37°

Membrane source	*Preincubation temperature*	*μmoles P_i/mg protein per h at 37°*					
		ATPase			*(Na^+-K^+)-ATPase*		
		Expt. 1	*Expt. 2**	*Expt. 3**	*Expt. 1*	*Expt. 2**	*Expt. 3**
Liver	20°	51.6	63.1	47.4	18.1	14.7	23.9
	40°	51.6			13.9		
	50°	51.1	61.4	46.7	5.1	0.0	6.9
		Expt. 4	*Expt. 5*		*Expt. 4*	*Expt. 5*	
Hepatoma	20°	6.2	13.8		11.7	19.0	
	40°	4.7	10.9		12.9	20.5	
	50°	2.0	7.2		10.0	18.3	

* See text.

TABLE III

EFFECT OF PREINCUBATING LIVER PLASMA MEMBRANES AT 50° ON THE ATPase AND (Na^+-K^+)-ATPase ACTIVITIES AT 37° AND 50°

Preincubation temperature	*Incubation temperature*	*μmoles P_i/mg protein per h*	
		ATPase	*(Na^+-K^+)-ATPase*
0°	37°	45.8	14.8
0°	50°	35.6	25.0
50°	37°	45.4	6.1
50°	50°	25.1	19.0

assay at 50° on the one hand, and by preincubation at 50° followed by incubation at 37° on the other, prompted experiments in which the effect of preincubation at 50° followed by incubation at 50° on the ATPase and (Na^+-K^+)-ATPase of liver membranes was studied (Table III). Although preincubation at 50° followed by incubation at 37° did not affect the ATPase activity (as compared with that of membranes kept at 0° and incubated at 37°), preincubation at 50° followed by incubation at 50° led to a pronounced impairment of the ATPase activity, the latter effect being more outspoken than that caused by incubation at 50° of membranes previously kept at 0°. These results show that a temperature of 50° during the enzyme assay is necessary for the ATPase inhibition to become manifest, whereas the preincubation at 50° *per se* causes only a potential inhibition which is not expressed when the membranes are subsequently incubated at 37° but is revealed in the potentiation of the inhibition caused by incubation at 50° *per se*. The data of Table III on the (Na^+-K^+)-ATPase demonstrate that the activation of this enzyme by incubating the membranes at 50° overrules the inhibition that is caused by preincubation at 50° (as expressed by incubation at 37°). The absolute increase of the (Na^+-K^+)-ATPase activity obtained in the 50° incubation relative to the activity at 37° was at least as high with

membranes which had been preincubated at 50° as with membranes which had previously been kept at 0°, while the percentage increase was significantly higher in the former than in the latter case. Thus, preincubation at 50° *per se* does not cause the measure of inactivation of the (Na^+-K^+)-ATPase that is suggested by the decrease of the latter's activity on subsequent incubation at 37°, the enzyme still being capable of responding to the activating effect of the 50° incubation. However, it is also seen that the 50° incubation did not completely compensate for the loss of the (Na^+-K^+)-ATPase activity induced by preincubation at 50°.

TABLE IV

EFFECT OF UREA AND Ca^{2+} ON THE ATPase AND (Na^+-K^+)-ATPase OF LIVER PLASMA MEMBRANES INCUBATED AT 30–50°

Urea, 2 M; $CaCl_2$, 5 mM.

Incubation temperature	*Addition*	*μmoles P_i/mg protein per h*	
		ATPase	*(Na^+-K^+)-ATPase*
30°	—	46.4	11.1*
	Urea	29.9	10.1
40°	—	52.6	10.6*
	Urea	26.3	12.6
50°	—	38.9	24.3
	Urea	16.0	4.1
	$CaCl_2$	38.7	2.8

* In the presence of 5 mM $CaCl_2$ the (Na^+-K^+)-ATPase activity was zero.

Effect of Ca^{2+} and urea on the ATPase and (Na^+-K^+)-ATPase activities at 50°

The high (Na^+-K^+)-ATPase activity of the liver membranes obtained at 50° was virtually abolished by addition of 5 mM $CaCl_2$ (Table IV) which had previously been shown[2] to have a similar effect on this enzyme at 37°. Ca^{2+} did not affect the ATPase at 37 and 50°. As illustrated in Table IV, 2 M urea (refs. 8, 9) inhibited the ATPase of the liver plasma membranes for 45 % at 30° and for 60 % at 50° but, while having no effect on the (Na^+-K^+)-ATPase at 30 and 40°, urea inhibited the latter enzyme for 85 % at 50°. Thus, calcium is a specific inhibitor of the (Na^+-K^+)-ATPase at any temperature, whereas urea loses its specific ATPase inhibiting effect at 50° to become an even more potent inhibitor of the (Na^+-K^+)-ATPase.

DISCUSSION

The following conclusions can be drawn from the present experiments.

(i) The 5'-nucleotidase activity of liver and hepatoma plasma membranes reacted similarly to the rise of the temperature of incubation from 10 to 40°. The temperature response of the 5'-nucleotidase was distinct from that of the ATPase and the (Na^+-K^+)-ATPase.

(ii) Irrespective of whether the membranes were assayed directly or after preincubation, the liver-membrane-ATPase and (Na^+-K^+)-ATPase activities reacted differently from the corresponding hepatoma-membrane enzymes to the temperatures

of (pre)incubation. This afforded a clear-cut distinction between the enzymes of the liver and those of the hepatoma membranes.

(iii) Each response obtained in the latter experiments was unique, depending not only on the type of enzyme and membrane, but also on the experimental conditions (direct assay *versus* preincubation). Preincubation of liver membranes at 50° prior to their assay at 37° showed that the ATPase activity was not impaired and, accordingly, that heat inactivation of the ATPase could not underlie the decrease of the latter's activity observed by assaying fresh membranes at 50°. Conversely, the decreased liver-membrane (Na^+-K^+)-ATPase and hepatoma-membrane-ATPase activities following preincubation at 50° and assay at 37°, suggested that these enzymes were to a large extent inactivated at 50°, which they were apparently not when the membranes were directly assayed at 50°. Subsequent experiments in which liver membranes were both preincubated and assayed at 50° showed that the decrease of the (Na^+-K^+)-ATPase after preincubation at 50° and assay at 37° could not have been due to heat-inactivation of the enzyme. Accordingly, the factors involved in the effect of temperature on the enzyme velocities appear to be complex and to differ for the conditions of direct incubation and preincubation.

The information provided by the present experiments does not allow one to establish the relative importance of the various factors, mentioned in INTRODUCTION, by which the temperature may affect the velocities of the membrane enzymes. However, the uniqueness of each temperature response may suggest that factors other than the intrinsic properties of the ATPase and (Na^+-K^+)-ATPase were also involved. If not, the conclusion must be drawn that the different temperature responses of the hepatoma- as compared with the liver-membrane enzymes are due to mutational alterations in the neoplastic enzymes.

A number of independent data point to the possibility that the membrane structure might be involved in the temperature response. The 5'-nucleotidase of the plasma membranes is a stable enzyme that is not inhibited by drastically changing the membrane structure (*e.g.* by lipid extraction) or by agents (oleic acid, vitamin-A alcohol, deoxycholate, ultrasound) capable of interacting with the membrane structure (-lipid), all of which do inhibit the ATPase and (Na^+-K^+)-ATPase of the plasma membranes[2,3]. Thus, if the temperature affects the membrane structure, the activity of the 5'-nucleotidase would not, whereas the ATPase and (Na^+-K^+)-ATPase activities might be influenced by a temperature-dependent structural change of the membrane. The types of temperature responses actually recorded for the various liver-membrane enzymes on direct assay are not contradictory to the latter assumptions. The non-uniform response of the ATPase and (Na^+-K^+)-ATPase of the liver membranes at 50°, *i.e.* the increase of the Na^+ activated at the expense of the basal ATP splitting, has previously been obtained[2] by exposing the membrane to certain chemical agents (low concentrations of detergents, bile, deoxycorticosterone acetate) which may interact with the membrane structure. This 'recoupling' of the sodium sensitivity of the ATP splitting suggests that the (Na^+-K^+)-ATPase and ATPase are inversely related through some kind of coupling mechanism which apparently also becomes manifest at 50° following the inhibition of the ATPase. However, since the ATPase inhibition is not automatically accompanied by (Na^+-K^+)-ATPase activation (*cf.* the effect of urea, Table IV), some particular condition seems to be required for the co-ordinated effect.

Although minute changes in the structure of the plasma membrane element as a result of the temperature may not be detectable, the demonstration[4,5] that the liver-plasma-membrane structure is changed at 37° whereas that of the hepatoma membranes is not, was considered to lend support to the supposed role of the membrane structure in the temperature response of the enzymes. The different responses of the ATPase and (Na^+-K^+)-ATPase of the hepatoma as compared with those of the liver-plasma membranes would be compatible with these morphological findings. However, the relevance of the latter to the present problem has been reduced very recently by the observation[10] that the temperature-dependent change of the membrane structure occurs in the zones of fusion between the liver membranes (tight junctions, zonulae occludentes, which are lacking in the hepatoma membranes[5]) for which there is no evidence that they contain the enzymes in question. Hence, the possibility should also be taken into account that other factors, such as endogenous or contaminating inhibitors or activators with activity depending on the temperature, may affect the enzymes directly or through an effect on the molecular structure of the membrane. In particular, these substances may have been active in the preincubation experiments in which there was more time available for their formation and/or interaction than when following direct incubation. The observed differences between the temperature responses of the liver- and hepatoma-membrane enzymes might then be related to differences in chemical and morphological composition between these membranes[5].

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