Distribution of Enzymes in Cell-Free Yeast Extracts

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The evidence for the existence of 'mitochondria' in micro-organisms has been growing, but the criteria for thus naming cytoplasmic inclusions are by no means clear. From the biochemical point of view, mitochondria are generally considered to be sites of oxido-reduction and of organized oxidations. The 'cyclophorase' complex in the mitochondria of animal cells studied by Green and his School (Green, Loomis & Auerbach, 1948) has never been demonstrated in micro-organisms, although it has recently been found in plants (Millerd, Bonner, Axelrod & Bandurski, 1951).

The failure to prepare cell-free, actively respiring yeast extracts contributes to the continuing controversy about the main respiratory mechanism of this micro-organism (cf. Krebs, Gurin & Eggleston, 1952; Martius & Lynen, 1950; and Foulkes, 1951).

Methods used so far for preparing yeast extracts are probably such that the intracellular organization of enzymes, and therefore organized oxidation systems, are destroyed during cell disintegration. We have developed an ultra-rapid mechanical shaker which disintegrates 2 g. yeast almost entirely within 90 sec. (Nossal, 1953*a*). We failed to prepared actively respiring yeast 'mitochondria' with this machine. However, the results reported here show that the distribution of enzymes in the cell-free extracts is not unlike that in extracts of animal cells: dehydrogenases are found largely associated with the granular fraction when the extracts are prepared by very brief disintegration.

While the work was in progress, Hirsch (1952) reported that fumarase and aconitase were found largely in the non-sedimentable supernatant obtained after spinning yeast extracts at 30 000 *g*. Hirsch's extracts were prepared with a Towers Ultra-Rapid Shaker at 560 rev./min. The shaker used here disintegrates micro-organisms about 100 times more rapidly. Hirsch's findings were therefore re-examined in the light of the results obtained for dehydrogenases. A preliminary report of the very high fumarase and aconitase activities of the granules in cell-free extracts, prepared by 10 sec. disintegration in our shaker, has appeared elsewhere (Nossal, 1953*b*).

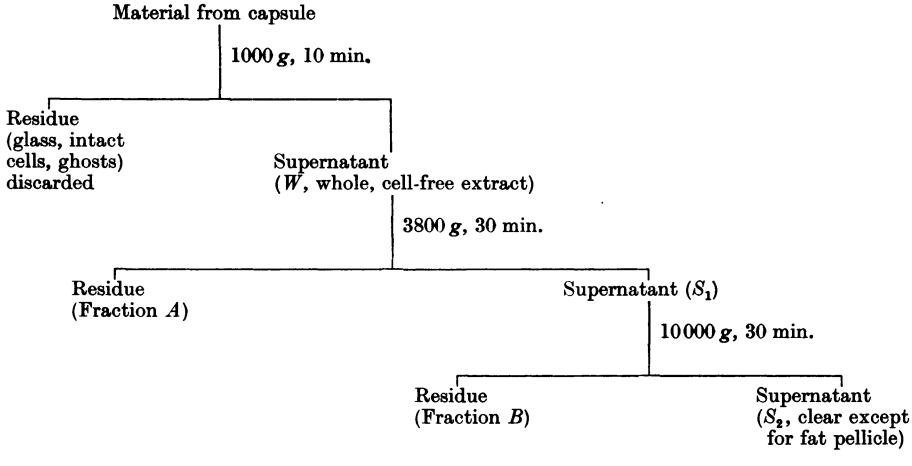
METHODS

Materials. Baker's yeast was obtained weekly from the Effront Yeast Co., South Yarra, Victoria, and kept in a humidor at 1°. Cakes may be kept thus without decolorization or loss of respiratory activity for up to 4 weeks.

Chemicals used were commercial samples, except 80% pure diphosphopyridine nucleotide (DPN), prepared from yeast by the method of Kornberg & Pricer (1951), and triphosphopyridine nucleotide (TPN) from liver (Kornberg & Horecker, private communication), either 10% pure and containing 9% DPN or 50% pure and free of DPN.

Mechanical disintegration was carried out as described previously (Nossal, 1953*a*): the stainless steel shaker capsule was charged with 2 g. wet wt. yeast, 10 g. Ballotini glass beads no. 12 (The English Glass Co., Leicester, England) and 10 ml. 0·9% KCl, cooled to 0° and shaken at 5600 rev./min. with an amplitude of $\frac{5}{8}$ in. (maximum acceleration about 300 *g*) for 10, 30 or 90 sec. at 1°.

Fractionation of the extract was effected by differential centrifugation as follows:



W was examined routinely and found to contain less than 0·1% of the original cells. This represents a negligible proportion when compared with disintegrated cells (see Table 2). The particulate fractions were washed once with as much 0·9% KCl as the original volume of *W*, unless otherwise stated. Dry weights were obtained by heating 2 ml. portions to 120° for 3 hr.

All manipulations were carried out as near 0° as possible, but at the end of 90-sec. disintegrations, the temperature of the capsular contents was about 25°; the capsular contents were cooled immediately. Dialyses were carried out at 0° for 15 hr., the dialysis sac being attached to a small magnet and rotated on a magnetic stirrer.

Activity measurements. Dehydrogenase activities were measured *in vacuo* in Thunberg tubes at 38°. The tubes contained 0·02 M-Na_2HPO_4/KH_2PO_4 buffer pH 7·0, 0·1 M substrates (neutralized if necessary), 10^{-4} M methylene blue, other additions as indicated and water to give a total fluid volume/tube of 3·0 ml.

Activities are expressed as $Q_{M.B.}$ values, which were calculated from 90% decolorization times. $Q_{M.B.} = \mu$l. methylene blue decolorized/mg. dry wt./hr. In some of the systems, the decolorization times were not linearly proportional to the amount of enzyme present. Therefore, for the purposes of comparison, equal amounts (on a dry wt. basis) of various extracts and fractions were taken for each experiment. Otherwise, the mean values obtained from several sets of experiments are cited.

Aconitase and fumarase activities were measured as rates of increase in optical density at 240 mμ. with citrate and L-malate, respectively, as substrates (Racker, 1950). The assay system contained enzyme, 0·01 M phosphate buffer pH 7·0, 0·01 M substrate and glass-distilled water to a final volume of 3·0 ml. Blanks contained no substrate. Room temperature was approx. 20°. No attempt was made to regulate the temperature in the spectrophotometer, but the temperature variation over a whole day was less than 2°.

For fumarase, the rate of change of optical density was directly proportional to enzyme concentration over a wide range (up to 0·1 density change/min.). For aconitase, the linearity held only for lower activities (up to 0·015/min.). Also, whereas for fumarase the rate of change of optical density was constant for at least 15 min., in the case of aconitase it declined after about 5 min. Therefore, specific activities (increases in optical density/mg. dry wt./min. $\times 1000$) were calculated from the mean of the first ten 30-sec. readings (0·5–5·5 min. after mixing) for both enzymes. Activities reported here, multiplied by 10, should be comparable with those of Hirsch (1952), whose results are expressed on a mg. protein N basis.

At first, measurements were made in 0·9% KCl to preserve granular structures in the extracts (under the phase contrast microscope the granules were better defined in 0·9% KCl than in water). It was found that this concentration of KCl inhibited fumarase activity up to 50% in all fractions. Aconitase was much less affected. Similar inhibition was observed when KCl was omitted and the buffer concentration increased to 0·1 M. Accordingly, all subsequent dilutions in the test system were made with glass-distilled water. The total salt concentration during measurements (including enzyme, buffer and substrate) was maintained below 0·04 M.

RESULTS

Activities in whole, dialysed extracts. Without dialysis, *W* and S_2 had high blank dehydrogenase activities. All results for dehydrogenases quoted in this work were obtained with dialysed *W* and S_2 or washed granules. The highest blank activities ($Q_{M.B.}$ values occasionally up to 4) were observed in the presence of impure TPN, containing DPN, in 30-sec. whole extracts. Otherwise the blank $Q_{M.B.}$ values were less than 1.

The dehydrogenase activities studied depended to some extent on the disintegration period used in the preparation of the extracts (Table 1). The ethanol, malic and glutamic dehydrogenases were little affected by the duration of disintegration, but the succinic, 'citric' and lactic dehydrogenase activities were much lower in 90-sec. extracts than in 10- or 30-sec. extracts.

Table 1. *Dehydrogenase activities in whole, dialysed yeast extracts*

Cell-free extracts were prepared by concurrent 10-, 30- and 90-sec. disintegrations from the same batch of yeast. 90% decolorization of methylene blue was measured as described under Methods. Dialyses were for 15 hr. against 50 vol. 0·9% KCl at 1°. $Q_{M.B.}$ values (μl. methylene blue decolorized/mg. dry wt./hr.) are the means of four complete series of experiments.

		$Q_{M.B.}$		
		10-	30-	90-
Substrate	Other additions	sec. disintegration		
None	0·1 mg. DPN 0·1 mg. TPN	<1	<4	<0·5
Succinate	—	6·6	7·1	<2·5
Citrate	0·1 mg. TPN 0·1 mg. DPN 0·001 M-$MnCl_2$ 0·001 M Adenosine 5-phosphate	8·7	10	2·8
Ethanol	0·1 mg. DPN	65	65	65
Lactate	—	20	19	10
L-Malate	0·1 mg. DPN	30	28	24
L-Glutamate	0·1 mg. TPN	17	18	14

Table 2. *Effect of disintegration period on the dry-weight distribution in cell-free yeast extracts*

Values given represent means of 20–40 samples for each disintegration period. The percentage disintegration was determined by direct counting of intact cells and ghosts on a haemocytometer at 400-fold magnification.

Disintegration period (sec.)	% disintegration	Fraction	Dry wt. (mg./ml. original extract)	% of dry wt. of whole extract
10	35	*W*	14·4	100
		Particulate, *A*	1·1	7·6
		Particulate, *B*	1·2	8·3
		S_2	11·3	78
		Combined washings*	0·8	5·6
30	60	*W*	22·1	100
		Particulate, *A*	2·4	11
		Particulate, *B*	2·7	12
		S_2	16·4	74
		Washings*	1·5	6·8
90	85	*W*	31·7	100
		Particulate, *A*	2·8	8·8
		Particulate, *B*	5·3	17
		S_2	21·7	68
		Washings*	2·5	7·9

* Sum of dry weights of 1 ml. each of the washings from particulate fractions *A* and *B*.

The activities given for citrate in *W*, S_2 and particles are much lower than the *iso*citric dehydrogenase content of these systems. The reason for using citrate as a substrate was the non-availability of *iso*citrate in sufficient amounts. The dehydrogenase activities measured therefore depended also on aconitase. This enzyme is notoriously unstable, especially to dialysis (see Hirsch, 1952). With the small amount of *iso*citrate available, it was shown that low concentrations of *iso*citrate gave much greater activities than high concentrations of citrate. With 0·01 M *iso*citrate, the highest $Q_{M.B.}$ value was 53 for a whole extract. This is in accordance with the high rates of citrate breakdown in yeast extracts reported by Foulkes (1951) and Hirsch (1952), and shows that *iso*citric dehydrogenase is one of the most active enzymes present in the extracts.

Effect of disintegration period on the relative proportions of particulate fractions. Examination of fresh yeast cells under phase contrast showed cytoplasmic granules with high refractive index and fairly uniform size. Similar findings were reported recently by Sarachek & Townsend (1953). It seemed possible that fraction *B* of yeast extracts was formed by mechanical disintegration of fraction *A*. The percentage of *B* should then increase with increasing disintegration period. This was found experimentally. 10- and 30-sec. extracts contained approximately equal amounts of fractions *A* and *B* (Table 2). In 90-sec. extracts, the sum of particulate fractions approximately equalled that in 30-sec. extracts, but only one-third of the material sedimented at the low speed and two-thirds at the higher speed. The increased weight of 30- and 90-sec. combined particles over 10-sec. combined particles was probably due to smashed cell-wall fragments not removed by centrifuging at 1000 *g*.

Effect of disintegration period on the enzymic activities of particles. While this work was in progress, Slonimsky & Hirsch (1952) reported in a brief note that the following enzymes predominated in the non-sedimentable supernatant of cell-free yeast extracts: lactic, malic, ethanol and *iso*citric dehydrogenases, fumarase and aconitase.

The dehydrogenase activities of granules in yeast extracts were here found to depend on the disintegration period employed in preparing the extract (Table 3). The particulate fractions of 10-sec. extracts had very high ethanol, malic and succinic dehydrogenase activities and also appreciable activities of the other enzymes. All activities were much lower in 90-sec. particles, the decreases being least for succinic dehydrogenase and greatest for ethanol and malic dehydrogenases. Glutamic and lactic dehydrogenases seemed to predominate in the supernatant. At present it is not possible to ascertain whether these enzymes really do occur mainly free in the cytoplasm of the intact cell, or whether they are the most easily removed from cytoplasmic granules. The extracts prepared by Hirsch (1952) and Slonimsky & Hirsch (1952) were made on a Towers Ultra-Rapid Shaker, which is about 10 times less rapid than ours. It is probable that after the much longer disintegration periods which had to be employed (60 min. as against 10 sec.), the more soluble enzymes appear largely in the supernatant. Even by a single washing with 0·9 % potassium chloride, particulate fractions lost weight and some dehydrogenase activities, which were recovered in the washing solution. Hirsch's disintegration medium was 0·1 M phosphate buffer, pH 7·3, and in 60 min. considerable amounts of dehydrogenases would be detached from the particles.

Table 3. *Effect of disintegration period on dehydrogenase distribution*

Washed particulate fractions *A* and *B* were prepared from 10-, 30- and 90-sec. extracts as described under Methods. Additions for each substrate as in Table 1. $Q_{M.B.}$ values are the means of the number of experiments shown.

Disintegration period (sec.)	Particulate fraction	No. of experiments	$Q_{M.B.}$ Blank	Succinate	Citrate	Ethanol	Lactate	L-Malate	L-Glutamate
10	*A*	3	<0·5	21	7*	49	9	27	5
	B	3	<0·5	14	3	33	7	33	1
30	*A*	9	<0·5	16	3	16	8	15	2
	B	6	0·4	16	3	23	8	14	1
90	*A*	8	<0·5	10	1	9	4	9	0·5
	B	5	<0·5	7	<0·5	5	3	5	<0·5

* The highest activity with 0·01 M *iso*citrate was 26.

Table 4. *The effect of disintegration period on the distribution of fumarase and aconitase in cell-free yeast extracts*

The specific activities (increase in optical density at 240 mμ./mg. dry wt./min. × 1000) are the means of three complete series for each disintegration period.

Disintegration period (sec.)	Activity measured	Specific activities of the fractionated extract: Whole extract	Particulate fraction *A*	S_1	Particulate fraction *B*	S_2	Washings of fraction *A*	Washings of fraction *B*
10	Fumarase	109	130	116	155	126	70	93
30	Fumarase	131	39	157	64	199	89	152
90	Fumarase	135	7	145	6	212	102	138
10	Aconitase	37	16	34	27	39	18	15
30	Aconitase	41	8	49	14	62	31	43
90	Aconitase	49	3	49	3	74	34	39

Table 5. *Effect of double washing on the enzymic activities of yeast granules*

10-sec. whole extracts were centrifuged at 10000 *g* to separate them into particles (fractions *A* plus *B*) and S_2. The particles were washed as indicated below. Activities refer to those of the combined particulate fraction. $Q_{M.B.}$ values are the means of two separate series, specific fumarase and aconitase activities means of three series.

Washing solution	No. of washings	$Q_{M.B.}$ Succinate	Citrate	Ethanol	Lactate	L-Malate	Specific activities: Fumarase	Aconitase
0·9% KCl	1	17	1·6	52	8	19	121	30
0·9% KCl	2	15	2·3	47	9	21	162	29
Water	1	11	4·7	50	6	18	110	42
Water	2	17	5·9	58	7	18	147	51

The diminished dehydrogenase activities of 30- and 90-sec. particles were largely accounted for by correspondingly increased activities in the supernatants, except for those enzymes which seemed to be denatured by prolonged disintegration (Table 1). Succinic dehydrogenase was found in 90-sec. supernatants and in the washings of 90-sec. granules. Both findings point to extensive disorganization of the particles.

The results for fumarase and aconitase were similar: the particles of 10-sec. extracts had fumarase activities at least as high, and occasionally twice as high, as those of the supernatant (Table 4). Their aconitase activity was usually lower than that of the supernatant. 30-sec. particles had fumarase activities between 25 and 50% of those of the corresponding supernatants, and still lower aconitase activities. The fumarase and aconitase activities of 90-sec. particles were less than 10% of those of the supernatant and often too low to measure.

Both particulate fractions from all extracts had a very similar enzymic composition. This, coupled with the probable conversion of fraction *A* into fraction *B* by prolonged mechanical disintegration, suggests that the fractions do not represent functionally or morphologically different particles, but that there is one type of granule, rich in respiratory enzymes, in yeast cytoplasm.

To rule out the possibility that the observed granular dehydrogenase and hydrase activities were due to contamination of the granules with supernatant, the effect of a second washing on the

activities of particulate fractions was examined. Both water and 0·9 % potassium chloride were used as washing agents, the former because it partly dissolved the granules; any supernatant adhering to the surface of granules should then be completely removed. Singly and doubly washed fractions had virtually the same dehydrogenase activities, whether saline or water were used for washing (Table 5). But for fumarase and aconitase, the opposite was found. Doubly washed granules had a higher fumarase activity than once washed granules, whether potassium chloride or water were used for washing. Aconitase activity was greatly increased by washing with water. Table 6 shows that all washings had both fumarase and aconitase activity. Thus, washing caused overall increases in the total activities of both enzymes. This is discussed further below.

Table 6. *Specific activities of washings from particulate fraction*

	Fumarase	Aconitase
First aqueous washing	38	20
Second aqueous washing	77	28
First saline washing	186	45
Second saline washing	Dry weights too low to measure but activities appreciable	

Table 7. *Coenzyme requirement of ethanol, malic, citric and glutamic dehydrogenases*

Dialysed S_3, from 10-sec. extract, was used. Values are means of three experiments.

	$Q_{M.B.}$		
Substrate	Without coenzyme	With 0·1 mg. DPN	With 0·1 mg. TPN
Ethanol	<1	59	11
L-Malate	10	23	15
Citrate	<0·5	1·0	3·5
L-Glutamate	<0·5	1·3	16

Table 8. *Effect of dialysis on the DPN requirement of malic and ethanol dehydrogenases*

Dialysed S_2 from various extracts was used.

		$Q_{M.B.}$	
Disintegration period (sec.)	Substrate	Without DPN	With 0·1 mg. DPN
10	L-Malate	2·7	10
30	L-Malate	8·3	15
90	L-Malate	8·0	15
90*	L-Malate	6·6	—
10	Ethanol	<0·5	34
30	Ethanol	<0·5	50

* Supernatant dialysed for 15 hr. and then for another 40 hr., each time against 60 vol. 0·9 % KCl.

Nucleotide-coenzyme requirement of the various dehydrogenases. The succinic and lactic dehydrogenases were independent of added coenzymes in all fractions tested.

The ethanol and malic enzymes were more active with DPN than with TPN (Table 7), whereas the citric and glutamic enzymes were more active with TPN than with DPN. All four enzymes, however, were activated by both coenzymes. Our assays showed that the DPN used for these experiments was free of TPN (Zwischenferment assay, Warburg, Christian & Griese, 1935); and that the TPN was free of DPN, there being no difference between chemical estimation of DPN+TPN (Colowick, Kaplan & Ciotti, 1951) and Zwischenferment assay. Whether TPN was enzymically converted into DPN in these preparations is not known, but this is possible according to the observations of von Euler & Adler (1938). The conversion of DPN into TPN is less likely since this process requires added adenosine triphosphate (Kornberg, 1950). Kornberg & Pricer (1951) have already reported two *iso*citric dehydrogenases in yeasts, specific for DPN and TPN respectively. The former also requires manganous chloride and adenosine 5-phosphate. In our extracts, these two substances were required for maximum activity in the presence of DPN, but the TPN-dependent enzyme seemed to predominate.

Dialysis of S_2 caused almost complete loss of ethanol, *iso*citric and glutamic dehydrogenase activities when these were tested without added coenzymes. However, only about 70 % or less of the total malic dehydrogenase activity was lost (Table 8). S_2, dialysed twice against a total of 120 vol. 0·9 % potassium chloride and tested without DPN, still retained almost half the activity shown in the presence of DPN. This suggests the occurrence of a DPN-independent malic dehydrogenase or firmer binding of DPN to this particular enzyme.

Evidence for the existence of bound coenzymes in the granules. In carefully prepared mitochondria from animal cells, dehydrogenases which in the soluble ('dissociated') form require pyridine nucleotide coenzymes, are fully active without added coenzymes. Green and associates have shown this to be due to bound coenzymes (Huenneckens & Green, 1950; Still & Kaplan, 1950). Yeast particles prepared from 10-sec. extracts also showed this property, but 30- or 90-sec. particles did not (Table 9).

The malic and ethanol dehydrogenases of 10-sec. particles were occasionally as active without added DPN as in the presence of DPN, but more often they showed only about half the full activity. The possibility of contamination by coenzymes from the supernatant was ruled out in two ways. One batch of 10-sec. particles was divided into three parts, one of which was washed as usual with the original

Table 9. *Coenzyme dependence of granular ethanol and malic dehydrogenases*

W was centrifuged at 10000 *g* and the combined particles were washed as shown below.

			$Q_{M.B.}$			
			Ethanol		L-Malate	
Expt.	System	Notes	Without DPN	With 0·1 mg. DPN	Without DPN	With 0·1 mg. DPN
1	10-sec. particles	Washed as usual	41	46	15	24
		Washed with 2 vol. KCl	46	46	14	20
		Washed with 3 vol. KCl	33	37	12	17
2	10-sec. particles	Washed as usual	46	60	11	21
		Washed twice	38	46	9	19
		Washed thrice	19	26	9	19
3	30-sec. particles	Washed as usual	3	15	1	12
	90-sec. particles	Washed as usual	<0·5	4	<0·5	5

Table 10. *Fumarase and aconitase balance of fractionated extracts*

Rates of activity refer to 1 ml. of the original undiluted extract. Fractions all calculated on this basis. Values are means of the number of experiments cited.

			Rate of change of optical density (240 mμ.)/min./ml. × 1000							
					Particulate fractions		Washings of particulate fractions			
Disintegration period (sec.)	Activity measured	No. of experiments	Whole extract (1)	S_2 (2)	*A* (3)	*B* (4)	*A* (5)	*B* (6)	(2)+(3)+(4)+(5)+(6) (7)	% increase over original whole extract
10	Fumarase	3	1513	1420	129	204	46	29	1828	21
10	Fumarase	3	1020	950	←250*→		←80*→		1280	25
30	Fumarase	3	3227	3573	94	185	121	121	4094	27
90	Fumarase	2	4540	4620	16	26	216	171	5049	11
10	Aconitase	3	369	375	←37*→		←20*→		428	16
30	Aconitase	3	983	1100	20	41	39	32	1232	25
90	Aconitase	2	1540	1480	9	21	63	44	1617	5

* In these experiments, *W* was centrifuged at 10000 *g*, so that the sediment contains combined fractions *A* and *B*; these were washed together.

volume of 0·9 % potassium chloride, the others with 2 and 3 vol., respectively. The dehydrogenase activities of all three were almost as high without added DPN as with DPN. Secondly, three portions of another batch of 10-sec. particles were washed once (as usual), twice and three times, respectively, with the original volume of 0·9 % potassium chloride. Again, all three had high activities without added DPN. 30- and 90-sec. particles had only very low activities without added DPN.

10-sec. particles lost almost entirely these coenzyme-independent activities (but not so much their dehydrogenase activities with DPN) by storage at 1° for 24 hr., and also by 15-sec. shaking without Ballotini in our disintegrator. Both observations are in accordance with those for mitochondria. Carefully prepared yeast particles therefore seem to resemble mitochondria from animal cells in containing bound coenzymes.

Quantitative studies with fumarase and aconitase. In an attempt to ascertain the contribution of the particulate fraction to the total enzymic activities of the extracts, we have measured the fumarase and aconitase activities of unfractionated extracts and of all the individual fractions. Table 10 shows that in 10-sec. extracts the sum of the activities of individual fractions exceeded those of the original extract by 25 %. Similarly, when a whole 10-sec. extract was reconstituted from the individual fractions, its activities again exceeded those of the original extract. The difference between the original activities of unfractionated extracts and the sum of the activities of individual fractions was much smaller in 90-sec. extracts.

The specific activities of 90-sec. whole extracts were found to be up to twice as high as those of 10-sec. extracts made from the same batch of yeast (Table 11).

These findings suggest masked fumarase and aconitase activities in 10-sec. extracts. Masked activities seem to be associated with extracts containing enzymically active granules, and were therefore not found in 90-sec. extracts, in which the granules are inert. It was noted above (Table 5)

Table 11. *Effect of disintegration period on the fumarase and aconitase activities of whole extracts*

Concurrent 10-, 30- and 90-sec. disintegrations were carried out with 2 g. portions of the same yeast sample. Values are the means of three such experiments.

Disintegration period (sec.)	Amount used for test (mg. dry wt.)	Activity measured	Change in optical density/min. × 1000	Specific activity
10	0·40	Fumarase	28	70
30	0·38	Fumarase	44	115
90	0·37	Fumarase	50	135
10	0·79	Aconitase	15	19
30	0·77	Aconitase	23	30
90	0·74	Aconitase	24	33

that double washing increased the activities of 10-sec. granules. We have also found that 24 hr. storage of 10-sec. particles in 0·9% potassium chloride at 2° increased both fumarase and aconitase activities. Various explanations to account for this masking are discussed below.

DISCUSSION

This work has shown for the first time that granules rich in enzymes other than succinoxidase and cytochrome *c* reductase can be prepared from a micro-organism. Increasing periods of mechanical disintegration cause progressive losses of all enzymes, but especially the more soluble dehydrogenases and fumarase and aconitase, from granules to supernatant. It seems, then, that the intact cell contains granules rich in all these enzymes, and that our particulate fractions contain the microscopically visible granules which have been called mitochondria by many workers on purely histological grounds (Lindegren, 1949; Mudd *et al.* 1951; Sarachek & Townsend, 1953; Mundkur, 1953). The particulate fractions of 10-sec. yeast extracts also resemble the mitochondria prepared from animal tissues in containing bound coenzymes. On the evidence presented here, it seems that yeast cells do not contain different types of granules rich in respiratory enzymes, such as the mitochondria and microsomes of animal cells. The similarity in enzymic content of particulate fractions *A* and *B* argues against such a distinction, as does the finding that increasing periods of mechanical disintegration apparently cause conversion of the more readily sedimentable fraction *A* into the less readily sedimentable fraction *B*.

The discrepancies between our results and those of Hirsch (1952) and Slonimsky & Hirsch (1952) can probably be explained by the differences in disintegration procedure. Whereas our best extracts were prepared by 10-sec. disintegration, the equivalent period on the Towers Shaker must have been of the order of 60 min. During that period, most of the more soluble dehydrogenases would be removed from the granules. Therefore, the granules in Hirsch and Slonimsky's extracts resemble our 90-sec. granules in being deficient in the more soluble enzymes and also in bound coenzymes.

The more quantitative results with fumarase and aconitase give some indication of the contribution of granules to the total enzyme content of the cell. In 10-sec. extracts, the particulate fraction accounts for about 15% of the dry weight, and the specific fumarase activity of the particles is up to twice that of the supernatant. The particulate fumarase activity is reduced threefold in 30-sec. extracts. It is reasonable to assume, therefore, that in the intact cell most of the fumarase activity is associated with granules. This may well apply also to the soluble dehydrogenases, and further strengthens the argument that yeast cells contain granules akin to the mitochondria of animal and plant cells.

Several sets of findings have indicated masked fumarase and aconitase activities in the extracts, and the masking is associated with the granular fraction. No natural inhibitors of fumarase and aconitase are known. It seems unlikely that the masked activities recorded are due to partial inhibition. Two other explanations cover the findings. The first is partial structural separation of enzymes and their substrates in carefully prepared granules; this could be due to a membrane such as that described for plant mitochondria by Farrant, Robertson & Wilkins (1953) or to orientation of the organized enzymes. Washing, especially with water, and storage could lead to deterioration of the granules and thus to increased activity. Increasing disintegration periods cause increasing mechanical disruption of particulate fractions, again liberating granular enzymes.

The second explanation is metabolic 'removal' of the products of fumarase and aconitase action, possibly by reduction. On the other hand, no enzymes of this type have been found to occur in yeast cells in activities even approaching those of fumarase.

We hope to carry out electron-microscopic studies of granular structure to examine further the first theory.

The high malic, glutamic, succinic and *iso*citric dehydrogenase, fumarase and aconitase activities of these extracts again suggest participation of the Krebs's cycle in yeast respiration. However, until actively respiring, cell-free extracts can be prepared, the main mechanism of the carbohydrate and alcohol oxidation remains controversial (see Weinhouse & Millington, 1947; Martius & Lynen, 1950; Foulkes, 1951 on one hand; and Krebs, 1949; Krebs *et al.* 1952 on the other).

SUMMARY

1. Cell-free yeast extracts were prepared by 10-, 30- and 90-sec. high-speed mechanical disintegration, and residual intact cells removed by centrifugation at 1000 *g*. The extracts were then fractionated into particulate fraction *A* (sedimented at 3500 *g*), particulate fraction *B* (sedimented at 10 000 *g*) and non-sedimentable supernatant.

2. The following substrates, in decreasing order of activity, caused methylene-blue reduction in Thunberg tubes with whole unfractionated extracts: ethanol, L-malate, *iso*citrate, lactate, L-glutamate, citrate and succinate. Fumarase was about three times as active as aconitase.

3. Increasing periods of mechanical disintegration cause a progressive migration of all enzymes tested from particles to supernatant.

4. The enzyme content of both particulate fractions is so similar that they are probably not separate entities.

5. Malic and ethanol dehydrogenase activities are maximal with DPN, 'citric' and glutamic dehydrogenase activities with TPN. All four enzymes are activated to some extent by both coenzymes. The other enzymes require no added coenzymes.

6. The particles of 10-sec. extracts are rich in all enzymes examined and contain bound coenzymes which are not removed by repeated washing, but are lost by 24 hr. storage at 2° or by mechanical treatment. The particles of 30- and 90-sec. extracts contain less, if any, bound coenzymes.

7. Several sets of findings point to masked fumarase and aconitase activities in unfractionated extracts, and the masking is associated with the particulate fraction. Various possible explanations are discussed.

8. It is suggested that in the intact cell the microscopically visible granules, which some authors have called mitochondria on histological grounds, are active sites of enzyme activities concerned with respiration.

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