the ionization schemes proposed by Calvin and Edsall.

4. Kinetic data permit accurate estimates of titration pK values to be made.

5. The problem of the determination of intrinsic dissociation constants is discussed with reference to cysteine.

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Studies on the Particulate Components of Rat Mammary Gland

5. COMPARISON OF LARGE PARTICLES FROM LIVER AND MAMMARY GLAND*

BY T. F. SLATER† AND D. N. PLANTEROSE‡
Department of Biochemistry, University College London, Gower Street, London, W.C. 1

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It is generally considered that the mitochondrial fraction of tissue suspensions contains the major part of the oxidative-enzyme systems of the disrupted cells. In liver tissue the mitochondrial fraction can be separated by differential centrifuging in 0·25 M-sucrose for approximately 100 000g-min. Similar fractions can be obtained, under more or less identical conditions, from a variety of other tissues. This paper emphasizes that large variations can occur between certain tissues in the sedimentation properties of the particles containing such oxidative enzymes as succinic dehydrogenase and cytochrome oxidase, and that the results obtained for sedimentation patterns in liver tissue are not always applicable to other tissues such as mammary gland.

Comparative studies on the mitochondrial fraction from various tissues, contrasting sedimentation properties with enzymic distributions, have seldom if ever been made. Cytological investigations (Weatherford, 1929; Dempsey, Bunting & Wislocki, 1947) and biochemical studies (Greenbaum & Slater, 1957c) on rat mammary gland have indicated a change in size of mitochondrial particles over the lactation cycle. Other reports (Dmochowski & Strickland, 1953; Tuba, Orr & Wiberg, 1955) have suggested a size difference between the mitochondria from liver and mammary gland. It was therefore of interest to centrifuge liver and mammary-gland suspensions under identical conditions, tissues from animals at various stages of the lactation cycle being used, to check for: (a) possible variations in the size of mammary-gland mitochondria over the lactation cycle; (b) possible variations in the size of liver mitochondria over the lactation cycle; (c) possible differences between liver and mammary-gland mitochondria.

* Part 4: Slater & Planterose (1958).

† Beit Memorial Fellow.

‡ Present address: Research Institute (Animal Virus Diseases), Pirbright, Surrey.

METHODS

The animals used were hooded Norwegian adult female rats (body wt. 180–200 g.) of the Medical Research Council strain. The litters of lactating animals were restricted to 6–11 young. Three stages of the lactation cycle were studied: late pregnancy (18–19 days of pregnancy), 3-days lactating and 18-days lactating. Animals were killed by cervical dislocation and the livers and mammary glands were quickly removed and placed in ice-cold aqueous 0·25 M-sucrose. Livers and mammary glands were homogenized for the same time by the method described by Greenbaum & Slater (1957a); after straining through muslin the suspensions were briefly rehomogenized with a plastic pestle (diam. difference, 0·005 in.). Tissue suspensions so prepared were finally diluted (1:5) with ice-cold aqueous 0·25 M-sucrose.

The tissue suspensions of liver and mammary gland from the same animal were centrifuged simultaneously in a MSE Angle 13 centrifuge by the procedure shown in Fig. 1.

Table 1. *Head-radii (r), $g_{max.}$ values and $g_{max.} \times min.$ for the centrifuging steps shown in Fig. 1*

See de Duve & Berthet (1953).

Fraction	$r_{min.}$ (cm.)	$r_{max.}$ (cm.)	$10^{-3} g_{max.}$	$10^{-4} g_{max.} \times$ min.
M1	7·7	10·1	4·25	5·15
M2	7·2	10·1	7·25	8·85
M3	6·7	10·1	11·75	13·95
M4	6·1	10·1	16·75	20·35
M5	6·1	10·1	19·65	33·90

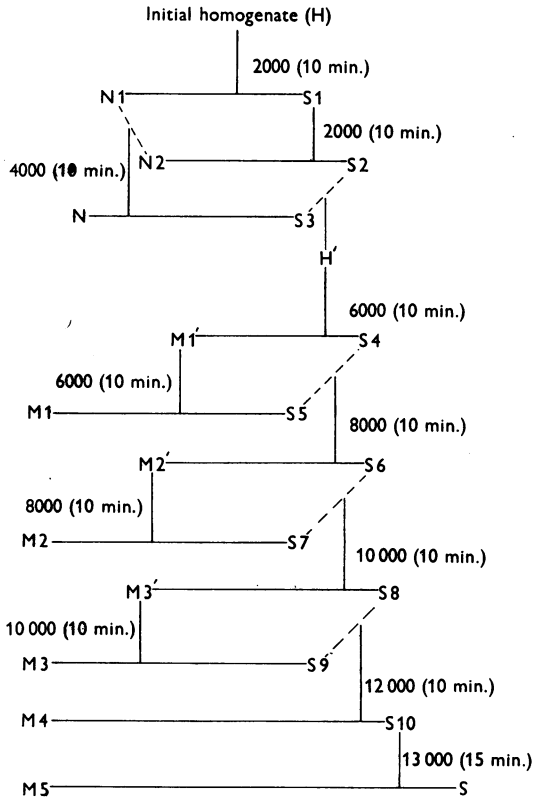

Fig. 1. Centrifuging scheme used for isolation of fractions H', M1–M5 and S from rat-liver and mammary-gland suspensions. For details see Methods. In the diagram, centrifugings are represented by vertical lines against which are placed the corresponding rev./min. and times used for each stage. The sediment and supernatant suspension resulting from each centrifuging are shown on the left and right respectively of the vertical line. Combination of two supernatant suspensions before recentrifuging is shown by a broken line (- - - -). All homogenate values referred to in the text are relative to that of H', which is taken as 100.

All operations were carried out at 0°; sediment and supernatant suspension were separated by suction pipettes with bent ends. The constants for each stage of the differential centrifuging are given in Table 1 (cf. de Duve & Berthet, 1953).

Each fraction was finally resuspended in 5 ml. of ice-cold 0·25 M-sucrose. For all assays fraction H' (Fig. 1) was used as the whole-homogenate standard (i.e. values for H' are taken as 100%); it can be seen from Fig. 1 that the nuclear fraction and cell debris have been removed from the original tissue suspension in the preparation of H'. Deoxyribonucleic acid phosphorus (DNA-P) measurements on H and H' showed that only some 10% of the DNA-P in the original suspension (H) came through the centrifugings at 2000 rev./min.

Total protein in the seven fractions H', M1–M5 and S was determined by the colorimetric method of Lowry, Rosebrough, Farr & Randall (1951). The mean recovery in M1–M5 and S was 105% of H'; results are expressed in terms of the 'recovered' protein and not in terms of the value obtained for H'. Lactose and DNA-P were determined on H' as reported previously (Greenbaum & Slater, 1957*b*).

Cytochrome oxidase was assayed by a modification of the method of Straus (1956). *NN'*-Dimethyl-*p*-phenylenediamine oxalate was found to be more convenient than the hydrochloride used by Straus. A solution of the oxalate was obtained by stirring 0·11 g. of the solid with about 50 ml. of water at 50–60°; the resulting pinkish solution was diluted to 100 ml. and kept at 0° until used. It was not necessary to neutralize the oxalate before use (as reported by Straus for the hydrochloride), provided that a strong buffer was used in the incubation mixture; recrystallized 2-amino-2-hydroxymethylpropane-1:3-diol (tris), 0·1 M, pH 7·2, was found suitable. Higher concentrations of tris buffer (pH 7·2) had no influence on the reaction. In agreement with Straus (1956), Na^+ ions were found to be inhibitory in the cytochrome-oxidase reaction. Blanks consisted of enzyme fraction, sodium azide, tris buffer, cytochrome *c* and *NN'*-dimethyl-*p*-phenylenediamine oxalate. Cold ethanol was used in all cases to stop the enzymic oxidation. It was found that sucrose inhibits the enzyme reaction by about 15% of a final concentration of 0·06 M. The same amount of sucrose must therefore be added to all tubes. Since the extinction at 540 mμ decreases relatively quickly with time (about 30% in 30 min.) it is essential to keep to a rigid time-table in stopping the reaction and measuring the extinction at 540 mμ.

The conditions adopted for measuring cytochrome oxidase were: a mixture of 1·0 ml. (or 0·9 ml. for the blanks) of tris (0·1 M, pH 7·2); 0·2 ml. of 0·04% cytochrome *c* (Sigma Chemical Co.; crystalline) and 0·2 ml. of enzyme in 0·25 M-sucrose was pre-incubated for 2 min. at 37°. A portion (0·7 ml.) of 0·11% *NN'*-dimethyl-*p*-phenylenediamine oxalate was added and the reaction was stopped (usually 3–5 min. later) by adding 2 ml. of cold ethanol. The tubes were kept in crushed ice until the extinction was measured. The extinction at 540 mμ was determined in a Unicam SP. 600; the path length was 1 cm. for all measurements

given in this paper. Blanks were obtained by a similar procedure except that 0·1 ml. of 0·1 M-sodium azide was added to the incubation mixture. Duplicate tubes were run in all cases.

Under the above conditions the relationship between the amount of tissue added (0–4 mg. wet wt.) and the extinction at 540 mμ was linear. The amount of tissue added was usually 0–2 mg. wet wt. The extinction at 540 mμ increased linearly with the time of incubation at least up to 5 min.; the extinction value increased with the volume of cytochrome *c* added, reaching a plateau value at 0·2 ml.; there was negligible colour formation in the absence of added cytochrome *c*. Boiling the tissue suspensions completely inhibited the reaction. In Table 2 cytochrome oxidase activity is expressed as the extinction at 540 mμ (E_{540}) obtained after 5 min. incubation per mg. wet wt. of tissue.

Succinate–neotetrazolium reductase was assayed by the method of Slater & Planterose (1960) in the presence of added vitamin K_3 and vitamin C. Aerobic incubation was at 37° and was usually for 10 min., except for the most active liver fractions, when a shorter time was used. At least two different concentrations of tissue were used in the assay procedure to check for linearity of response; an equal number of blanks were also measured with sodium malonate in place of sodium succinate in the incubation mixture [for experimental details see Slater & Planterose (1960)]. The activity of succinate–neotetrazolium reductase is expressed in Table 2 as the extinction produced at 510 mμ per min. per g. wet wt. of tissue, the formazan having been extracted into 4 ml. of ethyl acetate.

Succinic dehydrogenase was assayed by the ferricyanide method described by Bonner (1955), modified by a preincubation period before addition of substrate to allow for the osmotic swelling of intracellular particles. Under these conditions the addition of substrate produced a linear decrease in extinction at 400 mμ with time.

RESULTS

In preliminary experiments four mitochondrial fractions were isolated from mammary-gland homogenates and the succinic-dehydrogenase activity was measured. For two animals in late pregnancy the percentage of the total activity found in each fraction and the corresponding ***g***-min. used in the isolation of the fraction were as follows: M1, 51 500 ***g***-min., 54, 49%; M2, 88 500 ***g***-min., 12, 17%; M3, 109 000 ***g***-min., 6, 10%; M4, 440 000 ***g***-min., 7, 11%; soluble fraction remaining after sedimentation of M4, 20 and 14%. On other occasions mammary-gland homogenates were separated into large-particle fraction and soluble fraction by centrifuging at 13 000 rev./min. for 15–30 min. in 0·25 M-sucrose, and the soluble fraction was assayed for succinic dehydrogenase and for cytochrome oxidase. Individual values obtained from animals in late pregnancy (expressed as percentages of the homogenate value) were succinic dehydrogenase 21, 29, 18% and cytochrome oxidase 14 and 21%.

Soluble fractions from mammary-gland suspensions obtained by removing the large-particle fraction at 13 000 rev./min. for 20 min. were occasionally centrifuged again in a Spinco model L centrifuge at 40 000 rev./min. for 40 min. The microsomal pellet so obtained was assayed for succinic dehydrogenase, succinate–neotetrazolium reductase and cytochrome oxidase. It was found that approximately 20–30% of the activity appearing in the soluble fraction before high-speed centrifuging was sedimented by the centrifuging at 40 000 rev./min. (i.e. approximately 20–30% of the activity in fraction S of Fig. 1 is sedimentable at 3×10^6 ***g***-min.).

Table 2 gives the results obtained for the variation in total homogenate activity (succinate–neotetrazolium reductase and cytochrome oxidase) over the lactation cycle for both liver and mammary gland. It can be seen that for liver there is a tendency for succinate–neotetrazolium-reductase activity to increase over the lactation cycle whereas cytochrome-oxidase activity remains constant. In mammary gland, succinate–neotetrazolium-reductase activity increases rapidly over parturition ($P < 0.001$) and also during lactation ($P < 0.001$). Cytochrome-oxidase activity, on the other hand, is virtually unchanged over parturition but increases by a factor of two in lactation (P 0·02).

Table 2 also gives the total protein contents of liver and mammary-gland homogenates (H′); it can be seen that liver protein/g. wet wt. of tissue is constant over the period studied, whereas mammary-gland protein increases approximately twofold at parturition and a further twofold in lactation.

An interesting feature of Table 2 is the percentage of enzyme remaining in the soluble fraction S. Columns 7 and 10 show that only a small amount of each enzyme is in the soluble fraction of rat liver, whereas in mammary tissue the percentage is extremely high (about 40%) in pregnancy. This high value in pregnancy decreases throughout lactation to a value similar to that obtained for liver.

Columns 8 and 11 (Table 2) give the recoveries of both enzymes in the six fractions assayed (M1–M5 and S) compared with the value obtained for the whole homogenate (H′). Column 8 for succinate–neotetrazolium reductase shows that the recovery is between 80 and 90% for both liver and mammary gland. For cytochrome oxidase, however, liver values are between 70 and 80%, whereas mammary gland values are all greater than 100%.

Table 3 gives the percentage distribution of both enzymes among the five particulate fractions M1–M5. For both enzymes in liver there is no difference in distribution at the three stages studied. In mammary gland there is a tendency for both enzymes to become more associated with M1 and M2 as lactation proceeds. For instance, with cyto-

Table 2. *Protein content and activities of succinate–neotetrazolium reductase and cytochrome oxidase of rat-liver and -mammary-gland suspensions*

All values given in this table are the means of four animals ± standard error of the mean. For details see Methods section.

Tissue	Stage of lactation	Tissue wt. (g.)	Milk content* (% wet wt. of tissue)	Protein content (mg./g.)	Succinate–neotetrazolium reductase			Cytochrome oxidase		
					Activity†	Supernatant‡ activity	Recovery (%)§	Activity‖	Supernatant‡ activity	Recovery (%)§
Liver	Late pregnancy	9·2±0·2	—	249±13	5·74±1·42	10·3±1·7	87±4	90±11·4	4±0·6	68±1·0
	Early lactation	8·2±0·8	—	226±12	7·28±0·74	7·2±2·0	87±6	104±4·7	4±1·2	73±3·8
	Late lactation	9·1±0·6	—	263±43	9·25±1·30	6·7±1·5	88±3	98±2·8	0	77±3·2
Mammary gland	Late pregnancy	4·9±0·2	—	23±3·7	0·06±0·01	36±3·2	80±4	11±1·3	45±5·6	114±6·6
	Early lactation	5·1±0·4	54	41±3·2	0·27±0·05	23±3·0	86±5	9±1·0	27±2·8	112±1·4
	Late lactation	6·2±0·1	39	84±13	1·50±0·26	8±1·1	82±10	20±3·6	5±3·0	111±6·9

* Calculated from Greenbaum & Slater (1957*a*).
† Units: E_{510}/g. of tissue/min.
‡ Expressed as percentage of the activity recovered in all fractions (M1–M5 and S).
§ Activity recovered in all fractions expressed as percentage of homogenate activity (H′).
‖ Units: E_{540}/mg. of tissue/5 min. incubation.

Table 3. *Percentage distribution of activities of succinate–neotetrazolium reductase and cytochrome oxidase among fractions* M1–M5 *of rat-liver and -mammary-gland suspensions*

Total activity in M1–M5 = 100%. All values are the means of 4 animals ± standard error of the mean. For details see Methods section.

Tissue	Stage of lactation	Succinate–neotetrazolium reductase					Cytochrome oxidase				
		M1	M2	M3	M4	M5	M1	M2	M3	M4	M5
Liver	Late pregnancy	82±4·5	10±3·6	2±0·5	4±0·9	2±0·0	80±1·6	11±1·7	4±0·3	3±0·3	2±0·3
	Early lactation	87±1·7	7±1·1	3±0·6	2±0·4	1±0·0	75±1·4	14±0·8	5±0·5	4±0·7	2±0·0
	Late lactation	82±3·1	10±1·6	3±0·0	3±0·8	2±0·4	75±1·1	16±0·6	4±1·0	3±0·7	2±0·4
Mammary gland	Late pregnancy	50±6·2	25±6·4	13±6·4	8±4·0	4±3·6	46±5·2	22±1·2	11±0·9	11±1·7	9±2·0
	Early lactation	71±3·4	13±2·5	7±1·4	4±0·7	5±2·2	59±6·3	20±2·6	6±1·3	7±2·2	7±2·6
	Late lactation	68±6·9	21±5·5	3±1·0	4±1·8	4±1·2	58±4·1	20±10·9	7±0·9	10±5·5	5±5·9

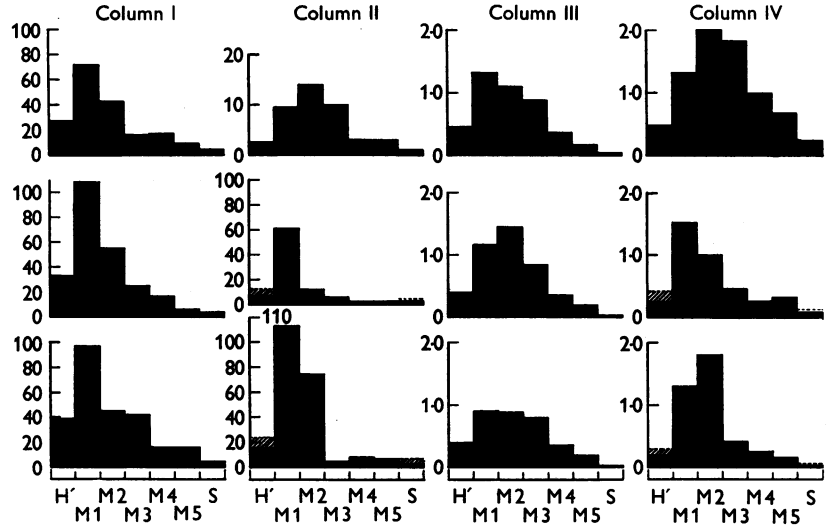

Fig. 2. Distribution of the specific activities of succinate–neotetrazolium reductase and cytochrome oxidase in rat-liver and -mammary-gland suspensions at three stages of the lactation cycle. The three histograms in column I, reading from top to bottom, represent liver succinate-neotetrazolium-reductase specific activity at late pregnancy, early lactation and late lactation respectively; column II, the corresponding values for succinate–neotetrazolium-reductase specific activity for mammary gland; column III, liver cytochrome-oxidase specific activities; column IV, mammary-gland cytochrome-oxidase specific activities. In each histogram the abscissa (reading from left to right) represents the seven fractions, H′, M1–M5 and S. The ordinate in each histogram represents specific activity (i.e. activity/protein content); enzyme activity is defined in Table 2. For mammary gland, the increases in specific activity after correction for retained milk protein are shown for early and late lactation animals by the cross-hatched areas. Each value is the mean of four animals.

chrome oxidase the percentage of the activity in M1–M5 found in fractions M1+M2 in late pregnancy is 68% compared with 78% in late lactation. For succinate–neotetrazolium reductase the corresponding values for M1+M2 are 75% compared with 89%.

Fig. 2 illustrates the variation over the lactation cycle of the specific activities of both enzymes (i.e. enzyme activity/protein content). There is no significant alteration in pattern for liver with either enzyme over the lactation cycle. With mammary gland, on the other hand, there is an accentuation of fractions M1 and M2 for both enzymes as the lactation cycle proceeds. The changes in the specific activities of mammary-gland enzymes are complicated by variations in the amount of milk retained by the gland. It is generally assumed that milk protein appears very largely in the soluble fraction after differential centrifuging so that only the specific activities of the whole homogenate and the soluble fraction would be greatly altered by the presence of retained milk protein. Corrections for retained milk protein made with protein values previously published (Greenbaum & Slater, 1957*a*) and the percentage of milk retained (Table 2, column 4) are shown in Fig. 2 for early and late lactation.

DISCUSSION

Protein content

There is no significant alteration in the amount or distribution of liver protein over the lactation cycle. The changes found for mammary-gland protein are comparable with those previously reported (Slater & Planterose, 1958).

Enzyme activities

There is no change in the cytochrome-oxidase activity of rat liver over the periods studied (Table 2, column 9); there appears to be a tendency for liver succinate–neotetrazolium-reductase activity to increase (Table 2, column 6), although this increase is not statistically significant.

With mammary-gland cytochrome-oxidase activity there is no increase over parturition but an increase occurs over the lactating period from 9 to 20 units (P 0·02). Similar increases in rat-mammary-gland cytochrome-oxidase activity over the lactation period have been reported by Tuba *et al.* (1955) and by Smith & Richterich (1958). Mammary-gland succinate–neotetrazolium-reductase activity increases enormously both over parturition and during lactation; these increases are far greater

than the increases previously reported for succinic oxidase as assayed manometrically (Greenbaum & Slater, 1957*c*; Tuba *et al.* 1955; Smith & Richterich, 1958).

Succinate–neotetrazolium reductase and cytochrome oxidase do not show a similar pattern of increase over the lactation cycle in mammary tissue. In late pregnancy the ratio of homogenate activities (cytochrome oxidase/succinate–neotetrazolium reductase) is 180, whereas in late lactation this ratio is 13. In liver the corresponding ratio is between 10 and 15 for all stages of the lactation cycle.

Enzyme recovery

The recovery of enzyme activity in the six fractions (M1–M5 and S) as a percentage of the activity found in H′ is between 80 and 90 % for the succinate enzyme in both liver and mammary gland (Table 2, column 8). This recovery is similar to the recovery of succinic oxidase from mouse liver (Schneider & Hogeboom, 1950) and the recovery of succinate–cytochrome *c* reductase from rat-liver fractions (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). Considering the relatively large number of centrifugings used (Fig. 1), a recovery of 80–90 % is satisfactory.

The situation is not so clear when the recovery of cytochrome oxidase (Table 2, column 11) is considered. The recovery for liver is lower than might be expected merely from a uniform loss of material during centrifuging. As a result of the complex fractionation used it was not possible to assay either enzyme until about 4 hr. after the start of the experiment. It is therefore conceivable that the isolated fractions deteriorated on standing at 0° more rapidly than the unfractionated homogenate. This seems the probable explanation for the low recoveries found with liver tissue; in several experiments in which only a simple fractionation scheme was used (i.e. separation of one mitochondrial and one microsomal fraction), and for which the assay was performed much sooner after the start of the experiment, the recoveries were all greater than 90 %. A similar explanation accounting for low recoveries of cytochrome oxidase in spleen has been proposed by Eichel (1957).

With mammary gland the apparent recoveries of cytochrome oxidase were consistently greater than 100 % even in experiments where a simple fractionation was used and in which the assay procedure was applied soon after the start of the experiment. Similar high recoveries of cytochrome oxidase have been reported by Brody, Wang & Bain (1952) for brain-tissue fractionations. It seems probable that the high recovery in mammary-gland suspensions is the result of the high fat content of the mammary-gland homogenates; a fat suspension obtained from mammary-gland homogenates has been shown to inhibit the oxidation of *NN′*-dimethyl-*p*-phenylenediamine oxalate in the presence of fresh homogenate, in other words to inhibit the apparent cytochrome-oxidase reaction. In the centrifuging scheme shown in Fig. 1, fat is largely removed during the first high-speed centrifuging at 0°, i.e. in the preparation of M1. Its major effect in the experiments reported in this paper would therefore be on H′, leading to high recoveries. The fact that the recoveries (Table 2, column 11) of cytochrome oxidase in mammary-gland suspensions are all virtually identical at the three stages studied indicates that this fat effect is a constant feature throughout the lactation cycle.

Enzyme distributions

The percentage distributions of both enzymes (Table 3) have been calculated in terms of the total activity recovered and not in terms of the activity of the unfractionated homogenate (see de Duve *et al.* 1955). In this manner comparison between different stages of lactation cycle and between the two tissues can be made with confidence since all assays were performed under identical conditions. Thus the distribution patterns obtained in the above manner for cytochrome oxidase in liver and mammary gland are strictly comparable with the respective distribution patterns for succinate–neotetrazolium reductase, although the percentage recoveries of these two enzymes in terms of the activity of H′ (Table 2, columns 8 and 11) are very different.

The distributions of both succinate–neotetrazolium reductase and cytochrome oxidase in the various intracellular fractions studied in rat liver are comparable with those found in many previous studies on the intracellular distributions of succinic dehydrogenase and cytochrome oxidase. The distributions found for succinate–neotetrazolium reductase and for cytochrome oxidase are indistinguishable from one another at all stages of the lactation cycle. This is also in accord with previous work on succinic dehydrogenase and cytochrome oxidase (see de Duve *et al.* 1955). It is now generally accepted that succinic dehydrogenase and cytochrome oxidase are almost wholly associated with the mitochondrial fraction in rat liver. Table 2 (columns 7 and 10) shows that there is little activity of either enzyme in fraction S. As can be seen from Table 3 (columns 3 and 8), both succinate–neotetrazolium reductase and cytochrome oxidase are largely concentrated in the particulate fraction M1.

In mammary gland, the distributions of both succinate–neotetrazolium reductase and cytochrome oxidase alter in a similar manner over the lactation cycle. In late pregnancy, for both enzymes there is a very high (36–45 %) proportion

of the recovered activity in fraction S (Table 2, columns 7 and 10); this has decreased to half its value by early lactation and is down to a value of less than 10% by late lactation. For each stage of the lactation cycle studied the distribution patterns of succinate–neotetrazolium reductase and cytochrome oxidase are very similar. It can be seen from Table 3 (columns 3 and 8) that fraction M1 in mammary gland does not contain so high a proportion of the total recovered activity as was found for fraction M1 in liver tissue.

The major difference between the enzyme distributions for liver and mammary gland, however, is in the percentage of the recovered activity associated with fraction S. In liver at all stages of the lactation cycle, the activity in fraction S is less than 10%, whereas in mammary gland at late pregnancy the value is around 40%. Since the overall activities of both enzymes in late pregnancy in mammary gland are low the significance of the activity ascribed to fraction S must be discussed with reserve. It will be convenient to consider the two enzymes assayed in turn. (*a*) Cytochrome oxidase. The constitution of the blanks rules out non-enzymic formation of colour as a source of activity in fraction S. Further, it has been shown that the activity apparent in fraction S is proportional to the amount of fraction S added, is dependent on the addition of cytochrome *c* and is destroyed by boiling the tissue suspension. Approximately 30% of the activity in fraction S can be sedimented by centrifuging at 3×10^6 *g*-min. Thus it would seem that the cytochrome-oxidase activity found in fraction S is real. (*b*) Succinate–neotetrazolium reductase. The same arguments can be made in favour of a real activity of succinate–neotetrazolium reductase in fraction S. The activity shown by fraction S is proportional to the amount added, is destroyed by boiling, is dependent on the presence of succinate and is inhibited by malonate (Slater & Planterose, 1960). Additional points can, furthermore, be made. First, the results of Greenbaum & Slater (1957*c*) showed that only some 55% of the total succinic oxidase activity of mammary-gland homogenates was sedimented after centrifuging for approximately 230 000 *g*-min. (approximately equivalent to fraction M5 in this investigation) at late pregnancy, whereas 85% was sedimentable during late lactation. It was in fact suggested that the results inferred a change in size of mammary-gland mitochondria over the lactation cycle (Greenbaum & Slater, 1957*c*); the activity in the supernatant from their experiments would presumably have been approximately 40% in late pregnancy and approximately 10% in late lactation, figures very similar to those obtained in this investigation by a colorimetric as opposed to a manometric method. Secondly, succinic dehydrogenase has been demonstrated in fractions during late pregnancy by the ferricyanide method of Bonner (1955). Thirdly, the distribution of succinic dehydrogenase determined by this method among four mitochondrial fractions and a soluble fraction from mammary glands during late pregnancy was strictly comparable with the results presented in Tables 2 and 3. Thus it would seem that the succinate–neotetrazolium-reductase activity in fraction S is real and that there are indeed large percentages of the total activity of cytochrome oxidase and succinate–neotetrazolium reductase in fraction S in late pregnancy. This activity in fraction S is gradually overshadowed by rapid increases in particulate enzymes as the lactation cycle proceeds.

Tissue differences

Although many reports have been concerned with variations in enzymic composition between mitochondria from different tissues (for instance see Holton, Hülsmann, Myers & Slater, 1957; Aldridge, 1957), no reports have appeared to our knowledge in which the sedimentation properties of mitochondria from liver and another tissue have been directly compared as in this investigation. The results show clearly how large the differences between liver and another tissue can be with respect both to the sedimentation behaviour of particles containing oxidative enzymes and to the composition of the mitochondria themselves. It can be deduced from the results that mammary-gland mitochondria show a more platykurtic distribution than liver mitochondria (i.e. enzyme activity is distributed over a greater range of *g*-min. in mammary gland). These conclusions confirm earlier reports of differences between liver and mammary-gland mitochondria (Dmochowski & Strickland, 1953; Greenbaum & Slater, 1957*c*). If it is assumed that there is no radical alteration in the density of the particles over the lactation cycle then it follows that the sedimentation changes found infer a change in size of the mitochondria in mammary gland over the lactation cycle. This conclusion is in agreement with the cytological results of Weatherford (1929), who reported that mammary-gland mitochondria increase in size from late pregnancy to late lactation.

SUMMARY

1. Liver and mammary-gland homogenates at three stages of lactation have been separated into five large-particle (mitochondrial) fractions. The distributions of succinate–neotetrazolium reductase, cytochrome oxidase and protein among these fractions were studied.

2. In liver there was a tendency for succinate–neotetrazolium reductase to increase from late

pregnancy to late lactation, whereas cytochrome oxidase remained constant. No change in the distribution of these enzymes or of protein was found at the three stages of the lactation cycle studied.

3. In mammary gland succinate–neotetrazolium reductase increased rapidly over parturition and throughout lactation; cytochrome oxidase showed a smaller increase during lactation. These increases were accompanied by changes in the enzyme distribution among the mitochondrial and soluble fractions, more activity being found in the larger particles as lactation proceeds.

4. Mammary-gland mitochondria appear to change in size over the lactation cycle, being smallest in late pregnancy and largest in late lactation. In late lactation they sediment at approximately the same *g*-min. values as do liver mitochondria.

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An Assay Procedure for a Succinate–Neotetrazolium-Reductase System

By T. F. SLATER* and D. N. PLANTEROSE†
Department of Biochemistry, University College London, Gower Street, London, W.C. 1

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There is a rapid production of reduced neotetrazolium chloride (formazan) by rat-liver suspensions in the presence of succinate, vitamin C and vitamin K_3 (Slater, 1959*b*). The enzyme system coupling the reduction of neotetrazolium chloride with the oxidation of succinate is termed succinate–neotetrazolium reductase. The adoption of the word 'reductase' in connexion with neotetrazolium chloride as final electron acceptor must, of course, be interpreted with reserve. It is not intended to imply that there is only one route for the reduction of neotetrazolium by succinate as it seems probable that the pathway operating in the unsupplemented homogenate is different from that which operates in the presence of added vitamin C and vitamin K_3. This paper describes suitable conditions for the assay of such an enzyme system in the presence of added vitamin K_3 and vitamin C; under the conditions described formazan production increases proportionately with the amount of tissue added.

* Beit Memorial Fellow.
† Present address: Research Institute (Animal Virus Diseases), Pirbright, Surrey.

METHODS

The animals used were hooded Norwegian rats of the Medical Research Council strain. They were adult females, body weight 180–200 g., and were fed on a diet described by Parkes (1946). Liver homogenates were prepared either in water or in 0·25 M-sucrose at 0° in a conventional-type homogenizer with a plastic pestle (diam. difference 0·005 in.). Mammary-gland homogenates were prepared in