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SUCCINATE-DEHYDROGENATING ACTIVITY AND CYTOCHROMES OF HEPATIC MICROSOMES

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SUMMARY

1. Microsomes prepared by four different methods and a microsomal fragment known as "microsomal electron-transport membranes" from bovine liver have been analyzed for their succinate oxidase, succinate (phenazine) oxidoreductase and cytochrome oxidase activities. From the differential activities of these enzymes, it has been concluded that microsomes possess succinate-dehydrogenating activity. This activity is constitutive to microsomes.

2. Microsomes also contain acid non-extractable flavin. The ratio of succinate phenazine reductase activity to acid non-extractable flavin parallels that in mitochondria.

3. Spectrally, the microsome samples used do not show detectable contamination of mitochondrial cytochromes, but exhibit well-documented cytochromes b_5 and P-450. However, P-450 is not reduced by succinate.

4. Addition of cholate to microsomes converts about 40–65% of the P-450 to P-420 and approx. 35–40% is retained as P-450. The total recovery is between 80 and 100%.

INTRODUCTION

It has been tactically assumed that hepatic microsomes do not contain succinate oxidase or cytochrome oxidase[1,2]. Recently, it has been reported[3] that a significant amount of acid non-extractable flavin occurs in microsomes from rabbit liver. In cardiac mitochondria, this partition of flavin can be equated to succinate dehydrogenase (*e.g.* ref. 4). Nonetheless, a number of articles including those presented at two recent symposia on the microsomal electron transport do not mention succinate-dehydrogenating activity[5,6,*]. On the other hand, some observations[7,8] have cast a doubt on the original postulate[1,2].

* D. MACLENNAN, A. TZAGOLOFF AND G. MCCONNELL, personal communication.

We are interested in succinate dehydrogenase in general and therefore the validity of the aforementioned assumption has been examined. This paper reports that liver microsomes possess succinate-dehydrogenating activity and contain acid non-extractable flavin. The CO-binding pigment (P-450) or cytochrome b_5 of microsomes is not, however, reduced by succinate.

EXPERIMENTAL PROCEDURE

Fresh bovine liver was collected in ice and used within 1 h, and usually within 30 min, after the death of the animal. Microsomes were prepared by four different methods. Differential centrifugation of liver homogenate in 0.25 M sucrose was used to yield microsomes at 105 000 × *g* by the method of SCHNEIDER AND HOGEBOOM[1,2]. Acid precipitation of the supernatant fraction obtained after removal of light mitochondria at 18 000 × *g* was also used to derive microsomes according to the method of STRITTMATTER[9] as modified by WILLIAMS, JR. AND KAMIN[10]. "Smooth" microsomes were prepared by the sucrose density-gradient centrifugation method of MASON *et al.*[3]. The method of D. MACLENNAN, A. TZAGOLOFF AND G. MCCONNELL (personal communication) was used to prepare microsomes, the "microsomal electron-transport membrane", and the residue (sediment) after removal of the "microsomal electron-transport membrane".

Succinate oxidase was assayed manometrically[11] at 37° with 0.2 ml of 10% KOH in the center well. Succinate phenazine reductase activity* was estimated at 37° using a constant level (3.0 mM) of phenazine methosulfate[12]. The cytochrome oxidase activity, in a system containing ascorbate and cytochrome *c*, was measured polarographically[13] at 23°. Acid non-extractable flavin[4] and protein[14] were estimated as usual.

Absorption spectra were measured in a Cary spectrophotometer, model 11, with cuvettes of 1-cm optical path. Thunberg-type optical cells were used when conditions required strict anaerobicity.

The millimolar extinction coefficients used were: 163 for cytochrome b_5 at 424 mμ with reference to 409 mμ for the difference spectrum of the dithionite reduced *minus* the oxidized[15], 91 for P-450 at 450 mμ with reference to 490 mμ for the difference spectrum of the CO- and dithionite-treated sample *minus* the dithionite reduced[16] and 111 (ref. 16) for the P-420 at 420 mμ with reference to 490 mμ for the difference spectrum as that for P-450. Selection of these wavelengths were justified as discussed in RESULTS.

RESULTS

In Table I are the protocols summarizing the results of analyses of the four different preparations of microsomes. For comparison the activities of mitochondria derived from the same batch of the homogenate were simultaneously estimated. Ca^{2+} at 1.0 mM was added to the mitochondrial preparations in the estimation of succinate phenazine methosulfate reductase activity to overcome permeability barriers, if any, for the dye. Ca^{2+} did not exert any effect on microsomal succinate phenazine

* The term succinate phenazine reductase is operationally defined by the activity determined with phenazine methosulfate as the acceptor for "succinate (acceptor) oxidoreductase."

TABLE I

SUCCINATE-DEHYDROGENATING ACTIVITY OF LIVER MICROSOMES

Methods I, II and III refer to references 2, 9 and 10 and 3, respectively, for the methods of preparation for microsomes. Method IV refers to the method reported by D. MACLENNAN, A. TZAGOLOFF AND G. MCCONNELL (personal communication).

Enzyme system	*Method I*		*Method II*		*Method III*		*Method IV*	
	Mitochondria	*Microsomes*	*Mitochondria*	*Microsomes*	*Mitochondria*	*Smooth microsomes*	*Mitochondria*	*Microsomes*
	*Activity (μmole/min per mg protein)**							
(1) Cytochrome oxidase	0.16	0.016	0.16	0.014	0.24	0.0132	0.20	0.0168
(2) Succinate oxidase	0.186	0.043	0.186	0.024	0.30	0.0346	0.22	0.052
(3) Succinate phenazine reductase	0.216	0.176	0.216	0.141	0.396	0.120	0.24	0.165
	Activity ratio							
(2)/(1)	1.2	2.7	1.2	1.7	1.3	2.6	1.1	3.1
(3)/(1)	1.4	11.0	1.4	10.1	1.7	9.1	1.2	9.8
(3)/(2)	1.2	4.1	1.2	5.9	1.3	3.5	1.1	3.2
(3)microsomes/(3)mitochondria	0.82		0.65		0.31		0.69	
	mμmole/mg protein							
Acid non-extractable flavin	0.057	0.041	0.057	0.039	0.076	0.046	0.063	0.042
	moles of succinate oxidized by phenazine per min per mole of acid non-extractable flavin							
True turnover number	3800	4300**	3800	3600**	5200	2400**	3840	3900**

* Expressed in the unit on the 2-electron equivalent basis.

** The true turnover numbers for microsomes were calculated by the formula $(X_t Y_s - X_s Y_t)/(X_t Z_s - X_s Z_t)$, where X, Y and Z represent the cytochrome oxidase, succinate phenazine reductase and the acid non extractable flavin, respectively, and subscripts t and s denote the mitochondrial and microsomal fractions Therefore, the turnover numbers represent the "true" activity for the oxidation of succinate by phenazine methosulfate; in other words, cytochrome oxidase was used as a marker enzyme for "mitochondrial contamination".

reductase. Hence, it was also routinely added to the assay mixture for microsomes.

The optimum pH for succinate phenazine reductase was found to be 7.6 in both microsomal and mitochondrial systems. Likewise, the activation energy of this reaction for both systems was about 5.8 kcal. The results for these experiments with microsomes are shown in Figs. 1 and 2.

Although the succinate oxidase and cytochrome oxidase activities varied somewhat in the different batches of mitochondria, a consistent relationship* between the two enzymes was observed and found to be approx. 1. A different relationship in

* It must be mentioned that the activity ratio of succinate oxidase to cytochrome oxidase, mentioned here and in Table I, is only for comparison under the conditions described. The numerical values of these ratios are of no quantitative significance because these two activities were determined at different temperatures. Nonetheless, after an approximate correction of temperature difference the results are expected; namely the activity of cytochrome oxidase is higher than that of succinate oxidase in liver mitochondria. But it was not true in microsomes (*cf.* Table I). This fact also suggests the conclusion that a non-cytochrome oxidase-linked succinate dehydrogenating system exists in microsomes.

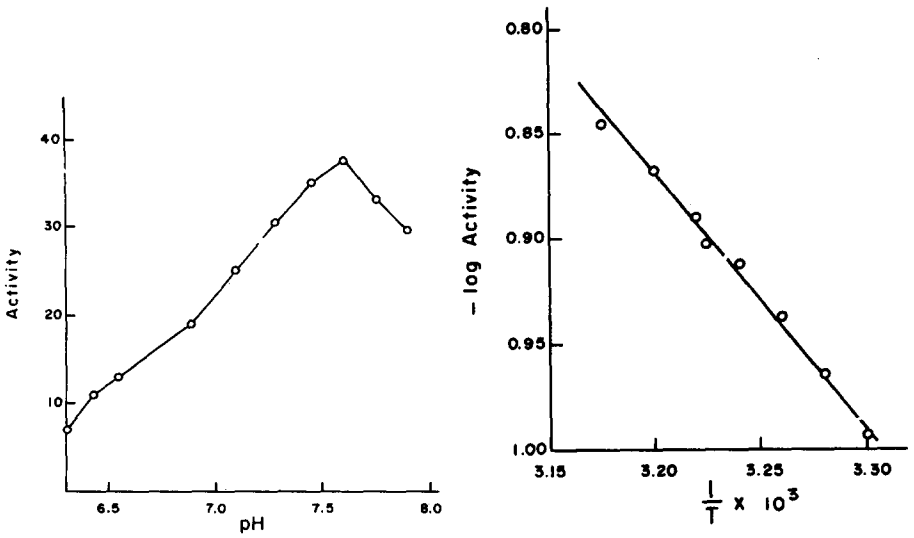

Fig. 1. The effect of pH on succinate phenazine reductase activity of liver microsomes. The ordinate is the activity in μl O_2 consumed per 8 min with system containing 0.738 mg protein. Phosphate buffer (Sørensen type) at 0.1 M was used.

Fig. 2. An Arrhenius plot of the effect of temperature on succinate phenazine reductase activity of liver microsome at pH 7.6, 0.1 M phosphate buffer. The activity was in the unit of μmoles of succinate oxidized per min per mg protein.

microsomes, however, existed; the ratio of succinate oxidase to the cytochrome oxidase activities in microsomes was always significantly higher than that in mitochondria as shown in Table I. This observation is in line with the findings of MOURY AND CRANE[7].

The ratio of succinate phenazine reductase to cytochrome oxidase activities in mitochondria followed a fairly constant relationship but it was more variable in microsomes. This ratio in microsomes, regardless of the method of preparation, was much higher than that in mitochondria. Moreover, this ratio in microsomes was, likewise, much higher than the ratio of succinate oxidase to cytochrome oxidase activity. On the basis of specific activity, the microsomal phenazine reaction was almost half of the mitochondrial system*.

"Microsomal electron-transport membranes" showed higher specific activity for the oxidation of succinate by phenazine methosulfate, sometimes as much as twice that found in the microsomes. This was in agreement with the observations of D. MACLENNAN, A. TZAGOLOFF AND G. MCCONNELL (personal communication) who reported a 1.6–2.4-fold purification for some of the other microsomal enzymes; they did not study succinate oxidation.

The acid non-extractable flavin varied between 0.03 and 0.05 mμmole per mg of microsomal protein of beef liver. The mitochondrial preparations were found to have a flavin content of 0.06–0.08 mμmole/mg protein. MASON *et al.*[3] have reported a value of 0.04 in rabbit-liver microsomes. It is interesting to note that both the succi-

* The "smooth" microsomes obtained by the method of MASON *et al.*[3] account for a little more than 30%.

TABLE II

MICHAELIS CONSTANTS FOR SUCCINATE (K_m) AND COMPETITIVE INHIBITION CONSTANTS FOR MALONATE (K_i) AT 37° FOR THE OXIDATION OF SUCCINATE BY PHENAZINE METHOSULFATE CATALYZED BY LIVER MITOCHONDRIA AND MICROSOMES

Fraction	K_m *(mM)*	K_i *(mM)*
Mitochondria	3.2 (± 0.7)	0.087 (± 0.007)
Microsomes	2.0 (± 0.268)	0.043 (± 0.003)

nate phenazine reductase activity and the flavin content of the microsomes are about half the values observed for mitochondria. But, whether all this partion of the flavin is due to succinate-dehydrogenating enzyme or there are other enzymes which contribute towards this type of flavin is yet to be investigated. Even assuming that all the acid non-extractable flavin is from succinate-dehydrogenating enzyme, the "true turnover numbers" (see second footnote of Table I) in microsomes are about the same as the value found in mitochondria.

The succinate oxidase activity in mitochondria and microsomes was found to be cyanide and antimycin A sensitive. More than 95% of the activity was inhibited by 1.5 mM cyanide or by approx. 1 μM antimycin A. Malonate competitively inhibited the oxidation of succinate by phenazine methosulfate. The Michaelis constants for succinate and the inhibition constants for malonate are summarized in Table II.

Spectral analysis of microsomal cytochromes

Liver microsomes contain two cytochromes: cytochrome b_5 and P-450. The latter is discovered by KLINGENBERG[17] and GARFINKEL[15]. P-450 is the CO-binding pigment which possesses a maximum at 450 mμ in the difference spectrum of CO-

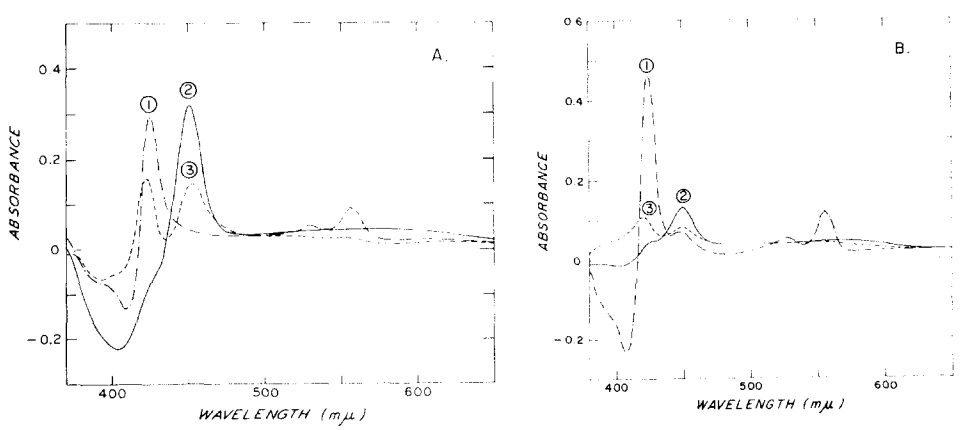

Fig. 3. Difference spectra of liver microsomes (A) and "microsomal electron-transport membranes" (B) prepared by the method D. MACLENNAN, A. TZAGOLOFF AND G. MCCONNELL (personal communication). Curve 1 (— · — · —), the dithionite reduced *minus* the oxidized form; Curve 2 (———), the CO-reduced *minus* the reduced form; and Curve 3 (- - - -), same as Curve 2 but cholate was added to 1%. The protein concentration was 3.3 and 1.85 mg per ml of 0.1 M phosphate buffer (pH 7.4) in systems of microsomes and "microsomal electron-transport membranes" respectively.

reduced *minus* the reduced sample. Our primary aim in studying microsomal spectra was two-fold. Firstly, comparison of the spectra of our preparations with those reported in literature would reveal the quality of the samples used. Secondly, gross contaminations of mitochondrial fragments, if any, could be easily revealed. As described in the following, the microsomes used for the present study exhibited the same spectral behavior as reported by other workers (*e.g.* refs. 5, 6). No contamination could be suggested from the spectra with respect to cytochromes *b*, *c*, c_1, *a* and a_3.

Although only the preparation made according to D. MACLENNAN, A. TZAGOLOFF AND G. MCCONNELL (personal communication) is shown in Fig. 3A, absorption spectra of the samples from other methods were similar to those given here. Dithionite immediately reduced both the CO-binding pigment and cytochrome b_5. However, addition of NADH or NADPH in the presence of air resulted in practically full reduction of cytochrome b_5 but left virtually all P-450 in the oxidized state. This observation confirms the report by OMURA AND SATO[16]. The spectral difference thus obtained between dithionite-reduced and NADPH-reduced microsomes should yield the spectral characteristic of the microsomal P-450. This spectrum was found in close resemblance to that reported previously[18], and exhibited two broad peaks around 440 and 550 mμ. Since reduced P-450 does not absorb in the region of 424 mμ, the absorption is entirely due to cytochrome b_5 and has been used[15] to compute the cytochrome b_5 concentration.

The CO difference spectra of P-450 in microsomes and in "microsomal electron-transport membranes" are depicted in Fig. 3. The CO adduct became evident when CO was bubbled through the sample after both the reference and the sample had been completely reduced.

Addition[19] of bile salts converts the P-450 to P-420 pigment; *i.e.*, the maximum shifts from 450 to 420 mμ in the difference spectrum. However, according to A. TZAGOLOFF AND D. MACLENNAN (personal communication), only deoxycholate, not cholate, is effective in this conversion. In fact, TZAGOLOFF AND MACLENNAN have used cholate to "solubilize" the microsomal P-450 and purified this pigment. In our studies we found that when cholate was added to either intact microsomes (Fig. 3A) or "microsomal electron-transport membranes" (Fig. 3B), the absorbance at 450 mμ decreased with a concurrent increase in the formation of a peak at 420 mμ.

TABLE III

CYTOCHROME CONTENT OF MICROSOMES AND MICROSOMAL FRAGMENTS

	Before cholate treatment		*After cholate treatment*			
	Cytochrome b_5	*P-450*	*P-420*	*P-450*	*P-420 (after cholate)*	*P-450 (after cholate)*
	(mμmoles/mg protein)		*(mμmoles/mg protein)*		*P-450 (before cholate)*	*P-450 (before cholate)*
Microsomes (Method I)*	0.57	0.51	0.33	0.16	0.63	0.31
Microsomes (Method II)	0.57	0.51	0.19	0.18	0.38	0.35
Microsomes (Method IV)	0.80	0.93	0.36	0.40	0.39	0.43
Microsomal electron-transport membranes	2.31	0.54	0.34	0.23	0.63	0.42
Sediment	0.65	0.34	0.28	0.069	0.82	0.20

* See legend of Table I.

After 1% cholate treatment of microsomes or "microsomal electron-transport membranes", about 35–40% of P-450 remained and about 40–65% P-420 concurrently emerged. The conversion took place within a few minutes, but even after 30 min the amount of these pigments remained the same without further change.

The concentration of cytochrome b_5, P-450 and P-420 in microsomes and "microsomal electron-transport membranes" are given in Table III. Cytochrome b_5 was enriched in the membrane fraction ("microsomal electron-transport membranes") to about three times as compared with microsomes, confirming earlier findings of D. MACLENNAN, A. TZAGOLOFF AND G. MCCONNELL (personal communication). On the other hand, decrease of P-450 on the per mg basis was observed.

The loss of the CO-binding pigment in "microsomal electron-transport membranes" may be due to denaturation and degradation. In fact, where the "microsomal electron-transport membranes" or the sediment was stored at $-25°$ for 20 days, both fractions completely lost their capacity to bind with CO in the presence or absence of cholate. Under the same conditions, a decrease of about 60% of P-450 was observed after a storage of 7 days. On the other hand, the cytochrome b_5 concentration remained unchanged for at least 20 days.

Even under the strict anaerobic conditions the difference spectrum of liver microsomes or "microsomal electron-transport membranes" in the presence of succinate and then saturated with CO against the succinate-treated sample did not show absorbance maximum at 450 mμ. This fact might be due to the unfavorable oxidation potential of P-450 for its reduction by succinate under the conditions tested*.

DISCUSSION

From more than 40 batches of bovine liver examined, some variations have been observed for the activities of cytochrome oxidase, succinate oxidase and succinate phenazine reductase as well as the acid non-extractable flavin content in the microsomes prepared by four different methods. Nevertheless, the microsome fraction always contains more succinate-dehydrogenating activities than cytochrome oxidase. These activities for succinate oxidation as well as for cytochrome oxidase may be explained due to the mitochondrial contamination. The argument becomes more specious in view of the apparent identity of optimum pH and activation energy for the microsomal and the mitochondrial oxidations of succinate by phenazine methosulfate, and the same sensitivity toward cyanide and antimycin A for the succinate oxidase activity.

However, quantitatively this explanation is not tenable. On per mg basis, *i.e.* the specific activity, the phenazine reduction in microsomes is more than half** that in mitochondria. If this reaction were, indeed, due to the contamination, then more than half of the microsome fraction must be mitochondria. This is impossible. Likewise, the spectra obtained do not suggest a contamination of mitochondria or mitochondrial fragments within the sensitivity of the spectral response. Moreover, not only the cytochrome oxidase in microsomes is less than one-tenth that in mitochondria but also the microsomal activity in the oxidation of succinate by phenazine

* Personal communication with Dr. H. S. MASON (1966).

** The "smooth" microsomes obtained by the method of MASON *et al.*[3] account for a little more than 30%.

approximates 10 times that of the cytochrome oxidase reaction. One may interpret these facts by the preferential contamination of mitochondrial fragments rich in succinate phenazine reductase but poor in cytochrome oxidase. On the statistical basis, the chances to account for this type of contamination of more than 50% by four different methods are not very likely. Furthermore, the kinetic constants as shown in Table II also seem to substantiate the difference between the mitochondrial and the microsomal oxidations of succinate.

With the present art for fractionation of cellular organelles, it is difficult to absolutely exclude the possibility of small contaminations. Nevertheless, even with the methods employed, it is untenable to explain the data presented by 50% mitochondrial contamination in the microsomal preparations. Therefore we are inclined to believe that the succinate-dehydrogenating ability is constitutive to microsomes. Future work will be concerned on the physiological role of this activity.

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