The Relationship between Pyridino-protein Dehydrogenases of Citric Acid Cycle and Electron Transport System※

By

Seishi KUWABARA

It is now generally recognized that the mitochondria of plant and animal cells catalyze the oxidation by molecular oxygen of all member of the citric acid cycle, of fatty acyl CoA's and β-hydroxyacyl CoA's and of other ancillary substances such as L-glutamate and L-proline. The system which emplements the machinery for energy production from oxidative process involving molecular oxygen is known as the terminal electron transfer system.[6]

During the five years Dr. GREEN's laboratory has been concentrating on the isolation and study of submitochondrial particulate units which contains the complete electron transport system for the aerobic oxidation of succinate and DPNH.[5] [6] [7] This unit of remarkably reproduciable composition has been called the electron transport particle or ETP which contains the classical heme chain. The electron transport particle does not carry out oxidative phosphorylation.

ZIEGLER[20] [21] extracted from the beef heart mitochondria a submitochondrial particle which catalyzed oxidative phosphorylation at the same efficiency as the starting mitochondrial suspension. Perhaps the most striking difference between this phosphorylating electron transport particle or PETP and ETP apart from the capacity for oxidative phosphorylation is that while ETP can not catalyze the oxidation of any member of citric acid cycle other than succinate ETP can catalyzed the oxidation of all member of citric acid cycle.

Tentative evidence suggests[16] [20] [21] that the dehydrogenases of the citric acid cycle are present in electron transport particle, but these enzymes are not capable of reacting with oxyzen. It suggests as working hypothesis that enzymes are arranged within the mitochondria as shown in Fig. 1.

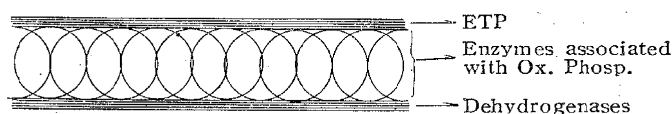


Fig. 1. Diagrammatic representation of morphology and enzymatic localization of sub-mitochondrial cristae in longitudinal section.

※ Contribution from the Shimonoseki College of Fisheries, No. 259 received May 2, 1958.
The following abbreviation will be used throughout this paper:
DPN, diphosphopridine nucleotide; DPNH, reduced DPN; TPN, triphosphopridine nucleotide; CoA, coenzyme A; CoASH, reduced CoA; ATP, adenosine triphosphate; GSH, reduced glutathione; BSA, bovine serum albumin; Tris, (*tris*) hydroxymethyl aminomethane; RNA, ribonucleic acid; RNA'ase, ribonuclease; DNA, deoxyribonucleic acid.

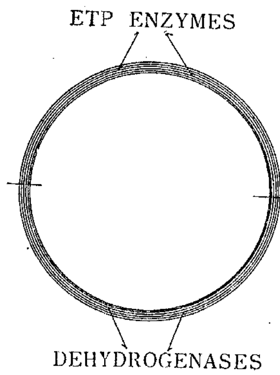


Fig. 2. Diagrammatic representation of electron transport particle (ETP)

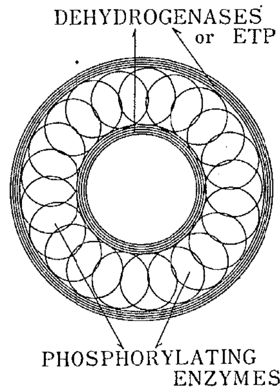


Fig. 3. Diagrammatic representation of phosphorylating electron transport particle (PETP).

Presentation of this structure (Fig. 2) is essential for closed system of oxidative phosphorylation added cofactor will not affect oxidative phosphorylation.

On the other hand, partly uncoupled system respond to added co-factor both for oxidation and phosphorylation. This system has undergone some structural change but it is postulated that co-factors are capable of recoupling the ETP-dehydrogenase-oxidative phosphorylating enzymes (Fig. 3).

The ETP and PETP are possible formed as shown in Figs. 4 and 5 respectively.

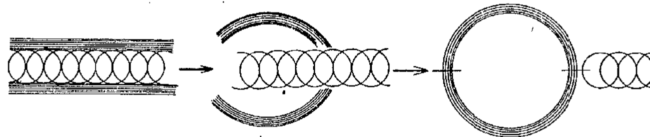


Fig. 4.

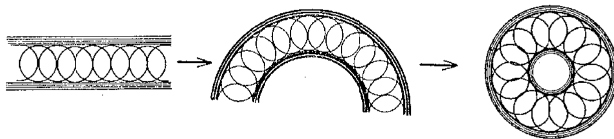


Fig. 5.

The present work is concerned the linkage by which the pyridino-protein enzymes are bound to the electron transport system in phosphorylating beef heart mitochondria. (BHM) BHM has been disintegrated by a variety of procedures to determine some of the factors governing the association of pyridino protein enzymes with the fragmented particle.

Materials and Methods

Preparation of ETP——The mitochondrial suspension was prepared in sucrose media essentially according to the standard procedure of Hogebon and Schneider. Such a suspension adjusted to pH 8.5 with 0.1 M KOH readjusted every 10min. to pH 8.5 for at least 45 min. at 15,000 rpm. Pour only red translucent material off top of tube (perhaps only a variable fraction of supernatant). Supernatant spin at 30,000 rpm for 30 to 50 min., washed twice with 0.25 M sucrose and resuspended in sucrose.

The whole problem of ETP has been to establish positively or otherwise that it is the repeating enzymic unit of which the BHM are compoesd. It has not been possible to find any real supporting evidence for this hypothesis prior to experiments using the Hughes press.

The yield of ETP can be made to vary very considerably depending upon the starting material but as no activity is created this does not lead support to repeating unit hypothesis, *e. g.*,

Unit	BHM	ETP	Residue	Soluble
(DPNH-cytochrom c activity, μmoles DPNH oxidized/min./mg. protein)	200	80	1.0	—
Specific Activity (μatomes O/min./mg. protein)	2.0	3.5	1.0	—

1st extract	(mg.)	100	23	70	—
Yield	(%)		20	70	10
2nd extract	(mg.)		20	40	—
〃 Unit			60	60	sol.
Specific Activity			3.0	1.3	—

A 3rd and 4th extractions may be necessary to completely extract the ETP but specific activity will be decreasing steadily and there will be no increase in units. Indeed if "light" BHM are repeatedly selected by centrifugation in sucrose, these "light" BHM can be converted to say 50% ETP on the first extraction and still more on 2nd and 3rd extractions. However there is still no creation of units and these mitochondria never give rise to PETP. The "heavy" BHM also selected by centrifugation yield only very small percent of ETP, say 5%.

Table 1. Effect of pH on ETP yield.

	Original			Residue				Supernatant			
	Vol. (ml.)	Protein (mg./ml.)	Total protein (mg.)	Vol. (ml.)	Protein (mg./ml.)	Total protein (mg.)	%	Vol. (ml.)	Protein (mg./ml.)	Total protein (mg.)	%
1#	27	20.6	556	11	22.6	349	45	22	13.8	304	55
2#	27	20.6	556	8.3	23.4	194	35	23	16.0	368	66
3#	27	20.8	582	6.7	25.0	167	28	25	18.1	453	77
				ETP				Soluble			
1#				—	18.9	—	—	50	1.49	75	13
2#				9.4	25.4	239	43	49	2.15	105	19
3#				9.9	24.2	240	41	46	2.0	98	17

1#—10 ml. BHM, 11 ml. 0.5 M sucrose, 9.1ml. KPO_4 0.5 M pH 6.1
2#—10 ml. BHM, 11 ml. 0.5 M sucrose, 9.0ml. KPO_4 0.5 M pH 7.1
3#—17 ml. BHM, 11 ml. 0.5 M sucrose, 9.0ml. KPO_4 0.5 M pH 8.1

Preparation of PETP— To make 30 ml. initial suspension, with 0.05 M $KHPO_4$, pH 7.0, 0.9 ml. and 95% ethanol 4.7 ml.

Coenzyme mixture 0.5 M versene 0.06 ml., 2.5 ml. CoASH 1mg./ml., 20 ml. 0.1 M DPN, 10 ml. 0.1 M GSH, 20 ml. 0.005 M cocarboxylase, 30 ml. H_2O, 0.2 ml. 0.1% TPN, 0.6 ml. 0.5 M pyruvate, 0.2 ml. 0.1 M ATP, 0.3 ml. $MgCl_2$.

Table 2. The yield of PETP.

Original			Residue				PETP			
Vol. (ml.)	Protein (mg./ml.)	Total protein (mg.)	Vol. (ml.)	Protein (mg./ml.)	Total protein (mg.)	%	Vol. (ml.)	Protein (mg./ml.)	Total protein (mg.)	%
120	27.2	3264	60	36.8	2808	68	20	17.1	342	11
			ETP				Supernatant			
			20	17.7	354	11	95	1.6	152	5

BHM 60 ml., 36 ml. KPO_4 (pH 7.8) 0.5 M, 18.8 ml. ethanol, 0.5 M. versene (pH 7.0), 1.2 ml. $MgCl_2$ 0.1 %, 1.2 ml. ATP 0.1 M, 10.0 ml. cofactor mixture (CoA 1mg./ml., 20 ml., DPN 0.1 M. 20 ml, GSH 0.1 M 10 ml, Cocarboxylase 0.005 M. 20 ml., H_2O 30 ml.), 0.8 ml. Pyruvate 0.5 M, 0.4 ml. Malate 0.5M, then make to 120ml. with sucrose.

Coenzyme mixture and BHM mixed to make *ca.* 16 ml. (BHM 1 : 1 in sucrose, protein *ca.* 60 mg./ml.), phosphate added, followed by alcohol precooled to −20°C. The whole is then homogenized at once at high speed and centrifuged at 15,000 rpm for 5 min. (*ca.* 4 min. to reach 15,000 rpm and about 4½min. to stop).

Pour off supernatant make to 30 ml. with 0.25 M sucrose spin at 30,000 rpm for 30 to 50 min. Pour off supernatant and discard. Slough off upper layer of sediment and transfer to another tube. Wash both sediments twice with 0.25M sucrose-10^{-3} M ATP, 10^{-3} M KPO_4, pH 7.1 (Table 2).

Preparation of ETP-PETP——This is distinct from alkaline ETP and phosphorylating PETP. This preparation phosphorylates poorly and differs from alkaline ETP in higher yield and contains the dehydrogenase of citric acid cycle. Yield of this material as for high phosphorylating PETP. Add BHM suspension, with 3.0ml 0.5M KPO_4, pH 7.4, 1.0ml 95% ethanol and 0.02ml. versene 0.5M, to marked 10ml. tube, transfer to homogenizer and homogenized at top speed. Spin in no. 40 Spinco at 17,000 rpm for 6 min., supernatant spin again at 40,000 rpm for 20 min. Wash quickly first sediment (residue) and second sediment (PETP-ETP) twice with 0.25 M sucrose and resuspended in same (Table 3).

Table 3. Some variation in earlier experiments on ETP-PETP.

1. Variation of pH.

pH	Residue (%)	ETP-PETP (%)	Supernatant (%)
7.0	72.5	22.6	12.3
7.8	61.0	28.0	12.1
8.5	50.0	23.3	12.5

3.2ml. 95% ethanol, 0.06ml. versene 0.5M, 60ml. 0.5M KPO_4 pH 7.0, 7.8, 8.5, BHM to 30ml. Mixture incubated at 0 for 30min. homogenized and fractionated.

2. Variation of alcohol content.

Alcohol (ml.)	Residue (%)	ETP-PETP (%)	Supernatant (%)
non	64.3	22.9	5.9
1.6	66.0	20.0	5.9
3.2	63.5	21.4	4.8
4.7	60.8	17.2	9.2
6.3	53.6	19.4	9.0

6.0ml. $KHPO_4$ 0.5M pH 7.8, 0.5M Sucrose 0.06 ml. BHM, variable alcohol, final volume 30.0ml.

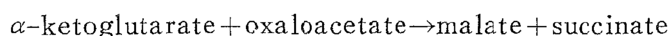
3. Variation of phosphate 95% 4.7ml., 0.5M versene 0.06 ml. BHM.

	Vol (ml.)	Residue (%)	PETP-ETP (%)	Supernatant (%)
tris 1.0M pH 7.8	15	90.0	1.6	6.3
KPO_4 0.5M pH 7.8	1.2	93.3	4.6	4.6
〃	6.0	71.8	24.0	8.1
〃	9.0	77.8	16.4	5.5
〃	12.0	66.9	15.2	5.5

Versene between 0.06ml. 0.05M and 0.3ml. 0.5M was without detectable effect. The inclusion of substrates(pyruvate, malate, α-ketoglutarate, as well as ATP, $MgCl_2$ does not effect the yield of ETP-PETP.

The only additive affecting the yield of ETP-PETP in the phosphate buffer, the pH, use of other buffer, inclusion of alcohol, substrate and cofactors do not significantly effect the yield. Ethanol is required only the preserve phosphorylating activity the substrates and cofactor assist in this process to varying degree; ethanol being the most important. Under the best conditions yield of ETP-PETP prior to Hughes press never exceeded 30% one single extraction and usually in range 10—25%. Sometimes however, only 5 % yield seem to depend upon starting material; one experiment yielding say 25%, other only 5 % by some precedure varying only in the starting BHM.

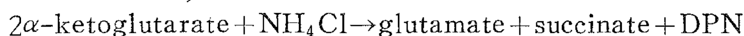
Estimation of Pyridino-protein Enzymes and Associated DPN Enzymes—— Some periods have been spent finding the most suitable methods for assaying the dehydrogenase present in BHM and various fractions isolated therefrom. 1) malic dehydrogenase:- DPN+oxaloacetate 2) isocitric dehydrogenase:- reduction of TPN[5)10)] 3) malic dehydrogenase:- reduction of TPN 4) β-hydrobutyric dehydrogenase:- reduction of DPN 5) α-ketoglutaric dehydrogenase:- anaerobic measurement of CO_2 evolution. Reaction carried out in $NaHCO_3$-$K_3Fe(CN)_6$ under atmosphere of N_2-CO_2 6) pyruvic dehydrogenase:- as yet this assay is unreliable. Using beef heart mitochondria the $Fe(CN)_6'''-HCO_3'$ method can be used with BHM with pyruvate alone or pyruvate+oxaloacetate as substrate, the rates being 0.012 μatomes CO_2/min./mg. protein and 0.030 μatomes CO_2/min./mg. protein (this latter corrected for oxaloacetate alone activity). 7) α-ketoglutaric dehydrogenase[9)]:- each manometer flask $K_3Fe(CN)_6$ 50 μmoles, $NaHCO_4$ 40 μmoles, $MgCl_2$ 20 μmoles, BSA, 10% 0.2ml., α-ketoglutarate 20 μmoles, BHM, water to 3.2ml. Rate of CO_2 evolution measured for 10min. late linear and proportional to added BHM, 5.4mg. and 10.8mg. respectively and 0.030 μmoles CO_2 per min. per mg. protein. The effect of varying concentrations of DPN (none added and 0.1ml. 0.01M DPN), $K_3Fe(CN)_6$ (10—50 μmoles), $NaHCO_3$ (20—40 μmoles) were all examined without any significant difference being observed, same was true for added cocarboxylase. The BHM were oxidized by incubated at 38°C for 13min. likewise without great change in the α-ketoglutaric dehydrogenase activity observed value 0.021. On attempted dismutation measurement,



α-ketoglutarate+oxaloacetate→malate+succinate

could not be measured anaerobically in $NaHCO_3$ buffer-N_2-CO_2 in the absence of $K_3Fe(CN)_6$.

Likewise,



2α-ketoglutarate+NH_4Cl→glutamate+succinate+DPN

in $NaHCO_3$ and absence of $K_3Fe(CN)_6$ was not detectable by this procedure. Acetone powder of BHM assayed zero for α-ketoglutarate and pyruvate using $NaHCO_3$-$K_3Fe(CN)_6$. On the other hand BHM in sucrose and washed with distilled water still assay some value for α-ketoglutarate. 8) pyruvic dehydrogenase in BHM:- each manometer contained $HaHCO_3$ 40 μmoles, DPN 0.1 μmole, $MgCl_2$ 20 μmoles, cocarboxylase 0.005 moles 0.1ml., 10% BSA 0.2ml., $K_3Fe(CN)_6$ 10 μmoles, pyruvate 20 μmoles, oxaloacetate 10 μmoles, water to 3.2ml. Under this assay conditions acetone dried BHM no activity towards pyruvate (0.012 μatomes CO_2/min./mg. protein) but an oxaloacetate decarboxylase was evidence in these preparations(0.03). α-kotoglutarate and the glutamate anaerobically oxidyzed by acetone dried BHM, the α-ketoglutarate

assayed glutamate 0.017. In combination of pyruvate+malate or be assayed to oxygen but not pyruvate+oxaloacetate. This small value (0.010) observed with fluffy ETP isolated during the preparation of ETP is attribute to contamination with PETP.

Extraction of RNA— An equal volume of cold 20% TCA(trichloroacetic acid) is added to nucleoprotein suspension in a graduated conical centrifuge tube and mixture is left in an ice bath for 15 to 30min. before centrifuging. The residue after centrifuging is washed 2 times with 10 volumes of cold 10% TCA, in order to remove the acid soluble nucleotides and sucrose. The washed TCA residue is suspended in water (about 1ml. per 20mg. protein of original sample) and mixed with four volumes of 95% ethanol at room temperature and centrifuged; the residue is washed 2 times with (3 : 1) ethanol-ether to extract the phospholipide which are discarded. The washed nucleoprotein residue is suspended in 5% TCA (approx. 1 ml. TCA per 25 mg. of protein) and heated for 15 min. at 90°C. and centrifuge. The clear supernatant is transferred to graduated conical centrifuge tube. The residue is washed once with 5% TCA (room tepmerature) and wash combined with hot extract to form the nucleic acid fraction. The washed TCA preparate constitutes the protein fraction and is reserved for the protein determination.

Determination of RNA-ribose——Tthe nucleic acid ribose in the extract is determined by the method of Mijbam. An aliquot of the extract is added to 1.5 ml. of the orcinal reagent and volume adjusted to 3.0ml. with 5% TCA. The color is developed by heating the tube in the boiling water bath for exactly 20min. The tubes are immediately cooled in an ice bath and read against a reagent blank at 666mμ. in the Beckman DU. The ribose concentration is calculated by referring the reading to a standard curve constructed from values obtained with crystalline arabinose. 1) **Orcinal reagent:-** a stock solution of 0.1% $FeCl_3 . 6H_2O$ in conc. HCl is prepared and stored in a tightly sealed glass stoppered bottle. The orcinal reagent is prepared by dissolving 100mg. of orcinal in 100ml. of the stock solution. 2) **Construction of the standard curve:-** because of its purity and availability arabinose is the prepared pentose standard 100mg. of crystalline arabinose are dissolved in 100ml. of glass distilled water. Since the color developed with the orcinal reagent is proportional to the amount of pentose between 1 and 20μg. and aliquot of the arabinose solution is diluted with H_2O and conc. TCA to give a fianl working standard containing 200μg. of arabinose per ml. in 5% TCA. 3) **Determination of protein:-** the protein fraction is suspended in 0.5 to 1.0ml. of H_2O and then dissolved by slowly adding 0.1 N KOH with constant stirring. The final volume of solution is noted and the protein determined by biuret method.

Results and Discussion

I. Fragmentation of Beef Heart Mitochondria

The Effect of DPN on Oxidative Phosphorylation by PETP and Residue—
Phosphorylating activity of mitochondria frozen and aged one day were assayed as the starting material (Table 4). Above mitochondria fractionated into PETP and residue respectively. Following alcohol PETP fraction recovered 3 fractions, composed of 10% fluffy layer, 20.7% PETP and 69.3% residue. The fluffy layer was essentially electron transport particle (ETP) and was discarded, the other fractions were assayed (Table 5).

Table 4. Oxidative Phosphorylation by intact mitochondria.

Substrate	△P μmole	△O μmole	P/O
Pyruvate-Malate	26.5	11.7	2.41
Succinate	27.0	15.7	0.81

Each flask contains 10mg. intact mitochondria, 100 μmoles glucose, 5 μmoles $MgCl_2$, 0.5 mg. yeast hexokinase, 5 μmoles ATP, 30 μmoles phosphate, pH 7.1 and where indicated 20 μmoles Na-pyruvate, 5 μmoles K-malate, 50 μmoles K-succinate, 0.25 M sucrose to a fianl volume of 3.2 ml. Incubated 30min. O_2 uptake measured by standard Warburg technique. Phosphate determined by method of Fisk and Subbarow.

The residue responds aerobically to DPN and the oxidation of DPNH is linked to phosphorylation, the observed P/O ratio in absence being 1.28 and in presence of DPN 1.73 μmoles P/min./mg. protein being 0.27 and 0.77 μatome O/min./mg. protein being 0.26 and 0.47 respectively.

Table 5. Effect of DPN on PETP and residue material.

Fraction	Substrate	DPN (1μmole)	P μmoles	O μmoles	P/O
Residue	Pyruvare-Malate	—	4.6	3.6	1.28
//	//	+	12.6	7.0	1.73
PETP	//	—	13.2	7.49	1.76
//	//	+	13.4	8.38	1.60

Conditions as in Table 4.

PETP was not greatly affected by added DPN but the oxidation or DPNH which did not couple to phosphorylation. The observed P/O ratio being 1.73, 1.76 in absence of DPN and 1.52, 1.60 in addition of DPN.

The Effect of an Alkaline Extract of Mitochondria on Phosphorylation dy PETP—— Mitochondria are washed suspended in 0.25M sucrose and pH adjusted to 11.0. The mixture is centrifuged at 40,000 rpm for 30 min., the sediment discarded; the supernatant constitutes the "alkaline extract". The effect of alkaline extract on

Table 6. The effect of an alkaline extract of mitochondria on phosphorylation by PETP.

Substrate	Alkaline Extract	△P μmoles	△O μatomes	P/O	μatomes O/min./mg. protein
Malate-Pyruvate	—	9.3	6.9	1.35	0.020
//	+	8.2	9.8	0.91	0.207

Each manometer contains 9.1mg. PETP and where indicated alkaline extract (0.8ml). Other conditions as in Table 4.

phosphorylation by PETP was decreased by added alkaline extract and increase in the rate of oxidation significantly (Table 6).

The Effect of Speed of Homogenization on Mitochondrial Activity—— Reostat setting on large blender 70. Heart tissue homogenized for 60 and 120 sec. Debris removed by centrifugation on International centrifuger with reostat setting 17, for

10 min. Mitochondria collected at 15,000 rmp for 15 min., washed once at 30,000 rpm for 5 min. The 60 sec. mitochondrial fraction appreciable contamination with muscle fragments which would probably by readily separated, by a large period of centrifuged at slow speed. The low concentration of protein in the manometers probably does not reflect the maximum activity of the preparation (Table 7). Additional protein in the form of serum albumin is probably desirable.

Table 7. Effect of speed of homogenization on mitochondrial activity.

Fraction	ΔP μmoles	ΔO μatomes	P/O
Mitochondria (60sec.)	11.8	5.1	2.31
Mitochondria (120sec.)	23.0	9.2	2.50

Substrate—Pyruvate-Malate.

Reostat set on 70 (½ total scale) and the whole mixture (30 ml. BHM, suspended in 0.02ml. versene 0.5M, 2.5ml. pyruvate 0.1M, 0.1ml. malate 0.5M, 3.0ml. GSH 0.1M, 0.1ml. $MgCl_2$ 0.1M, 43.5ml. sucrose 1.0M plus 23.3ml H_2O) blended continuously for 4min. with aliquots being with down at 10, 30, 40, 60, 90, 120, 150, 180, and 240 sec. Temperature was 0 to 4°C. Frothing was reduced (eliminated) by placing a cover over the surface of the liquid. The blended samples (240sec.) was centrifuged at 15,000 rpm for 15min. The sediment appeared to be unchanged compared with

Table 8. Effect of low speed blending on mitochondria.

Blending time (sec.)	0	10	30	40	60	90	120	150	180	240
O	9.3	8.2	8.8	7.7	9.3	9.0	8.9	8.2	6.8	7.1
P/O	2.31	3.01	2.93	3.36	2.74	2.83	2.83	2.86	3.60	2.76

original with little or no protein solubilization (Table 8).

BHM suspended in mixture blended at maximum speed for 240 sec. aliquot with down at given intervals, frothing was eliminated as previous experiment, temperature was 0 to 10°C. Then effect of high speed blending on BHM were estimated (Table 9). The decrease in the rate of oxidation and phosphorylation decreasing some what

Table 9. Effect of high speed bledning on mitochondria.

Blending time (sec.)	0	10	30	60	120	180	240
Phosphorylation (μmoles P/min./mg.)	0.234	0.222	0.205	0.188	0.115	0.142	0.117
Oxidation (μatomes O/min./mg.)	2.69	2.85	2.77	2.86	2.50	2.44	2.49

was repeatedly than that for oxidation. Three hours after the above experiments, the 240 sec. blended material was reassayed with cofactor mixture 0.5 ml. (contain DPN, cocarboxylase, CoASH, and GSH) to determine whether the reduced rate of oxidation with this sample is reflection of inactivation or depletion of co-factor (Table 10). The blending treatment has the effect of depleting the BHM of cofactor and also probably

uncoupling the phosphorylation. Oxidation induced by added cofactor is not coupled to phosphorylation.

Table 10. Effect of cofactor on blended mitochondria.

Substrate	Cofactor	P/O	Phosphorylation (μmoles P/min./mg.)	Oxidation (μatomes O/min./mg.)
Pyruvate-Malate	−	1.42	0.132	0.096
〃	+	2.45	0.117	0.048

Cofactor mixture—1μmole DPN, 0.5μmole cocarboxylase, 0.1 μmole CoASH.

Fragmentation of BHM in a Hughes press——BHM centrifuged and resuspended to make a concentrated paste in a mixture composed of-(20ml. M sucrose, 0.01ml. of 0.5M versene, 1.25ml. 0.1M pyruvate, 0.05ml 0.5M malate, 1.4ml. 0.1M GSH, 0.05ml. 0. 1M $MgCl_2$, water to 50ml.). The mitochondrial paste (about 6 — 8 ml.) was placed in a Hughes press precooled about −10 to −15°C for 2 to 5 min. then pressed. The crushed BHM were collected and resuspended in the above mixture. The procedure was repeated on a second sample of BHM only this time the Hughes press was precooled to −30 to −35°C. These fractions were then assayed for oxidative phosphorylation of the original BHM, the −10 to −15°C fraction and hard freeze fraction (Table 11).

Table 11. Effect of cofactor on fragmentation of beef heart mitochondria in a Hughes press.

Fraction	Cofactor	Phosphorylation (μmoles P/min./mg. protein)	Oxidation (μatomes O/min./mg. protein)
BHM	−	0.180	0.075
〃	+	0.175	0.197
Soft Freeze (−10°to−15°C)	−	0	0
〃	+	0	0.057
Hard Freeze(−30°to−35°C)	−	0	0
〃	+	0	0.014

The oxidative activity of the frozen samples[18] is reduced to zero except in the presence of cofactor supplements (DPN, cocarboxylase, GSH, CoA). Phosphorylative activity was completely destroyed by this precedure. In both instances(−10 and−30°C) the Hughes press completely disintegrated the BHM. The normal BHM and both pressed samples were then subjected to alkaline phosphate-alcohol-versene fraction. Residue and PETP-ETP fractions were isolated from all three samples 1# control mitochondria, 2# soft frozen (−10 to −15°) 3# hard frozen (−30 to −35°)(Table 12).

The residue of 1#, and 2# were re-extracted to yield a further 21.8% ETP for 1# and 24.8% ETP for 2#. Thus total extraction of 2# ETP can be made to approach 80% (74.8%). It was previously always being possible to extract further material from residue but never with increase in units. All of the cytochrom c independent activity has been extractable over a series of re-extraction but never with increased (increasing activity).

Total Extracted Activity——The residues from 1# and 2# above were re-extracted and re-assayed. The results of both assays were pooled and put on a % basis for directc omparision (Table 13). The unit created by the Hughes press exceeds 100%.

Table 12. Oxidative activity of fragments of beef heart mitochondria in Hughes press.

Fraction	Protein (mg.)	Unit (%)		Absolute Unit (%)	Specific Activity	
		no cytochrom c	cytochrom c added		no cyt. c	cyt. c added
1# BHM	158	182	109	291	1.15	0.69
1# ETP-PETP	40.2	135	6	141	3.20	0.15
1# Residue	120	70.8	73	285	0.59	0.61
2# BHM	132	185	62	247	1.4	0.47
2# ETP-PETP	70.2	273	0	273	3.85	0
2# Residue	49	38.8	47	86	0.79	0.96
3# BHM	182	56.5	187	241	0.31	1.10
3# ETP-PETP	48	118	43.7	133	2.45	0.31
3# Residue	104	43.7	183	182	0.42	1.33

1#, control mitochondria; 2#, soft freeze (−10 to 15°C); 3#, hard freeze (−30 to −35°C). All units expressed as μmoles DPNH or succinate oxidized/min./mg. protein at 37.5°C.

Table 13. Total extracted activity in a Hughes pressed and normal materials.

Fraction	Total Protein (mg.)	Units of Activity(%)	
		DPNH Oxidase no cytochrom c	cytochrom c added
Normal BHM	100	115	69
Pressed BHM	100	115	69
Normal Residue	76	45	46
Pressed Residue	23	12	12
Normal ETP-PETP	26	21	6
Pressed ETP-PETP	26	124	0

There is put on insignificant increase in unit by methods not utilizing the Hughes press. Previously fractionation of BHM in Hughes press (cf. Table 11 and 12) has probably carried out at too low a temperature to permit several of appreciable activity.

Table 14. Effect on cofactors on fragmentation of beef heart mitochondria in Hughes press.

Fraction	Co-factor	ΔP (μmoles)	ΔO (μatomes)	P/O
Normal BHM	−	16.1	8.36	2.12
〃	+	17.1	7.61	2.03
−5°C Pressed BHM	−	7.75	5.51	1.50
〃	+	4.1	5.67	1.08
−3°C Pressed BHM	−	0	0	0
〃	+	4.1	7.61	0.72

Attempted this experiment (Table 14) to rupture mitochondria at − 3 and − 5°C. Sedimented BHM resupended in sample mixture to make a paste. Paste added to Hughes press at −3°C and allowed to stand 5 min., then pressed. Precedure repeated at −5°C except material not allowed to stand but pressed after about 30sec.

The Hughes press procedure in these instances is much milder, oxidative activity

can be fully restored in one of the instances upon the addition of cofactor while in the later the restoration is a about 70% of maximum in the presence of cofactors. Phosphorylating activity is presented to some extent. Althogh the P/O ratio of the -5° sample goes down on the addition of cofactor thers is nevertheless some increased rate of phosphorylation. The sample frozen at -3°C had considerably less activity than that frozen at -5°C; this is probably attributable to depth of the treatment which will need to be standerized. Two factors will be important the temperature and the period to equilibrate before pressing which will determine how far the freeze is. Another important factor is that cooling of the Hughes block varies considerably from one point to another. An acetone dry ice bath set at any given temperature would be a standard temperature throughout the block. It follows that the -3° and -5°C setting recorded in this experiment mean very little in time of the temperature of the block as whole, these temperatures refer only the temperature of hole in the block.

Normal BHM and -5°C sample were carried through alcohol-phosphate fraction (KPO_4 0.5M, pH 8.7, 3.0ml., 95% ethanol 1.6ml., 0.5M versene 0.02ml.)samples added to 10.0 ml. Both homogenized and centrifuged at 40,000 rpm for 20 min., sediment retained and washed twice with 0.25 M sucrose, as was the first residue. This precedure yielded 4 fractions and 2 residues, one each from the BHM and -5° Hughes press samples and ETP-PETP samples, again one each from the BHM and the Hughes press samples (Table 15). The percentage gain in units obtained with the Hughes press is not rearly so high as in the previous experiment (cf. Table 14).

Table 15. Recovery of DPNH oxidase of beef heart mitochondrial fractions in Hughes press at -5°C.

Fraction	Total Protein (mg.)	Units of Activity(%)		Total Units recovered (%)
		no cytochrom c	cytochrom c added	
Normal BHM	174.0	146	96	243
ETP	34.7	132	0	132
Residue	113.0	66	72	132
Pressed BHM	169.0	192	32	226
Pressed ETP	85.3	205	0	205
Pressed Residue	76.8	54	38	92

This may be attributable to two courses; high temperature used at Table 12 compared with Table 14, also the latter was pressed in form of sluggishly flowing liquid whereas the former was free flowing being more dilute.

In previous exteriments with the Hughes press the temperature at which the material was pressed was only approximately controlled. In these experiments the metal block was placed in a acetone dry ice bath hold at -5 and -11°C. After the block had equilibrated with the bath, the samples of BHM were placed in the block left for 5 min. to equilibrate and then pressed (Table 16). The original BHM showing ratios of only 1.77 (unsupplement) with a rate doubling on supplementation and P/O of only 1.52 indicated that the original BHM possesed only both lots of pressed material showed significant phosphorylation. It will be of interest to press the BHM in the presence of alcohol which has been shown to preserve the phosphorylative activity of PETP.

Table 16. Oxidative phosphorylation by Hughes pressed beef heart mitochondria.

Fraction	Cofactor	△P µmoles	△O µatomes	P/O	Oxidation µatomes O/min./mg. protein
BHM(normal)	−	12.0	7.26	1.52	0.152
〃	+	14.1	7.98	1.77	0.091
BHM(pressed −5°C)	−	4.2	8.07	0.52	0.071
〃	+	0	0	0	0
BHM(pressed −11°C)	−	5.1	7.11	0.72	0.058
〃	+	0	0	0	0

Effect of Blending BHM with Ballotive Beads— BHM were suspended in 2 volumes of (M sucrose 80ml., 0.1 M pyruvate 3.0ml., 0.5M malate 0.5ml., 0.1 M $MgCl_2$ 0.15ml., GSH 3.0ml., 0.5 M versene 0.4ml., 0.01 M DPN 10ml., 0.1 M ATP 1.0ml., water to 60ml.). Two lots of 75ml. suspended BHM plus 14ml. ballotive beads were blended for 20sec. and 60 sec. at top speed in a waring blender with a cover so adjusted as to permit no frothing. Temperature rose only 0 to 4°C. The two blended samples 2# (20sec.), 3# (60sec.) and a normal suspension of BHM 1# were centrifuged three at 10,000 rpm for 10min. in Spinco no. 30 head. The samples were homogenized in 0.25M sucrose and centrifuged at 10,000 rpm for 10min. again without detactable separation. The phosphorylations of 1#, 2# and 3# were assayed (Table 17).

Table 17. Effect of blending BHM with ballotive beads on oxidative phosphorylation.

Preparation	P/O	Oxidation (µatomes O/min./mg. protein)	Phosphorylation (µmoles P/min./mg. protein)
1#	1.88	0.103	0.187
2#	1.666	0.132	0.219
3#	1.777	0.081	0.142

1#, Normal suspension of BHM; 2#, 20sec. blending BHM; 3#, 30sec blending BHM.

The blending procedure decreased in the rate of reactive in 3# but not significantly in 2#, It is noteworthy that the observed P/O values are not significantly altered. The three samples were then fractionated with phosphate-alcohol into, residue and PETP-ETP without any gross change in pattern being observed; although some significant increase may have been observed in the yield of ETP *e. i.*, 19.0→24.5→27.8%.

Effect of Blenidng BHM in Phosphate on the Yield of PETP-ETP—— BHM (30ml.) mixtured with PETP fractionation coenzyme mixture plus desoxycholate to 0.03% and 30ml. $KHPO_4$, pH 7.0 final volume made up to 100ml. Mixture blended for 3 min. and then fractionated without any added alcohol.

Blending Time (min.)	Residue (%)	ETP-PETP (%)	Supernatant (%)
0	72	14.2	11.7
3	42	38	16

Combination of phosphate and blending yield increased amount of PETP-ETP assay of activity (DPNH, no cytochrom c) show some creation of units but not as great as with Hughes. Analysis of all fractions not completed, as a rough estimation of activity the following values were obtained:-

	ETP specific Activity (μatomes O/min./mg. protein)	Total Unit (mg. %)
BHM (normal)	2.9	41
BHM (blended in KPO_4)	3.42	130

Effect of Hughes Pressed BHM in Sucrose-Phosphate on the Yield of ETP-PETP—— BHM were pressed as paste at $-12°C$. The block was equivalent to $-12°C$ in a alcohol-dry ice bath, the paste added and left for 3 min., the material was then pressed. Initially one lot of BHM was washed with 0.25M sucrose 0.15M KPO_4, pH 7.8 and the sediment obtained in the centrifuge tube was transferred directly to the Hughes press. A second lot of BHM was washed on the centrifuge with sucrose-cofactors (as given in page 17) the sediment was pressed, following fractions have been obtained:- 1#, control BHM; 2#, BHM pressed in sucrose-cofactor; 3# BHM pressed in sucrose-phosphate, pH 7.8.

Table 18. The protein values of BHM pressed in sucrose-cofactor and in sucrose-phosphate.

Fraction	Original			Residure				ETP				Soluble			
	Vol. (mg.)	Protein mg./ml.	Total Protein (mg.)	Vol. (ml.)	Protein mg./ml.	Total Protein (mg.)	%	Vol. (mg.)	Protein mg./ml.	Total Protein (mg.)	%	Vol. (ml.)	Protein mg./ml.	Total Protein (mg.)	%
1#	30	16.8	504	9.3	24.3	230	46	11	12.6	142	28.2	55	1.54	85	17.9
2#	30	22.3	669	10.0	15.3	153	23	11.5	25.3	291	45.3	49	2.72	134	20.0
3#	30	23.3	696	6.3	15.8	100	14.4	15	23.7	356	51.2	50	3.0	150	21.6

1#, control BHM; 2#, BHM pressed in sucrose-cofactor, 3# BHM pressed in sucrose-phosphate pH 7.8.

The above protein values (Table 18) in the supernatant were obtained by precipitating the protein in 10ml. supernatant with TCA (5 %) redissolving the precipitate in KOH-DOCA and assaying using the buiret. The under-lined values were obtained by a direct assay on 0.3ml. of supernatant. These values are probably high due to the conglomeration of compounds in the supernatant.

Succinoxidase Activity of Hughes Pressed BHM —— It was not possible to obtain a steady rate for succinate oxidation in the original mitochondria, so that A and B in Table (19) refer to observed rate of succinate oxidation (specific activity) between 0 — 5 min. and 5—10 min. *i.e.*, caluculated from the 5 min. and 10 min. recordings respectively. This is not very satisfactory[10] but it is at present the only method at our disposal. In several different assay at 30 and 38°C the mitochondria did not show a steady rate of succinate oxidation. The difference in specific activity between the original for 1 — 2# and 3# in Table 19 is accountable for in terms of the phosphate present. 1 # — 2 # are same material — just mitochondria suspended in sucrose whereas 3# is mitochondria suspended originally between pressing in sucrose- 0.15M KPO_4, pH 7.8.

The salient point appears to be that phosphate brings about extensive changes in the material irrespective of the Hughes press. The phosphate appears to contribute considerably to the destruction of the mitochondria. The Hughes press alone leads to the production of 44% ETP which in the succinoxidase assay responds to the extract of 43% in the presence of added cytochrome c. The Hughes press plus mitochondria in sucrose-phosphate led to 51% ETP which responded to the extract of 28 % in

Table 19. Succinoxidase activity of BHM pressed in sucrose-cofactor and in sucrose-phosphate (pH 7.8).

Fraction	1# no cyt. c	1# cyt. c	2# no cyt. c	2# cyt. c	3# no cyt. c	3# cyt. c
Residue						
Specific Activity	0.18	0.69	0.14	0.88	0.05	0.68
Total Unit	41.4	158.7	21.4	134.6	5.0	68
ETP						
Specific Activity	1.60	2.20	1.27	2.21	0.72	2.00
Total Unit	227.2	312.4	369.6	643.1	356.3	712
Initial Unit in Original A	317.5	534.2	431.5	695.8	459.4	548.8
Specific Activity	0.63	1.04	0.63	1.04	0.66	0.79
Change in Unit	−48.9	−53.1	−30.5	−81.9	−198.1	−230.2
Total Unit Change	−102		−50.4		−32.1	
Initial Unit in Original B	221	345	281	445	341	313
Specific Activity	0.42	0.68	0.42	0.68	0.49	0.45
Change in Unit	−57	−138	−110	−322	−80	−467
Total Unit Change	−195		−432		−384	

Assay Procedure:— Manometer contains 0.2M $KHPO_4$ pH 7.1 0.2ml., 0.5M succinate 0.2ml. (in side arm) 6 N KOH 0.2ml., 1% cytochrom c 0.1ml (where added) enzyme (0.7−1.5mg. protein) +about 0.9−1.4mg. for residue and 0.7−1.0mg. for ETP water to 3.2 ml. Manometer gased with O_2 for 3−5min. after temperature equilibration (5 min.) the succinate is tipped and the zero time reading is taken 2min. thereafter. The rate is determined at 5min. interval experiments are repeated to a suitable dilute. Specific activity was expressed as μatomes O/min./mg. protein.

presence of added cytochrom c. The normal ETP yield was 28% with a 21% response to added cytochrom c.

These results highlight the effect of added phosphate. The increased yield of ETP from the phosphate-Hughes procedure is only an apparent increase, in so far as the activity is less than from the Hughes press alone on a weight from weight basis.

II. On the Relationship between the ETP Enzymes and Associated DPN Enzymes

Oxidative Capacity of Residue and ETP —— As a first step in the study of component of dehydrogenase it was of interest to localize the some of the enzymes of citric acid cycle in supernatant, residue, and electron transport particle.

Table 20. Oxidative capacity of ETP and residue.

Fraction	Protein (mg./ml.)	Units of Activity DPNH Oxidase		Succinoxidase	
		no cyt. c	cyt. c added	no cyt. c	cyt. c added
ETP	25.8	3.34	3.94	0.88	1.67
Residue	38.4	0.52	1.49	0.34	0.95
Fluffy Residue	18.1	1.21	1.81	0.54	1.02

All units expressed as μmoles DPNH or succinate oxidized per minute per mg. protein at 38.5°C.

Washed beef heart mitochondria through the Hughes press in the presence of cofactors. The pressed material without dilution was centrifuged at 40,000 rpm for 30min., the supernatant was collected. The sediment was then twice washed at 30,000 rpm with sucrose and then resuspended in sucrose. The suspension was split at 15,000 rpm for 5 min., ETP supernatant reserved, as was loosely packed sediment from the normal type sediment. All three fractions were then collected separately by centrifugation. The most important catalystic constants for ETP and residue are summarized in Table 20. The rate of oxidation of pyruvate-malate is proportional to the concentration of supernatant (Table 21).

Table 21. Effect of concentration of supernatant on oxidation of pyruvate-malate by ETP.

Fraction	Supernatant added (ml.)	Oxidation (μatomes O/min./mg. protein)
ETP	—	0.010
〃	0.02	0.012
〃	0.10	0.036
〃	1.00	0.060
Supernatant		0.130

Each flask contains 20μmoles Na pyruvate, 5μmoles K malate, 100μmoles glucose, 5μmoles $MgCl_2$, 0.5mg. yeast hexokinase, 5μmoles ATP, 30μmoles phosphate, pH 7.1, 10% BSA, 1μmoles DPN, 0.5μmoles cocarboxylase, 0.1μmoles CoASH, and where indicated ETP 10mg, supernatant 10mg., 0.25moles sucrose to a final volume of 3.2ml. Incubated 37.5°C. O_2 uptake measured by standard Warburg technique.

Influence of Supernatant on the Rate of Oxidation of Fumalate, Citrate, and Isocitrate—— In this particular case (Table 22) the supernatant requirement for isocitrate can be replaced by TPN[2] (cf. Table 23). Hence isocitric dehydrogenase must

Table 22. Effect of concentration of Ist supernatant on oxidation of pyruvate-malately ETP.

ETP (mg.)	Supernatant (ml.)	μatomes O/10min.
10	—	1.0
〃	0.5 (boild)	1.2
〃	0.02	1.6
〃	0.1	3.6
〃	0.4	6.5
—	1.0	13.0

Manometer contains as in Table 21 (Protein content in supernatant is 10.7mg./ml).

be present in high concentration in the ETP. It must be bore in mind this ETP was prepared wholly by different centrifugation with the use of the alkalic, phosphate or alcohol-phosphate procedures. The observation in the case of isocitrate does not, however, suggest any explanation for the results obtained with malate-pyruvate, other than possibility that supernatant contains condensing enzyme, malic or pyruvic dehydrogenase.

Further Observation on the Effect of Supernatant Fraction on Oxidative Capacity of ETP—— A quantity of ETP was prepared from BHM by the phosphate. The isolated ETP was twice washed with sucrose and store. The first supernatant

Table 23. Influence of supernatant on oxidation of fumalate, citrate, isocitrate and pyruvate-malate by ETP.

Fraction	Supernatant	TPN	Substrate	Oxidation (μatomes O/10min.)
ETP	−	−	Pyruvate-Malate	0.9
//	+	−	//	8.8
//	−	−	Citrate	0.1
//	+	−	//	9.8
//	−	−	Isocitrate	0.3
//	+	−	//	7.3
//	+	+	//	5.6

alone the sedimented ETP was kept and one fraction (200ml.) was saturated with $(NH_4)_2SO_4$, stirred for one hour, centrifuged, precipitated, suspended in water and dialyzed for 40 hours against distilled water. A second fraction of supernatant was adjusted to pH 7.2 and stored. Another source of supernatant was formed some BHM through the Hughes press. Thus there were four fractions:- ETP (28.4mg. protein/ml.), supernatant from Hughes press (3.02mg. protein/ml.), (1) phosphate containing supernatant from ETP (3.06mg. protein/ml.) and anmonium sulphate precipitated from supernatant above (1). Both supernatants were effective in stimulation for pyruvate-malate oxidation and the rate of oxidation independent strictly upon the concentration of added supernatant (cf. Table 22).

Table 24. Influence of supernatant on oxidation by ETP prepared by phosphate procedure.

Fraction	Supernatant		TPN	Substrate	Oxidation (μatomes O/14 min.)
	Hughes press	Phosphate containing			
ETP	+	−	−	Pyruvate-Malate	13.0
//	−	−	−	//	3.8
//	−	+	−	//	5.0
//	−	+	−	//	8.1*
//	−	−	−	Citrate	0.1
//	−	−	+	//	0.1
//	+	−	+	//	15.1
//	+	−	+	//	13.1
//	−	−	−	Isocitrate	1.6

Each manometer flask contains 6.4mg. ETP, and where indicated 1.0mg. Hughes press supernatant, 0.5mg. phosphate containing supernatat. Another assay conditions as in Table 21.
* Contained 1.0mg. phosphate containing supernatant.

This preparation of ETP unlike the previous one prepared from the Hughes press contains no isocitric dehydrogenase. This may be an important observation in that, the Hughes pressed ETP (no alcohol, no phosphate, no alkalic) contains isocitric dehydrogenase, the phosphate (0.15 M) ETP contains no isocitric, PETP (alcohol-phosphate-cofactor) contains isocitric dehydrogenase—what of ETP? This latter preparation of ETP I would predict to contain isocitric dehydrogenase.

At present these observations are not explicable in simple terms. The ETP

contains high concentration of malic dehydrogenase so that unless the supernatant is supplying pyruvic dehydrogenase which seems unlikely, at this juncture the function of the supernatant is obscure.

The phosphate supernatant which had been precipitated on previous experiment by full saturation with $(NH_4)_2SO_4$, the precipitated collected and dialyzed for 40 hours against water assayed. This supernatant contained only a small amount of activity, most of the activity has been lost during the handling. Likewise dialyzed phosphate supernatant (from standard ETP preparation) was indicated.

A supernatant prepared by homogenizing BHM in the Virtis supernatant was dialyzed over night against distilled water. Dialysis againit distilled water results in a distinct reduction in activity but dialyzed material was still highly active. (Table 25).

This minimum blank oxygen would be 2.9 μatomes O/30 min. for all of the above as ETP alone is capable of such an oxygen uptake. This precipitated dialyzed supernatant activity appears to higher than was suspected on previous experiment (Table 25) with which supernatant prepared by homogenizing BHM in the Vrtis blender.

Table 25. Effect of dialyzed supernatant on oxidation of pyruvate-malately ETP.

Conditions	Oxidation (μatomes O/30min.)
ETP (4.26mg.)	2.9
〃 + 1.0ml. Virtis Super.	29.9
〃 + 0.6ml. 〃	20.6
〃 + 1.2ml. Dialyzed Super.	10.7
〃 + 0.9ml. 〃	8.6
〃 + 1.0ml. $(NH_4)_2SO_4$ ppt. Super.*	8.7

* Ammonium sulphate precipitated phosphate supernatant from standard ETP preparation dialyzed for 40 hours. Virtis super., *ca.* 8mg. protein/ml.; dialyzed Virtis super. *ca.* 5mg. protein/ml.

An experiment was carried out on the effect of added TPN on the rate of oxidation of pyruvate-malate in the presence of ETP plus virtis supernatant and ETP plus dialyzed Virtis supernatant. This experiment was an attempt to evoluated the effect of the isocitric dehydrogenase known to be presented in the supernatant.

It was considered that the supernatant could be influencing the rate of pyruvate-malate oxidation by the removal of citrate-isocitrate possibly formed by the ETP and inhibitory the further oxidation of pyruvate-malate as this ETP contains no isocitric dehydrogenase. The experiement was inconclusive, added TPN increasing the rate of oxidation of pyruvate-malate, citrate and isocitrate by ETP-supernatant by some 40—80% in all cases. It was found that if the Virtis supernatant dialyzed against phosphate buffer (0.25M KPO_4, pH 7.1) then there are no loss in activity. An ammonium sulphate fraction (40—60%) obtained from heart muscle was found to be inactive even up to concentration of 36mg. per manometer cup. Clearly the above supernatant has the capacity to oxidized malate alone without added pyruvate, which suggests an oxidative decarboxylation of malate is occuring. Assay for TPN malic-decarboxylation in the Beckman DU shows concentration of malic carbox. = 0.17 μmole TPN produced/min./mg. protein.

Location of Pyruvic Dehydrogenase—— So far it has not been possible to pin point the location of pyruvic dehydrogenase. The dehydrogenase is present in PETP but with ETP it is necessary to have an aliquot of Virtis supernatant present to

demonstrate any reaction. Both sueprnatant and ETP contain considerable concentration of malic dehydrogenase (cf. Table 26).

Table 26. Location of pyruvic oxidase.

Fraction	Substrate	mμatomes CO_2/min./mg. protein
ETP	Malate	7
//	Pyruvate	0
//	Pyruvate-Malate	7
Virtis super.	Malate	17
//	Pyruvate	0
//	Pyruvate-Malate	17
ETP-irtis super.	Malate	17
//	Pyruvate	37
//	Pyruvate-Malate	73

Each flask contains 30μmoles $K_3Fe(CN)_6$, 40 μmoles $NaHCO_3$, 20 μmoles $MgCl_2$, 1μmoles DPN, 0.1μmole CoASH, 10% BSA, 30μmoles phosphate, pH 7.1, and where indicated 7.0mg. ETP, 3.0mg, Virtis supernatant, 3μmoles K-*l*-malate, 20 μmoled Na-pyruvate. Water to final volume of 3.2ml. Incubated 13mim. at 38°C. CO_2 uptake measured anaerobically in $NaHCO_3$ buffer-N_2-CO_2 in the presence of $K_3Fe(CN)_6$.

This experiment strongly suggests that the pyruvic oxidase has been split into two halves. Malate oxidation rate alone closely approaches that of the pyruvate-malate most probably due to the rapid formation of pyruvate by the malate decarboxylase present in the supernatant. However it seems clear that the pyruvate is being oxidized in addition to malate by ETP plus supernatant. Otherwise supernatant alone would measure the straight decarboxylation of malate. Similarly as the ETP and supernatant contain malic dehydrogenase neither system alone can be capable of oxidizing pyruvate.

Dehydrogenase Composition of ETP and Supernatant——ETP assayed negatively for α-ketoglutarate, glutamate. ETP plus Virtis supernatants were assayed for aerobic activity towards pyruvate, malate, α-ketoglutarate and glutamate (Table 27). Malate was not oxidized in the absence of TPN.

Table 27. Effect of phosphate-ethanol treatment on ETP preparation.

Fraction	Substrate	Oxidation (μatomes CO_2/ 5min.)
ETP	α-Ketoglutarate	0.9
//	Pyruvate	0.1
// + Supernatant	α-Ketoglutarate	3.5
// + //	Pyruvate	0.1
Supernatant	α-Ketoglutarate	2.7
//	Pyruvate	0.2

Assay conditions as in Table 6.

Absence of α-Ketoglutaric Dehydrogenase from ETP—— ETP prepared in phosphate and ETP isolated during alcohol-phosphate-cofactor-substrate preparation of PETP both contain on α-ketoglutaric dehydrogenase (Table 28). Aerobically rate of α-ketoglutarate[17] with 1.0ml supernatant 3.5mg. and 4.5mg. ETP losed on ETP

protein rate was 0.101 μatomes O/min./mg. protein, *R. Q.* for α-ketoglutarate in the

Table 28. Effect of phospahte treatment on oxidative phosphorylation by residue and ETP.

Fraction	Oxidation (μatomesO/min/mg. protein)	P/O
BHM	0.095	0.24
Residue 1	0.013	0.51
Residue 2	0.014	1.51
ETP	0.004	0

BHM—Three times washed with sucrose, 4.4mg./ml.
Residure 1 —BHM blending in Virtis blender for 4 min. in the absence of phosphate then fractionated into a residue, 10.9mg. protein/ml.
Residue 2 — BHM blended in Virtis blender for 4 min., then homogenized in 0.15M KPO_4, pH 7.1 then fractionated into a residue, 10.8mg. protein/ml.
ETP—Prepared from Residue 2, 13.2mg. protein/ml.
Substrate—Malate-Pyruvate, another assay condition as in Table 24.

presence of ETP plus supernatant was 1.14 $\left(\frac{27.7\ \mu l\ CO_2}{24.2\ \mu l\ O_2}\right)$, these changes observed over a time period of 22min.

Distribution of Dehydrogenase in Residue, ETP and Soluble —— Probably the most significant observations were:- 1) blending of the BHM which producing considerable quantitives of soluble dehydrogenase[8] does not result in the initial complate solublization of these dehydrogenases observed when phosphate (0.15M KPO_4) is added either after blending or before comming blend the BHM suspension. 2) no ETP particles which are produced except in the presence of phosphate could perhaps be accounted for in term of spliting out phospholipid;— the lipid content of supernatant (S_1) was 14.7% while that of (S_2) was 15.4%. This represents no significant difference, the method of analysis could not distinigush a 10% difference between values. Perhaps same types of salts likage is involved in the disintegration of the residue.

Table 29. Dehydrogenase composition of ETP and supernatant.

Substrate	DPN	Oxidation (μatomes O/min/mg. protein)
α-Ketoglutarate	—	0.0672
Glutamate	—	0.193
Oxaloacetate	—	0
Oxaloacetate-Pyruvate	—	0
Malate	—	0
Malate-Pyruvate	+	0.460
Malate	+	0.460

Each flask contains 4.5mg. ETP—3.5mg. Virtis supernatant, 100μmoles glucose, 5 μmoles $MgCl_2$, 0.5mg. yeast hexokinase, 5 μmoles ATP, 0.5μmoles cocarboxylase, 0.1μmoles CoASH, and where indicated 20μmoles K α-ketoglutarate, 50μmoles oxaloacetate, 5 μmoles K-malate, 20μmoles Na-pyruvate, 1 μmole TPN. 0.25 M sucrose to a final volume of 3.2ml. Incubated 30 min. at 37.5°C. O_2 uptake measured by standard Warburg technique.

In view of the markedly difference dehydrogenase content of R_1 (residue) and R_2

the oxidation and phosphorylative activity of similar freshly prepared fractions were compared as shown in Table 29. The above results are an experiments carried out previously. However, it was not expected to find residue (R_1) and (R_2) with identical oxyzen rate nor (R_2) to show twice the phosphorylative activity of (R_1) the experiment was repeated. Another experiments give the identical rate to these recorded above for both oxidation of dehydrogenase in(R_1)they are not capable of reaction with oxygen. The other interesting point is the greater phosphorylative activity of (R_2) compared with (R_1). ETP still present in the no phosphate treated (R_1), appears to be so much dead wood.

III. Conditions of Recovery to Detach the Pyridino-protein

New Procedure for the Separation of BHM Types or Fragments—— Heparin has been recently reported as an effective agent for the dispersal of morphological entities of the liver. Electron micrographs of BHM[20] currently in use show that these preparations are gross mixture of mitochondrial type and fragments. Heparin (0.035%) or sucrose 0.25M was used in an attempt to separate some of these types.

Fresh non frozen BHM were separated into heavy and light mitochondria by the usual *tris*-sucrose technique. The light mitochondria where then homogenized in sucrose-heparin and centrifuged for 5 min. at 15,000 rpm. Three fractions were separated and assayed for DPNH-cytochrom c oxidase, and oxidative phosphorylation(Table 30, and 31).

Table 30. Oxidative phosphorylation by different BHM types.

Fraction	Cofactor	P/O	Oxidation (μatomes O/min./mg. Protein)	Phosphorylation (μmoles P/min./mg. Protein)
Bottom layer	—	2.48	0.250	0.633
〃	+			
Middle layer	—	2.55	0.255	0.395
〃	+	2.22	0.207	0.460
Upper layer	—	1.67	0.016	0.026
〃	+	0.72	0.041	0.030

Each manometer contains 6 N KOH 0.2 ml., 100μmoles glucose, 5 μmoles $MgCl_2$, 0.5mg. yeast hexokinase, 5 μmoles ATP, 30 μmoles phosphate, pH 7.1, 10% BSA, where added substrates were pyruvate 20μmoles, malte 5 μmoles, CoA 0.1 μmole, DPN 1 μmole, GSH 1 μmole, cocarboxylase 0.5μmole, and cofactor mixture (as in Table 2) 0.5ml. then sucrose to 3.2ml.

Table 31. DPNH-cytochrome oxidase activity by different BHM type.

Fraction	DPNH-Cytochrom C Oxidase Rate	
	no cytochrom c	cytochrom c (added)
Bottom layer	0.34	0.43
Middle layer	1.44	1.90
Upper layer	3.00	3.96

Assayed at 38°C and pH 7.4 with or without the addition of 0.1mg. cytochrom c in a total volume of 1.0ml. 0.2ml. KPO_4 0.2M 0.01ml. versene 0.01M 0.04ml. DPNH 0.2%, 0.01ml. cytochrom c. Enzyme(10—20mg.)about 2 O.D change *ca.* 0.040 per min. with no added cytochrom c, added cytochrom c increases rate 2—4 X. Measurement at 340 μ. All rates are expressed as μ moles DPNH oxidized min/mg. protein$\left(=\frac{\text{O.D change/min./mg. protein}}{6.2}\right)$.

Activity and Yield of PETP from Different Mitochondrial Types—— Using the sucrose-*tris* procedure BHM were separated into three fractions heavy, medium and light BHM. The attempt to prepare PETP from all three fractions was then made (Table 32). All three mitochondrial fractions phosphorylated at a rapid rate more than 0.350 μmoles P/min./mg. protein and give P/O ratios less than 2.3.

Table 32. The yield of PETP from different mitochondria type.

	Original			Residue				PETP			
	Vol. (ml.)	Protein (mg./ml.)	Total Protein (mg.)	Vol. (ml.)	Protein (mg./ml.)	Total Protein (mg.)	%	Vol. (ml.)	Protein (mg./ml.)	Total Protein (mg.)	%
1#	26	10.7	278	12	18.8	226	82	3.0	7.4	22	8
2#	26	13.9	361	12	23.9	287	80	4.6	11.9	55	16
3#	26	20.4	540	13	24.2	315	60	8.6	17.6	152	29

1# Heavy BHM, 2# Medium BHM, 3# Light BHM.

Attempted Conversion of Heavy Beef Heart Mitochondria to Light Mitochondria—— It appears that only what may be denoted "medium" and "light" BHM are capable of yielding PETP-ETP. The question arises as to what is the origin of light and medium BHM. Taking oxidative phosphorylation as an index "intactiness" HBHM would be closest to native BHM.

It seems likely that HBHM are converted to light and medium BHM either by specialized mechanical change or enzymically. The mechanical damage appears to be specific as it is so far impossible to duplicate the damage using isolated HBHM. Blending, freezing and thawing and treatment with distilled water does not convert HBHM to medium or light. Blending up the heart tissue in Potter-Elvehjem type homogenizer would not be as severe as blending tissue. Indeed it is assumed that the Potter-Elvehjem type homogenizer in view of previous observations with other tissues could not mechanically damage the BHM. These two methods of disintegrating tissues were compared.

To examine possible enzyme conversion of heavy—medium—light, minced heart mucles were, allowed to autolyze for 24 hours at 5°C and 48 hours at 5°C then 2 hours at 23°C BHM isolated from fresh tissue and compared with BHM isolated from fresh tissue. Also the minced heart muscle was homogenized in Potter-Elvehjem and allowed to stand 4 hours at 5°C then BHM isolated. The experiment may be conclusive that 1) the P.-E. type homogenizer has no advantage over the blender. The BHM obtained with either piece of apparatus has the same particle to yield ETP. 2) ETP does not appear to arise enzymically autolysis in whole tissue or homogenates does not effect ETP yield. 3) It would appear that as the tissue is disintegrated by either the homogenized or the blender the BHM are evidently two from between the muscle fibrils, and mitochondria.

Effect of Cholate on Phosphorylation, and Oxidation by HBHM—— As a preliminary to study of conditions recovery to detach the pyridino-proteins from the heme chain the effect of different cholate concentrations on oxidation and phosphorylation were examined (Table 33).

Using a level of cholate which did not affect phosphorylation *viz.*, 0.2mg. protein, the effect of varying salt concentrations was examined. HBHM (7.5ml.) plus 3.5 mg.

Table 33. Effect of different cholate concentration on oxidation and phosphorylation by BHM.

Cholate (mg.)	P/O	Oxidation (μatomes O/min./mg. protein)	Phosphorylation (μmoles P/min./mg. protein)
0	2.70	0.264	0.0560
0.5	2.48	0.216	0.487
1.0	0.69	0.107	0.074
3.0	—	0.077	0
4.5	—	0.058	0

Each manometer contains 2.9 mg. BHM 0.2 ml. 6N KOH, 0.5 ml. cofactor mixtuer (1μmole DPN, 0.5μmole DPT, 0.1μmole CoASH), 0.5ml. of (100μmoles glucose, 5μmoles $MgCl_2$, 0.5mg. yeast hexokinase, 5μmoles ATP, 30μmoles phosphate,(pH 7.1), 0.1ml. KPO_4, pH 5.0, K *l*-malate 5μmoles, Na-pyruvate 20μmoles, and where indicated cholate. Incubated 30min. at 37.5°C. O_2 uptake measured by standard Warburg technique. Phosphate determined by method of Fisk and Subbarow.

Na-cholate (3 mg./ml.) then prepared following:-

1) as above+0.9ml. 0.5M KPO_4, pH 7.0, sucrose to 30ml.

2) as above+3.0ml. 〃

3) as above+9.9ml. 〃

This procedure very clearly split the material into 3 distinct fractions at phosphate 9.5ml. 0.5M The block pellet which virtually exclusively constituted the residue while evident to same extent in 1) and 2) appeared to have been quantitatively split out from 3). The oxidative and phosphorylative activity of these fractions were determined (Table 34). Above assay in the presence of BSA and serum albumine

Table 34. Effect of cholate treatment in the presence of cofactor-substrate on oxidative phosphorylation by residue material.

Fraction	Oxidation (μatomes O/min./mg. protein)	Phosphoryltion (μmoles P/min./mg. protein)	P/O
Residue 1#	0.143	0.277	1.92
Residue 2#	0.127	0.127	1.00
Residue 2#-dark residue	0.150	0.174	1.16

1#, made to 30ml. with sucrose, 2#, 4.5ml cholate (20mg./ml.) added, made to 30ml. with sucrose. During fractionation the small dark tightly packed bottom was separated from 2# and made up 4.0ml. This does not estimate for protein, both residue 1 # and (which must be merely washed HBHM) and 2# was washed 3 times with sucrose.

Two 30ml. tubes were made up to contain the PETP, cofactor (0.5 M KPO_4, 90% ethanol, 0.5M versene, pH 7.0 $MgCl_2$ 0.1 %, 0.1 M ATP, 0.5 M Na pyruvate, 0.5 M K L-malate, CoASH 1 mg./ml., 0.1 M DPN, 0.1 M GSH, cocarboxylase 0.05 M and substrate mixture. BHM to 15 mg./ml., 500μmoles KPO_4, pH 7.0.

these procedure compared with previous assay lead to substantial losses in oxidative and phosphorylative activity. The effect of similar treatment in the presence of co-factor substrate were determined.

HBHM seems to undergo some change during the washing the oxidative activity at this stage being near halved so it the phosphorylation. Cofactor and substrate do not appear to protect residue (1) from loss of phosphorylative activity during this procedure.

Effect of Phosphate on the Phosphorylative Activity of HBHM—— Table 35

shows that residue (2) (67 μmoles phosphate/15mg. protein/3mg. cholate) is essentially devoided of phosphorylative activity, whereas residue (1) (16μmoles phosphate/15mg. protein/3mg. cholate), contains a considerable amount of phosphorylative activity. The dialyzed supernatant has somehow picked up an inhibitor the non-dialyzed supernatant appeared to neither inhibite or stimulate oxidative phosphorylation was increased some 40 percent.

Table 35. Effect of phosphate on the phosphorylation by HBHM.

Condition	△O (μatomes)	△P (μmoles)	P/O
Residue 1#	6.1	6.84	1.02
Residue 1# + no dialyzed super. 0.5ml.	9.1	6.98	0.77
Residue 1# + dialyzed super. 0.5ml.	10.86	4.01	0.37
Residue 1# + dialyzed super. 1.0ml.	11.25	0	0
Residue 1# + boiled dialyzed super. 1.0ml.	0	0	0
Residue 2#	8.08	1.77	0
Residue 2# + dialyzed super. 1.0ml.	10.46	0	0

PETP mixture+4.0ml. cofactor mixture+HBHM 15mg./ml. volume to be 30ml.
1# as above—80mg. Na-cholate+500μmoles KPO_4, pH 7.0
2# as above—for 1# except+2000μmoles KPO_4, pH 7.0.
After separation of the supernatants both residues were twice washed with PETP cofactor mixture due to cysteine which reduces Cu to CuO_2. Residues were frozen over night then assayed, supernatants were dialyzed over night against water plus 10ml. conc. solution of PVP.

Effect of Phosphate Concentration on Activity of Dilute HBHM Suspenion—— If the results obtained with 1 mg. protein/0.2mg. cholate/25μmoles KPO_4/ml. can be equated with 15mg. protein/3mg. cholate/25 μmoles KPO_4/ml. appreciable protein solublization will have taken place (<3%). To examine this possibility and note the effect of solubilized material:- 2.5ml. 0.1M $MgCl_2$, 50ml. 1.0M glucose, 25ml. 0.1M ATP, 35ml. 0.5M KPO_3, pH 7.1 added 250mg. dialized hexokinase, sucrose to 250ml., 2.5ml—(CoASH 20ml. 1 mg/ml., DPN 0.1M 20ml., water to 30ml.), 0.5ml 0.1M pyruvate, 0.4ml. 0.01M malate, 2.6ml. cholate (20mg./ml), 0.065ml. KPO_4 0.5M, pH 7.0. mixture homogenized, halved and centrifuged for 5 min. at 15,000rpm. The

Table 36. Effect of phosphate concentration in activity of diluted BHM suspension.

Fraction	Oxidation (μatomes O/min./mg.)	Phosphorylation (μmoles P/min./mg.)	P/O
1	0.147	0.271	1.87
2	0.160	0.290	1.85
3	0.162	0.282	1.73
4	0.115	0.035	0.30
5	0.114	0	0

Fraction 1; control sample, precipitated from mg. HBHM/0.2mg. cholate/KPO_4 25μmoles/ml. 2#; control sample centrifuged resuspended in own supernatant 3#; control sample centrifuged, supernatant discard, sedimentes resuspended in fresh coenzyme-cofactor-cholate-susbstrate solution. 4#; 14mg. HBHM/3mg. cholate/25μmoles KPO_4/ml., centrifuged resuspended in own supernatant 5#; 15mg. HBHM/3mg. cholate/25μmoles KPO_4/ml., centrifuged, supernatant discard. Sediments resuspended in fresh coenzyme-cofactor-cholate-susbtrate solution.

residue of one half has been resuspended in the supernatant, while the residue of the second was suspended in sucrose. The two samples were assayed, 0.2ml. aliquots added to manometer containing standard solutions, zero time phosphates were determined (Table 36).

Six ml. of samples mixture, 6.0ml. coenzyme mixture as discribed in previous experiment, 6.0ml. 0.1M KPO_4 pH 7.0, 4.8ml. 0.1M pyruvate, 2.4ml. 0.005M malate, 0.2ml. 0.005M versene, 0.024ml. 0.2M sucrose, 12.6ml. Na-cholate (20mg./ml.), 0.23ml., mix, remove 6.5ml added 6.5ml. HBHM (30mg. protein/ml.). To two spinco tube, and 6.5 ml. centrifuge, resuspended one residue in its supernatant resuspended other in the fresh cholate-substrate-cofactor solution 2.8ml. aliquots precipitated into manometer containing 0.2ml. 10% BSA, zero time phosphates were determined (Table 37).

Clearly the results obtained with dilute protein solutions can not be equated with more concentration of protein solutions. In dilute solution 1 mg. protein—0.2mg. cholate—25 μmoles KPO_4/ml. little of no damage results from the presence of cholate and phosphate. If the protein concentration and cholate concentration are increased 15 times per ml. extensive protein solubilization takes place with abolition of phosphorylation and some 40 percent reduction in oxidation.

Table 37. Effect of cholate-phosphate-alcohol on oxidative phosphorylation by HBHM fragments.

Fraction	Ethanol	Cholate	Phosphate	P	μatomes O/min./mg.	P/O
HBHM	—	—	—	22.5	0.114	2.17
〃	+	+	—	21.2	0.109	2.15
〃	+	+	—	0	0	—
〃	+	+	+	0	0.045	—
HBHM(centrifuged)	+	+	+	0	0.044	—
Super. sucrose washed with residue	+	+	+	0	0.044	—

Fraction—5.3mg HBHM (30mg./ml)+2.1ml., 0.5M KPO_4, pH7.0, 1.5ml. Cholate (20mg./ml.), 0.2ml. K L-malate 0.05M, 0.2ml. Na-pyruvate 0.5M, 0.5ml. coenzyme mixture, 0.5 ml. cofactor mixture, 0.05ml. versene 0.5M, 1.1ml. ethanol 95%,…this mixture was homogenized 0.2ml. aliqout removed for estimation, the rest was halved and centrifuged. One lot of residue was resuspended in this own supernatant, the second residue was washed with sucrose then assayed.

The Fragmentation of HBHM in Presence of Cholate-Phosphate-Alcohol—— In the presence of 10% ethanol very high concentration of phosphate is required to fragment the HBHM. Below 75μmoles there is no significant protein solublization.

The ethanol used in experiment (Table 38) has some effect on presencing the oxidative capacity of cholate-phosphate treated mitochondria but it does not contribute to preservation of phsphorylative activity. Interesting to note that ethanol does not inhibit phosphorylation or oxidation at a concentration of 330 μmoles/manometer cup.

It would be appeared from the results of Table 37 and 38 tha two different batches of HBHM differ in the easy with which they can be degraded with cholate-phosphate solution. The HBHM prepared in Table 37 are very dark in color, are comparatively easily disintegrated in cholate-phosphate treatment of the HBHM retain much of their oxidation and phosphorylative activity. However the HBHM prepared in Table 38 are much lighter in color, do not split out a small dark-brown to black pellet as did preparation in Table 36, and are comparatively resistant to cholate-

phosphate solution. The levels of cholate-phosphate required to solubilize some of the protein HBHM in Table 36 read to a complete loss of phosphorylative activity and marked reduced of oxidative activity.

Table 38. Effect of alkaline extract on oxidation and phosphorylation by HBHM.

Condition	Oxidation (μatomes O/min./mg.)	Phosphorylation (μmoles P/min./mg.)	P/O
HBHM*	0.121	0.179	1.48
HBHM+0.5ml. alkaline extract of alcohol-PO_4 contented BHM	0.141	0.150	1.03
HBHM**+1.0ml. alkaline extract of alcohol+ PO_4 contented BHM	0.176	0.114	0.65
HBHM+0.5ml. alkaline extract of BHM	0.167	0.117	0.70
HBHM***	0.118	0.222	1.88
HBHM+1.0ml. alkaline extract of alcohol +PO_4 contented BHM	0.213	0.230	1.07

HBHM*+1.0ml. alkaline extract of alcohol-phosphate contented BHM.
HBHM**+34.7mg./ml. HBHM+0.5ml. Na-cholate (20mg./ml.)+1.5ml. 0.1M BHM+KPO_4 pH 7.0, sucrose to 4.0ml.
HBHM***+as for HBHM except 0.4ml. 0.1M KPO_4 pH 7.0.
Alkaline extract was prepared with devided following fractions:- one was a pH 11.0 extract of BHM, the other a pH11.0 extract BHM which had prior to alkaline treatment been extracted twice with alcohol-phosphate.

The first supernatant obtained from HBHM in alcohol-phosphate-cofactor then centrifuged was dialyzed for 18 hours. The dialyzed solution was added back to residue (3) and oxidative phosphorylation rates were determined. The supernatant was found to increase the rate of oxidation of pyruvate-malate but there was no phosphorylation by the residue above or residue plus dialyzed supernatant.

HBHM degrated with cholate (0.2mg. potein/ml.) plus phosphate (560μmoles/ml.) in the presence of cofactor mixture (DPN, CoASH, GSH, cocarboxylase), $MgCl_2$, pyruvate-malate, versene retained more of the oxidative capacity. If the HBHM are incubated for a short period at 38°C in the absence of substrate then they degrade more readily on subsequent treatment with cholate-phosphate and versene can protects against this effect if present when HBHM are incubated at 38°C.

Further Studies on the Effect of Cholate on HBHM—— Previously some suggestion of a factor(s) present in an alkaline extract of BHM residue stimulating the rate of phosphorylation in PETP has been obtained. The effect was only marginal but consistant. It was considered that perhaps PETP was the wrong assay system in so far as oxidation cannot be greatly increased as the dehydrogenases are limiting and phosphorylation can be only increased within the range 2—3 as the ratio is near optimal. A possible better system was considered to be one of the fractions (HBHM-Na-cholate-KPO_4, pH 7.0,2.0ml.—sucrose to 4.0ml.) are showing ratios below theoretical (0.032 μatomes O/min./mg. protein, 0.020 μmoles P/min./mg. protein).

Effect on Alkaline Extract on HBHM—— Preparation of alkaline supernatant; a sample of washed HBHM and BHM were made up in sucrose to approximately 40mg./ml. and then pH of the samples adjusted to 11.0. When the pH remained constant the samples are centrifuged. The neutralized supernatant constitutes the so-called alkaline supernatant.

The inhibition of phosphorylation only alkaline extracts of BHM and failure to observe the expected increase in phosphorylation may be due to the starting material used to prepare the extract. Previously where activations were observed in residue was used as the sucrose material. Alkaline extracts of BHM and residue were estimated for their effect. Clearly an inhibitor of phosphorylation present in extract of BHM. If BHM are first extracted with alcohol-phosphate as in or ETP the inhibitor is either destroyed as extract. The alcohol-phosphate residue extracted at pH 11.0 shows some margianl stimulation of phosphorylation. The effect of the alkaline extract on preparation was examined (Table 38).

Two alkaline extracts were assayed; one was pH 11.0 extracted of BHM, the other a pH 11.0 extract BHM which had prior to alkaline treatment been extracted twice with alcohol-phosphate. No oxidation is detected under measuring oxygen uptake with supernatant plus 3# and 5# in the absence of added substrate. The above supernatants contain no appreciable quantities of malic or isocitric dehydrogenase and malate alone is not oxidized by that supernatant plus 5#; the alkaline extract of whole BHM more so than the other supernatant. The above results show both supernatants inhibit fraction 5#; Both supernatants while effective to some extract against 3# are less so than 5# (cf. Table 38).

Table 39. Effect of TPN on oxidation and phosphorylation by HBHM in the presence of alkaline supernatant.

Fraction	Boiled Alkaline	Extract	TPN	Oxidation (μatomes O/min./mg.)	Phosphorylaiton (μmoles P/min./mg.)	P/O
HBHM*	—	—	—	0.076	0.109	1.43
//	+	—	—	0.086	0.148	1.72
//	+	+	+	0.100	0.121	1.12

*HBHM added Na-cholate (20μmoles/ml.)—0.5ml, 0.1mole KPO_4, pH 7.0 sucrose to 4.0ml.

The pH 11.0 alkaline extract containing both activatior and inhibitor was dialyzed for 48 hours against two changes of 0.005M *tris* pH 7.4, in the ratio of 20ml. of extract to 7l of *tris* solution. Frozen alkaline extract (0.8ml.) is equivalent to 1.3ml. dialyzed extract on a volume basis. The dialyzed extract almost competely inhibits phosphorylation. It is noteworthy that the boiled dialyzed solution, is still inhibitory but not to such an extract as the non-boiled material which suggests that boiling does not completely destroy the inhibitor, so that stimulation of phosphorylation by boiled material may be regarded as minimal.

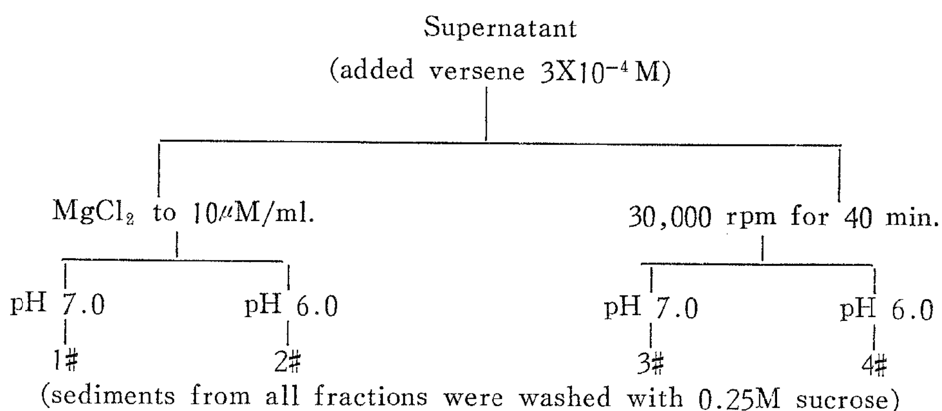
The possibility was considered that stimulation of phosphorylation by the alkaline extract might be due to presente of TPNH in the extract. This was shown as in Table 39, TPN inhibited phosphorylation by some 40%. The presence of alkaline supernatant served partially reverse this inhibition.

IV. Nature of Linkage of Pyridino-protein to Electron Transport System

The present work is concerned with the linkages by which the pyridino-protein enzymes are bound to the electron transport system in phosphorylating beef heart mitochondria (BHM). Oxidative phosphorylation is abolished whenever the BHM are fragmented by any procedure which leads to the solublization of the pyridino-protein. Mechanical damage to the BHM, induced by a high speed Virtis blender, in the

presence of phosphate leads to essentially complete solubilization of the malic and isocitric dehydrogenases. The α-ketoglutaric, glutamic and pyruvic dehydrogenase are solublilized but to a less extent. In the absence of dhosphate during the blending a large portion of the pyridino-protein dehydrogenases remain associated with the paticulate fraction. BHM have been distintegrated by a variety of procedures to determine some of the factors governing the association of pyridino-protein enzymes with the fragmented particles.

Experimental 1 ——Blending in Waring blender for 3 min. heart mince suspended in 4 vol. 0.02M KPO_4, pH 7.4, centrifuged for 10 min. at 20,000 *g*. supernatant row pH 6.9 adjusted to 7.4 and centrifuge for 10min. at 30,000 rpm, recovered supernatant was optically clear or near so, devoid of visible particle.

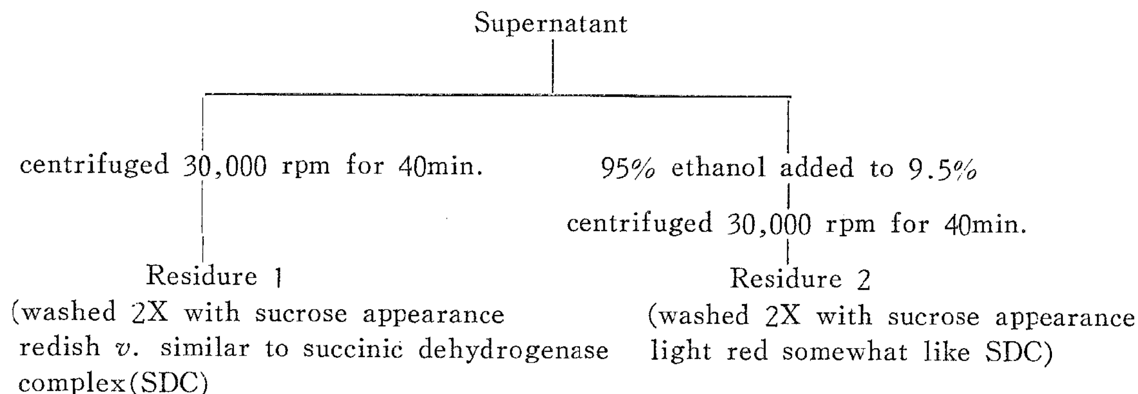


Enzyme assay

Fractions	Malic Dehydrogenase (μM DPNH oxidized/min./mg.)	α-Ketoglutaric Dehydrogenase (μMCO_2/min./mg.)	Pyruvic Dehydrogenase (μMCO_2/min./mg.)
1#	3.20	0.138	0.024
2#	0.30	0.124	0.026
3#	3.29	0.127	0.022
4#	1.76	0.124	0.018

None of the preparations contained any DPNH oxidase.

Experimental 2 —— Heart muscle minced suspended in 30vol. 0.02 M KPO_4, pH 7.4—0.004M versene. Waring blender was filled to include all air and then the suspension was blended for 5 min. Suspension centrifuged for 10min. at 2000*g*. residue discarded, then supernatant centrifuged for 5 min. at 30,000 rpm, residue discared.



Enzyme assay

	α-Ketoglutaric D. (μM CO_2/ min./mg.)	Pyruvic D. (μM CO_2/ min./mg.)	Pyruvic-MalicD. (μatome O/ min./mg.)	Succinic D. (μMCO_2/ min./mg.)	Malic D. (μM DPNH/ min./mg.)
Residue 1	0.048	0.012	0.017	0.408	4.3
Residue 2	0.029	0.005	0.005	0.349	—

This experiment does not represent a fair test of the use of ethanol in this procedure as it was found after the addition of the ethanol the temperature was−10°. The presence of an active succinoxidase[9] and DPNH oxidase is most probably due to employing too short a period of centrifugation at 30,000rpm for the preparation of the starting supernatant. The use of versene might also be a contributing factor in terms of preventing oxidation of nital ligards holding the dehydrogenase complex to the heme chain. Aeration with or without versene may be an important factor.

Experimental 3 —— Heart muscle minced suspended in 4vol. of 0.02M KPO_4 pH 7.4

a) suspension blended for 3 min. (600ml. in 1 *l* Waring blender)

b) versene added to $2X10^{-4}$M to 600ml. suspension then blended for 3 min. in 1 *l*.

c) versene added to $3X10^{-4}$ M to 600ml. suspension then blended for 3 min. in 1 *l* so that blender filled to capacity with no air space.

d) 1000ml. suspension blended for 3 min. Blender filled to capacity with no air space

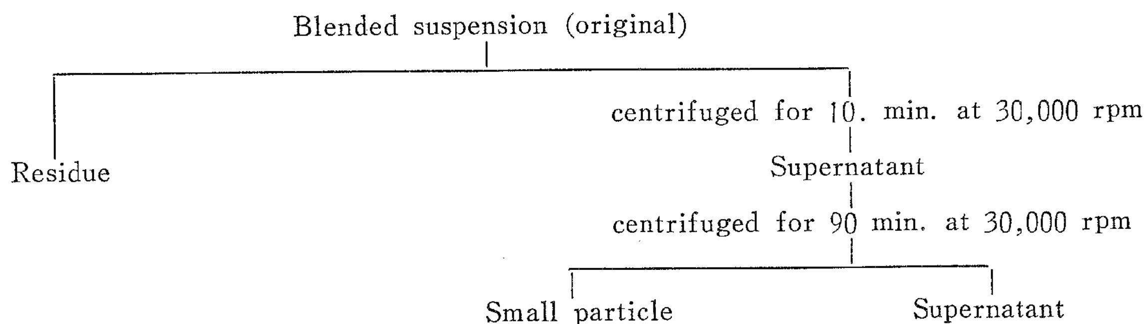
(a) (b) (c) (d) centrifuged for 15min. at 30,000 rpm. Supernatant centrifuged for 10min. at 30,000 rpm, pH of supernatant 7.0 versene added to (a) and (b) to $2X10^{-4}$M.

Supernatants centrifuged for 60min. at 30,000 rpm. Residue collected and washed once with 20ml. 0.02M KPO_4 pH 7.4 at 40,000 rpm for 60min.

Enzyme assay

	Malic D. (μM DPNH/min./mg.)	α-Ketoglutaric D. (μMCO_2/min./mg.)	Pyruvic D. (μMCO_2/min./mg.)
(a) ⅔ full	1.34	0.101	0.023
(b) ⅔ full+versene	0.94	0.153	0.019
(c) full	2.56	0.160	0.019
(d) full+versene	1.32	0.151	0.021

Experimental 4 —— Pooled aged HBHM were 2X washed with sucrose then suspended in 0.02M KPO_4, pH 7.4—10^{-4}M versene. The suspension contained 25mg. protein/ml.; this material was blended for 3 min. in the Virtis blender (cup full to capacity) with the reostat set on 85.



Enzyme assay

	α-Ketoglutaric D.	Pyruvic D.
Original	0.060	0.013
Small particle	0.150	0.024
Supernatant	0.108*	0.028

*These figures are questionable as rate of CO_2 evolution decreased *v.* rapidy (halved) after first 3 min. The supernatant plus small particle showed a finer rate of CO_2 evolution and the rates were addictive.

	Macli D.	Isocitric D.
Original	6.19	0.47
Small particle	5.53	0.64
Supernatant	53.5	1.30

Experimantal 5 —— Whole mitochondrial suspension in 0.02M KPO_4 pH 7.8+3X 10^{-4}M versene blended for 3 min. in Virtis blendor, with the cup of the blender filled to capacity. Blended suspension centrifuged for 2 min. at 30,000 rpm, residue discarded, supernatant then fractionated as showen.

Supernatant
|
15min. at 30,000 rpm
|
- Residue "ETP" washed once with 0.02 M KPO_4 pH 7.8 (R_1)
- Supernatant adjusted to pH 6.0
  - Ethanol to 15% added centrifuged at 30,000 rpm (R_2)
  - Centrifuged at 40,000 rpm for 60 min. (R_3)

Enzyme assay

	α-Ketoglutaric D.	Pyruvic D.	Succinic D.	Isocitric D.
R_1	0.032	0.119	2.22	—
R_2	0.008	0.024	0.11	0.44
R_3	2.12	0.018	0.018	—

	Malic D.	β-Hydroxybutyric D.	Bounded DPN (μM/mg. protein)	Ribose(RNA detn.)
R_1	—	—	—	—
R_2	3.49	0,07	0.5	7.01
R_3	—	—	—	—

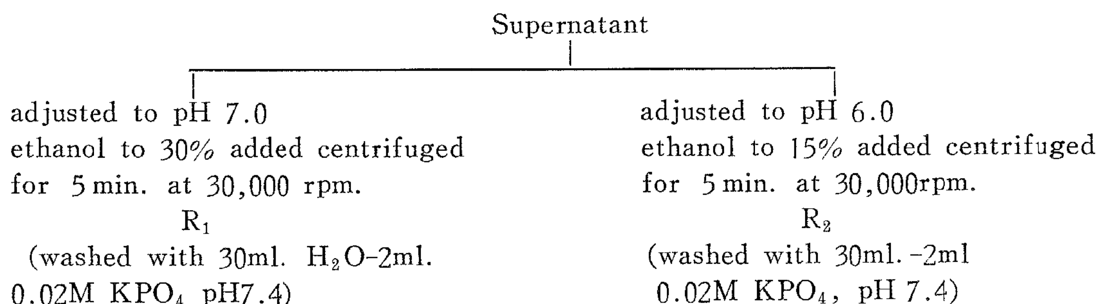
Experimental 6 ——Washed BHM suspended in 0.02 M KPO_4 pH 7.8 blended for 3 min. in Virtis blender; reostat set on 85, Virtis cups filled to capacity. Blended suspension, centrifuged for 5 min. at 30,000 rpm residue discard. Supernatant pH 7.4 adjusted to pH 7.9, centrifuged for 20min. at 30,000 rpm, residue discard.

Supernatant
|
- adjusted to pH 7.0 ethanol to 30% added at 30,000 rpm (R_1) (washed with 30ml. H_2O-2ml., 0.02M KPO_4, pH 7.4)
- Supernatant adjusted pH 6.0 ethanol to 15% added centifuged for 5 min. at 30,000 rpm (R_2) (washed with 30ml. H_2O-2ml. 0.02M KPO_4, pH 7.4)

Enzyme assay

	α-Ketoglutaric D.	Pyruvic D.	Lipid analysis
R_1	0.120	0.016	20.5 (%)
R_2	0.083	0.013	14.4

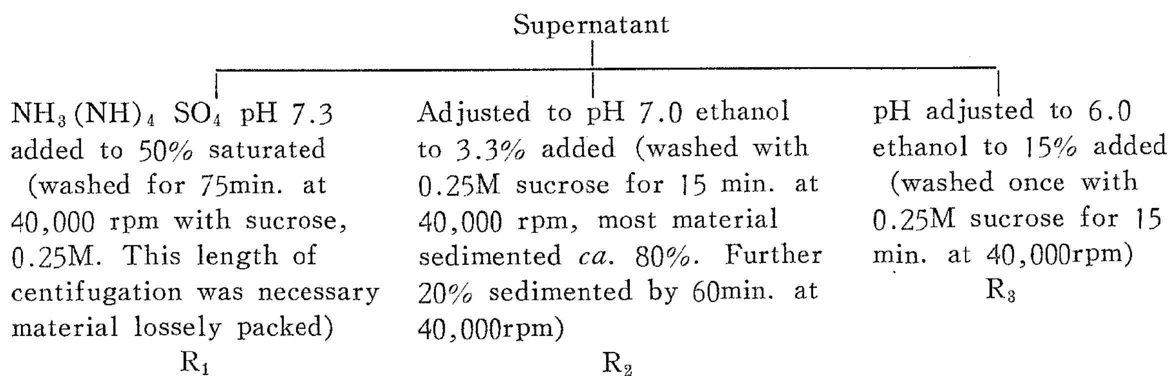
Experimental 7 —— Washed BHM suspended in 0.02M KPO_4, pH7.0 blended for 3 min. in Virtis blender; reostat set on 85, Virtis cups filled to capacity. Blended suspension, centrifuged for 5 min. at 30,000 rpm residue discard. Supernatant pH 7.4 adjusted to pH 7.9, centrifuged for 20min. at 30,000 rpm, residue discard.



Enzyme assay

	α-Ketoglutaric D.	Pyruvic D.	Lipid analysis (%)
R_1	0.083	0.013	14.4
R_2	0.120	0.016	25.0

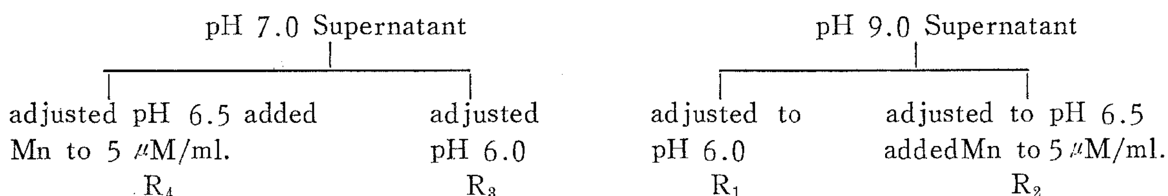
Experimental 8 —— BHM suspended in 0.25 M sucrose-$3X10^{-4}$ versene, pH adjusted to 7.9. Suspension blended for 3 min. in Virtis blender cup fulled to capacity Suspension centrifuged for 2 min. at 30,000 rpm then for 30min. at 30,000 rpm. Residue discard in each time.



Enzyme assay

	α-Ketoglutaric D.	Pyruvic D.	Succinoxidase (in O_2 with cytochrom c)
R_1	0.132	0.055	1.200
R_2	0.094	0.025	0.423
R_3	0.120	0.019	0.323

Experimental 9 —— Washed BHM suspended in sucrose. suspension *ca.* 25mg./ml. Versene to $5X10^{-3}M$ added Suspension halved and adjusted to pH 9.0 and 7.5. Suspension blended for 3 min. in Virtis blender, reostat on 85. Suspension spin for 10min. at 30,000rpm then 45min. at 30,000 rpm, residue discard.



(centrifuged for 30min. at 30,000rpm)

Washed all 60min. at 40,000 rpm. Material did not wash readily, this period of centrifugation was necessary.

Enzyme assay

	α-Ketoglutaric D.	Pyruvic D.	RNA (Ribose μg./mg. protein)
R_1	0.063	0.010	—
R_2	0.112	0.014	—
R_3	0.113	0.019	2.2
R_4	0.190	0.116	3.6

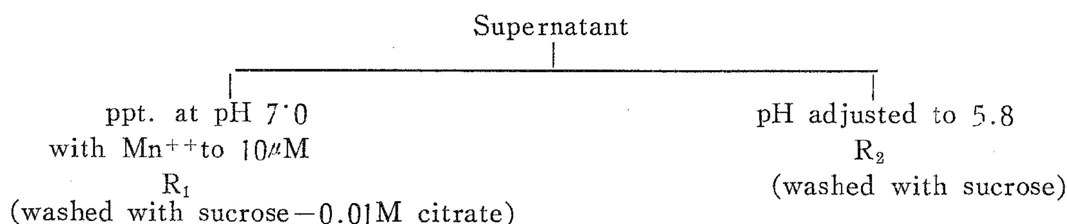
Experimental 10 —— Washed BHM suspended in 0.25M sucrose$-5X10^{-3}M$ versene. Suspension blended for 3 min. in Virtis reostat setting 85, cups full. Suspension centrifuged for 5 min. at 30,000 rpm then 30min. at 30,000 rpm, residue discarded. To the final supernatant adjusted to pH 6,3, $MnCl_2$ to 10 μM/ml. was added. This precipitate, was collected by centrifugation for 1 min. at 30,000 rpm. In an attempt precipitate to remove the Mn^{++} from the precipitate, the precipitate was washed with 0.08M versene by centrifugation for 60 min. at 40,000 rpm. Much of the precipitate dissolved. The washed particles are here dennated R_1 and the supernatant washing R_1 Supernatant.

Enzyme assay

	α-Ketoglutaric D.	Pyruvic D.	Malic D.	RNA (ribose μg/mg.)	Succinic D.	DPN (mμM/mg.)
R_1	0.212	0.029	0.78	3.9	0.292	0.2
R_1 Supernatant	0.018	0.009	7.38	4.5	—	—

Experimental 11 —— BHM in 0.25M sucrose$+2X\ 10^{-5}M$ sucrose$+3$μM pyruvate/ml.$+1$ μM malate/ml, ATP $2X\ 10^{-4}M$, DPN 1 μM/ml, 20ml. cofactor mixture (CoA 1mg./ml, 0.1M DPN, GSH 0.1M, cocarboxylase 0.005M) blended for 3 min. in Virtis reostat 85, cups full.

Centrifuged for 10min. at 30,000 rpm, supernatant sufficently clear did not centrifuge again.



	Enzyme assay			
	α-Ketoglutaric D.	Pyruvic D.	Succinic D.	DPN
				(mμM/mg. protein)
R_1	0.134	0.222	0.253	—
R_2	0.121	0.026	0.386	0.54

The Mn^{++} particle R_1 [13] washed with 0.01M citrate-sucrose does not, unlike the Mn^{++} particle washed with 0.008M versene, show a significant increase in concentration of α-ketoglutarate and pyruvate over the pH 6.0 particle.

Experimental 12 —— Washed BHM suspended in sucrose no versene. Suspension blended for 3 min. in Virtis, reostat on 85, suspension centifuged for 5 min. and 30 min. at 30,000 rpm. Supernatant adjusted to pH 5.8, precipitates recoverd. The precipitate was suspended in sucrose and pH adjusted to 6.6. Much of the precipitate dissolved, leaving a sediment R_1 (washed), the supernatant was collected and the pH adjusted to 4.9, the precipitate was covered as R_2. The first supernatant obtained at pH 5.8 after the removal of R_1 was precipitated with NH_3-$(NH_4)_2SO_4$ to 0.8, precipitate disolved and dialyzed 1 hour against distilled water.

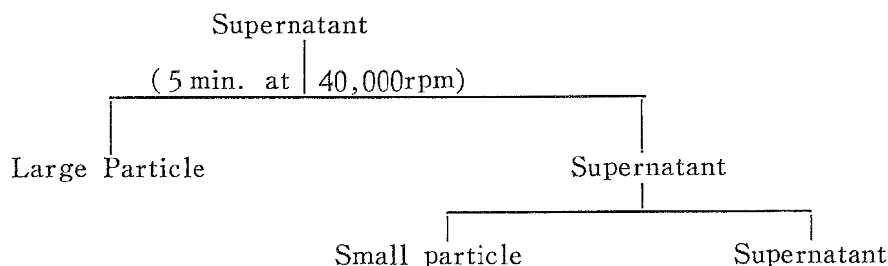
	Enzyme assay	
	α-Ketoglutaric D.	Pyruvic D.
R_1	0.176	0.027
R_2	0.162	0.052
NH_3-$(NH_4)_2SO_4$ ppt. (N)	0	0
NH_3-$(NH_4)_2SO_4$-R_1	—	0.044

Appears as previous experimence would have suggested that the pyruvic dehydrogenase is again being split into two components. The unusually high pyruvic dehydrogenase is puzzling in so far as previously acid pH values produced low pyruvic dehydrogenase values in the particle.

V. Initial Baseline Distribution of Whole Series of Dehydrogenase and Associated DPN Enzymes

The Effect of the Sonic Ocillation Time on Disruption——This experiment was trial run on the sonic ocillator to examine the effect of ocillation time on BHM.

BHM (40mg. protein/ml) sonicated separately for 4 min. and 15 min. hereafter denoted 4 and 15. 48 ml. dialyzed to 75ml. 6 ml. set aside for analysis of original material. Then centrifuged for 5 min. at 16,000 rpm (no. 40 roter Spinco head) to give the large particle, 30 min. at 30,000 rpm yield small particle and supernatant.



(Composed only material which packed firmly the rest was pored off with the supernatant)

Table 40. Effect of ocillation Time on disruption of beef heart mitochndoria.

Fraction	α-Ketoglutric dehydrogenase				Pyruvic dehydrogenase			
	S. A.	Total Unit	Unit (%)	Total Unit(%)	S. A.	Total Unit	Uint (%)	Total Unit(%)
4′ large particle	0.044	2.640	28		0.012	0.72	33	
4′ small particle	0.009	0.135	1	68	0.066	0.090		63
4′ supernatant	0.230	3.680	38		0.036	0.576	4	
15′ large particle	0.015	0.525	6		0.007	0.250	26	
15′ small particle	0.008	0.272	3	62	0.005	0.170	11	
15′ supernatant	0.218	4.58	53		0.039	0.819	8	56
4′ sonicated unseparated material	0.095	9.50			0.022	2.200	37	
15′ sonicated unseparated material	0.086	8.60			0.220	2.200		
BHM untreated	0.170	17.00			0.035	3.500		

A. S. (specific activity) expressed as 4μmolos CO_2/min./mg. protein.
Assay conditions; each manometer contains $K_3Fe(CN)_6$ 50 μmoles, $NaHCO_3$ 40 μmoles, $MgCl_2$ 20 μmoles, BSA 10%, 0.2 ml. and where indicated K α-ketoglutarate 20 μmoles Na pyruvate-oxaloacetate (20 μmoles-10 μmoles), and each fraction. Water to a final volume of 3.2 ml. Incubatod 30 min., at 37.5°C. CO_2 uptake measured Warburg techique anaerobically in $NaHCO_3$ buffer-N_2-Co_2 in the presence of $K_3Fe(CN)_6$.
Unit expressed as μmoles DPNH oxidized/min./mg. protein adition of 0.1 mg. cytochrom c in a total volume of 1.0 ml. 0.9 ml. KPO_4 0.2 mole, pH 7.4, 0.01 ml. versene 0.01 M, 0.04 ml. 0.2% DPNH, 0.01 ml. cytochrom c. Enzyme (10—20 mg.) about 2 mg., measred at 340 mμ.

$$\left(\text{Unit} = \frac{\text{O.D change/min./mg./protein}}{6.2}\right)$$

The small particle and the large particle were washed with 30 ml. 0.25 M sucrose then resuspended in fresh sucrose. HBHM original not sonicated (22.4mg./ml. protein). Activity date for this experiment is shown in Table 40.

In order to test on partial incubation of α-ketoglutaric dehydrogenase on storage and pH 6.0 precipitate, HBHM suspension sonicated for 15min. then centrifuged for 5 min. at 40,000 rpm hereafter denoted 15min. supernatant (freshly prepared), 15min. supernatant (48 hours old,) pH 6,0 precipitated for 24 hours old, 15min. supernatant (ppt. then stored 24 honrs), and 15min. supernatant (48 hours old) after removal of pH 6.0. Freshly prepared assayed for α-ketoglutaric dehydrogenase 0.088 (μmoles CO_2/min./mg. protein) after 24 hours further storage (pH 6.0) the material assay 0.056. Clearly pH 6.0 precipitate plus 15min. supernatant (48 hours old) after removal of pH 6.0 precipitate are not giving a simple summation expected specific activity for this combination 0.085 whereas observed specific activity is 0.113.

Table 41. Partial incubation of α-ketoglutaric dehydrogenase on sotrage and pH 6.0 precipitate.

Fraction	α-Ketoglutaric Dehydrogenase Specific Activity (μmoles CO_2/min./mg. protein)
15min. super. (freshly prepared)	0.218
15min. super. (48 hrs. old)	0.159
pH 6.0 ppt. from 24 hrs. old 15 min. super.	0.056
15 min. super. (48 hrs. old) after removal of pH 6.0 ppt.	0.110
pH 6.0 ppt. from 24 hrs. old 15 min. super. 15 min. super. (48 hrs. old) after removal of pH 6.0 ppt.	0.113

Protamine Sulphate Precipitation——Two aliquots of 50ml. HBHM (40mg. /ml.) were separately sonicated for 4 min. one at an energy input correspond to 1.1 amps, the other at 0.8 amps. Hereafter denoted 8 A and 11A. About 47ml. from each ocillation was removed and diluted to 75ml. fractionation as carried out previously. The 8 A and 11A supernatants were pooled there being on obvious difference between these fractions. To 82ml. of combined supernatant protamine sulphate (25mg./ml.) was slowly added with stirring, 1.0ml. of this solution, was added to give a slight excess, no further precipitated was collected and washed once with sucrose. The

Table 42. Reactivation of protamine sulphate precipitation.

Fraction	Total Protein (mg.)	α-Ketoglutaric Dehydrogenase				Pyruvic Dehydrogenase			
		S. A.	Units	% Units	Recovery (%)	S. A.	Units	% Units	Recovery (%)
Combined 5A-11A super.	458	0.104	10.40			0.030	3.00		
Protamine ppt.	213	0.186	8.2	80	87	0.031	1.36	45	45
Super. after removal of protamine ppt.	320	0.011	0.73	7		0.00	—		

combined supernatants were taken as 21 percent of total protein. On Table 42, it is shown that following protamine precipitate 55 percent of the pyruvic dehydrogenase units are lost and the surving is concentrated solely in protamine precipitate. This may suggest the presence of a factor in the supernatant or else pyruvic dehydr odgenase inactivation.

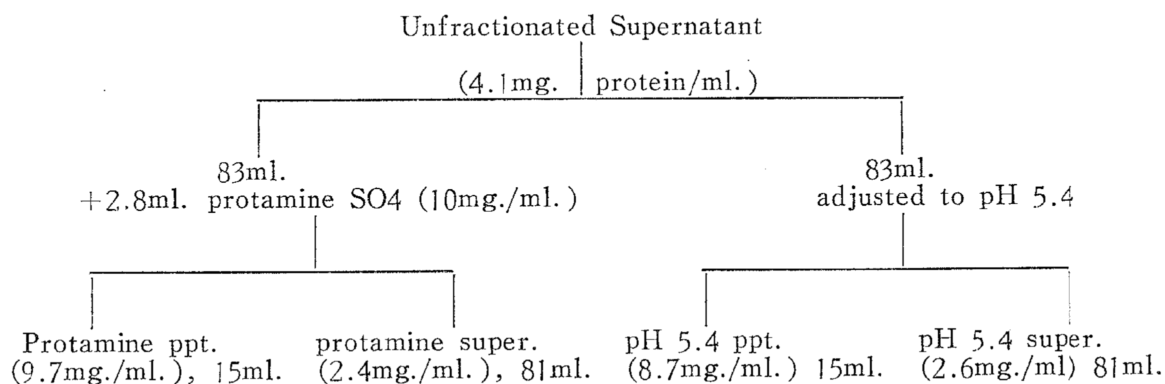
The dialyzed whole unfractionated supernatant and the the dialyzed supernatant after the removal of protamine precipitate, both showed no activity over the blank. The whole supernatant showed only a change of 15 units/8 min. while the other supernatant showed 5 units/8 min. Most likely both supernatants had lost their activity of alternately were too dilute. The protamine precipitate assayed 0.026 (μatomes 0 /min./mg. protein) towards pyruvate. The supernatant after removal protamine precipitate or anmonium sulphate precipitated fractions 0—45%, 45—65%, 65—95%, solution all were inactive alone towards pyruvate and also contribution with the protamine precipitate.

Recovery of Pyruvic Dehydrogenase Units between Protamine Precipitate and Supernatant after Removal of Protamine——Protamine sulphate (1.4ml./10mg./ml.) was added to unfractionated supernatant (42ml.) to give a slight excess. The precipitate was collected and washed with sucrose (30ml.). Supernatant recovered 41ml. The 7.0ml. protamine precipitated recovered 0.5ml. used per manometer, specific activity towards pyruvate was obtained 0.0034 (μatomes 0 /min./mg. protein). The precipitate free supernatant obtained protamine precipitate (0.3ml.) plus supernatant (1.8ml.) specific activity is in determinations: -0.079, 0.057, 0.051, and 0.049 μatomes/min./mg. protein towards pyruvate. The combination of protamine sulphate precipitate plus supernatant free of protamine precipitate present in the unfractionated supernatant as showen thus. Total volume of protamine precipitate is 7.0ml; 0.3ml. aliquot as used in previous determinations; so that it means enough material for $\frac{7.0}{0.3}$, 23 determination. Total volume of protamine precipitate free supernatant is 41ml. 1.8ml. aliqout was used in the pervious determination so that the experiment has enough material for $\frac{41}{8}$, 23 determinations.

Effect of Dialysis against Phosphate, pH 7.4 and 24 Hours Aging——An aliquot of the protamine sulphate precipitate free supernatant was dialyzed for 18 hours, against 80 volumes 0.005M KPO_4, pH 7.4; the dialyzed material was then compared for activity with a non-dialyzed supernatant. This small difference observed between the dialyzed and non-dialyzed supernatant has lost much of its activity since the previous day.

Precipitation at pH 5.4——Washed BHM were sonicated for 10min. and the supernatant recovered. The supernatant was then adjusted to pH 5.4, the precipitate collected. The pH 5.4 precipitate (1) was adjusted to pH 7.0, then to pH 5.4 to yield pH 5.4 precipitate (2), and 5.4 supernatant (2). This procedure was repeated to yield pH 5.4 precipitate (3) and 5.4 supernatant (3). The 5.4 precipitate was reprecipitated three times without any appreciable solubilization. Recombinations of any of the 5.4 supernatant (1) with the 5.4 precipitate (4) did not affect the activity of the 5.4 precipitate (4). The activity of the 5.4 precipitate was very low—0.010 μatomes O/min./mg. protein.

Comparison of Effectivities of pH 5.4 Precipitate and Protamine Sulphate in Separation of Pyruvic Dehydrogenase Components—— Washed HBHM were sonicated for 15min. then separated into large particle (30,000 rpm for 20min.) and a small particle (40,000 rpm, for 30min.) and supernatant.



The pH 5.4 and protamine sulphate supernatant of previous experiments were freezed tried and the solids redissolved in 10ml. H_2O—a 15 fold concentration of material,

Table 43. Comparison of effectivities of pH 5.4 precipitates and protamine sulphate precipitates in separation of pyruvic dehydrogenase components.

Conditions	Pyruvic Dehydrogenase Specific Activties (μmoles CO_2/min./mg. protein)
Initial super.	0.035
5.4 ppt. (1.7 mg.)	0.049
5.4 ppt.+5.4super.	0.080
Protamine ppt. (1.9 mg.)	0.034
Protamine ppt.+protamine super. (5.0 mg.)	0.082*
Protamine ppt.+lyophilized protamine super. (11 mg.)	0.083
Protamine ppt.+lyophilized protamine super. (22 mg.)	0.117
Lyophilized super. (22 mg.)	0.000

lyophilized solution after removing undisolved material contained 34.3mg. protein/ml. It is evident comparing the above values* in Table 43, that about 50 percent of the response activity has been lost on lyopilized. Protamine precipitate is essentially unchanged in activity. It is also appearent that large excess of protamine supernatant relative to the initial concentration is required to set the system.

DPNH Reduction in the Pyruvate System —— Cuvettes contained 0.1ml. BSA 10%, 10 μmoles KPO_4, pH 7.0,/3 μmoles $MgCI_2$, 0.02ml. KCN 0.1M, 0.5 μmole DPN. Purified lactic dehydrogenase and 5 μmoles latate, optimal dencity (O.D) change 0.350→0.620 to read equilib. DPT, CoA, protamine precipitate added linear continuous change recorded 0.972→0.935→0.14, These values have to be halved to account for the lactic dehydrogenase. Pyruvic dehydrogenase specific activity to DPN 0.07. This represents considerably higher activity than recorded to Fe $(CN)_6'''$. The Fe $(CN)'''_6$ reaction a ssays around 0.040 but as we obtain $3CO_2$/moleculae pyruvate for comparison with the DPN assay 0.040 must be divided by 3. It is not possible to conclude

Table 44. DPN-Pyruvic dehydrogenase distribution in protamine precipitate.

Fraction	Total Protein (mg.)	Total Units	Total Units (%)	Pyruvic Dehydrogenase Specific Activity (μmoles CO_2/min./mg. protein)
Protamine ppt.	813	172		0.021
Protamine super.	196	150	88	0.077
Protamine super.-protamine ppt.	614	0	0	0.000

Assay conditions;—0.2ml. KPO_4 0.5M, pH 7.4, 0.01ml. BSA 10%, 0.003ml. $MgCl_2$, 0.4ml. H_2O, 0.005ml. DPT, 0.005M and 0.1ml. DPN incubated at 38°. Then added 10μl. lactic dehydrogenase (crystalline), 0.03ml. Lactate 0.2M, incuvate in cuvette untill reaction comes to equilibrium (*ca.* 4min.), then added enzyme 0.015ml. KCN 0.01M, 5μl PTA (phosphotransacetylase). 0.02ml. CoA (1.0mg./ml.). Mix, read at 340mμ. A small amount of KCN is essential even through the preparation may be devoided of DPNH-oxidase.

the protamine in which the lyophilate and large particle showed be added back to each other (Tables 44 and 45).

Comparison of the two Tables (44 and 45) shows that all the measurable DPN-pyruvic dehydrogenase unit can be recovered in the protamine precipitate. However the $Fe(CN)_6$-pyruvic dehydrogenaes units are split between the two fractions, protamine precipitate and protamine supernatant. It may be conclusive that the series of reactions leading to DPN reduction[14] are still in one unit while the $Fe(CN)_6$ reaction requires two protein components one of which is not present in the protamine precipitate. It is also perhaps relevant to recall previous experiment on which demonstrates the complete localization in the protamine precipitate of the preliminary dehydrogenase and attendant reactions associated with factor formation.

Attempts to Demonstrate a Lipoic Acid Dehydrogenase or Pyruvate — DPN Reaction —— BHM sonicated, supernatant, collectcd and lyophilized. Dry powder dissolved in 15ml. 0.01 mole KPO_4, pH 7.4 and dialyzed against 300 vol μmoles 0.01 mole KPO_4, pH 7.4. There was no evidence of any denaturation resulting from this procedure. Using the standard pyruvate-DPN assay[15], this dialyzed material showed specific activity of 0.222 μmoles DPN/min./mg. protein. Al_2SO_3 treatment was stirred at room tempreature for 5 min. with Al_2O_3 (230 mg.) cooled to 0°, centifuged supernatant collected. The supernatant showed no less in protein. However there was a 75% less in activity to 0.005. This activity was not increased by oxidized Na-lipoate

0.4, 2.0, 7.0, 40 μg/cuvette nor did incubation at 0 (1.6mg. protein/2.0μg. lipoic acid), affect activity. Incurlsion of 1—3μmoles GSH in the reaction cuvette or prior to incuvation of the enzyme with GSH followed by lipoate 0.5—2.0 μg./3.0mg. protein, was likewise without effect.

Effect of Sonication of the Distribution of the Citric Acid Cycle Dehydrogenase and Associated DPN Enzymes——HBHM in 0.25M sucrose were sonicated separately for 2 min. and fractionated. The sonicate was centrifuged for 5 min. at 15,000 rpm (No. 30 Spince head) to give the large particle. The supernatant was centrifuged at 40,000 rpm for 30 min. to yield the small particles were washed with a small volumes of sucrose and wahsing combined with the initial supernatant. The large and small particles where then washed with a large volume of sucrose and washing discard. Recovery of material based on dry weight is 99% for 2 min. sonicates and 91% for 10 min. sonicates. All the protein determinations were repeated using 5 min. aliquot for the original, large and small fractions and 0.2ml. for the supernatant. Total protein recovery was 101% for 2 min. sonicated and 92% for 10min. sonicates. The supernatant from the 2 and 10min. sonicated were centrifuged at 40,000rpm for 90min. The spellet was an opalesent red in appearence indicating some heme was present.

Table 45. $Fe(CN)_6$-Pyruvic dehydrogenase distribution in protamine precipitate.

Fraction	Total Protein (%)	Unit	Unit (%)	Pyruic Dehydrogenaes Specific Activity (μmoles CO_2/min./mg.)
Unfractionated super.	100	3.00	—	0.030
Protamine ppt. (3.0mg.)	24	1.06	35	0.044
Protamine super. (lyopilized)(3.0mg.)	74	0	0	0
Protamine ppt.+protamine super.(26.7mg.)	—	—	—	0.139
Protamine ppt.+protamine super.(26.7mg.)	—	—	—	0.114

The supernatants were completely clear. To each of the supernatants, protamine sulphate was added to a final concentration of 0.3mg./ml., and preciptitate removed with centrifugation. The precipitates from the 2 min. and 10min. sonicates were combined and washed once with 20 volumes of 0.25M sucrose.

Table 46. Effect of sonication of the distribution of pyridino-protein dehydrogenase in TCA cycle and associated EPN enzymes.

Fraction	Protein (%)	Malic Dehydrogenase			Isocitric Dehydrogenase		
		S. A.	Total Unit	Total Unit (%)	S. A.	Total Unit	Total Unit (%)
Original super.	17	54.0	986	131	4.85	83	76
40,000 rpm protamine.	3.4	trace	—	—	trace	—	—
40,000 rpm super.	12	73.4	881	125	6.0	72	67
Protamine super.	8.2	100	820	118	8.50	70	65
Original super.	18.0	—	—	—	—	—	—
40,000 rpm particle	2.7	—	—	—	—	—	—
Protamine ppt.	2.9	trace	—	—	trace	—	—

Fraction	β-Hydrooxybutylic D.			α-Ketoglutaric D.			Pyruvic D.		
	S. A.	Total Unit	Total Unit (%)	S. A.	Total Unit	Total Unit (%)	S. A.	Total Unit	Total Unit (%)
Original super.	0.106	1.8	93	0.913	1.3	39	0.04	68	38
40,000 rpm protamine.	0.270	0.92	47	0.15	0.5	14	0.01	34	19
40.000 rpm super.	0.16	0.2	10	0.06	0.74	20	—	—	—
Super. Protamine	—	—	—	—	—	—	—	—	—
Original super.	0.87								
40,000 rpm particle	0.31								
Protamine ppt.	trace	0	—	0.18	0.54	14	0.09	0.28	17

Each flask contains 0.2 ml. BSA 10%, 0.1 ml $MgCl_2$ 20 μmoles, 0.1 ml. DPN 1 μmoles, 0.3 ml. KPO_4 20 μmoles, pH 7.0, 35 μmoles acetoaldehyde(neutolyzed), 0.2ml. H_2SO_4 2N(center well), H_2O to 3.2ml. and where indicated 30 μmoles substrates. The manometers were gassed with N_2 and at completion of 30min., H_2SO_4 was tipped to obtain values for the total CO_2 evolved.
S. A. (specific activity) = μmoles CO_2/min. /mg. protein
Unit = DPNH oxidized/min./mg. protein

In the Table 46 all on the % are expressed in terms of the original whole sonicate. Reduction of DPN was used to assay the pyruvic dehydrogenase in this Table, and there may be some question as to the validity of the assay of the protamine precipitate as there may have been a blank rate, or a sufficently high amount of CoA. Addition of CoA[1) 3)] did not stimulate the rate of DPN reduction as it did in other pyruvic assay. Present experiments may be conclusive that α-ketoglutaric dehydrogenase and pyruvic dehydrogenase can be centrifuged out at high speed in some and any enzyme left often this treatment can be precipitate with protamine sulphate. Malic and isocitic dehydrogenases were recovered almost 100% in the protamine supernatant.

Table 47. Oxidation of substrates of citric acid cycle to molecular oxygen by intact beef heart mitochondria

Substrate	Oxidation (μatomes O/min./mg.)	Phosphorylation (μmoles P/min./mg. protein)	P/O
Isocitrate	0.007	—	0
Citrate	0.015	—	0
Lactate	0.009	—	0
Malate	0.013	—	0
l-Glutamate	0.068	0.114	1.67
Pyruvat-Malate	0.100	0.278	2.78
β-hydroxybutyrate	0.111	0.210	1.95
Succinate	0.177	0.200	1.33
α-Ketoglutarate	0.050	0.140	2.83

Each flask contains 100 μmoles glucose 5moles $MgCl_2$, 0.5mg. yeast hexokinase, 5μmoles ATP, 30μmoles phosphate, pH 7.1, 1μmole DPN, 0. 1μmole CoASH, 0.5mg. TPN, 4mg. BSA, 2.0moles sucrose, 0.14μmoles $MnCl_2$ 0.2mg. purified cytochrom c and 20μmoles substrates. Incubate 20min. at 38°C. O_2 uptake measured by standard Warburg technique. Phosphate determined by method of Fisk and Subbarow.

Two preparations of morphorogical intact mitochondria were prepared in 0.88 % sucrose and they were assayed for oxidative phosphorylation[11]. The oxidative rate of isocitrate, citrate, lactate, malate, and pyruvate were zero in 11 experiments in the Tables 47, and 48.

Table 48. Oxidation of substrates by intact mitochondria after sonication.

Substrate	Rate of Activity	
	Cytochrom c added	Percent for Original
Isocitrate	0.127	∞
Citrate	0.146	∞
Lactate	0.060	∞
Malate	0.024	∞
L-glutamate	0.068	57
Pyruvate.malate	0.052	65
β-Hydroxybutyrate	0.114	100
Succinate	0.261	159
α-Ketoglutarate	0.068	144

VI. The Effect of RNA′ase Digestion of Sonicated HBHM

Determination on Ribonucleic Acid (RNA) in BHM——The estimation of RNA is based on the color reaction giving by its ribose moiety on treatment with orcinol in HCl. Many substrates other than RNA give color reaction with orcinol, and it is necessary to first separate the RNA from the interferring compounds with mitochondorial suspeusion. This is readily accomplished by selective extraction with hot TCA.

The first part of the method will therefore be concerned with the extraction procedure for nucleic acid and second part with the determination of ribose with orcinol.

Concentration of RNA′ase Required to Digest the RNA in Sonicated HBHM on α-Ketoglutaric and Pyruvic Dehydrogenase——Two pilot experiments have been carried out to determine the concentration of RNA′ase required to digest the RNA in sonicated HBHM. Whole HBHM 10ml. which had been sonicated for 80 sec. plus 1 mg. RNA′ase, incubated at 38°C, aliquotes for RNA determination removed after 10min. and 30min. In Table 49, a very sizeable residue of RNA, remaines; it was concluded that higher concentration of RNA′ase were required to effect of speedy

Table 49. RNA digestion in heavy beef heart mitochondria.

	μg ribose/mg. protein	Reduction in ribose(%)
Whole HBHM sonicated no added RNA′ase	0.63	—
10min. incubation with RNA′ase	0.48	24
30min. incubation with RNA′ase	0.38	40

digestion of total RNA, and also either a portion of the RNA was inaccessible to the enzyme or that a considerable amount of DNA was present(Fig. 6)

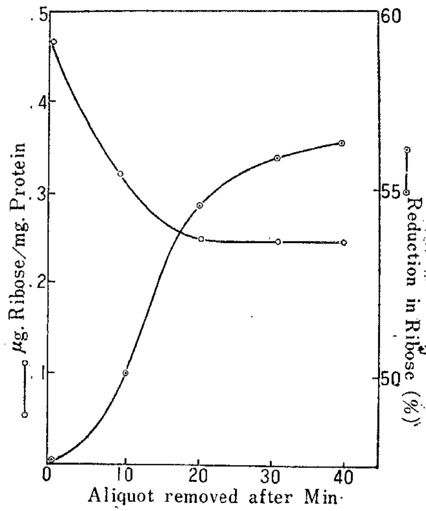


Fig. 6. The concentrations of RNA'ase required to digest the RNA in sonicated heavy beef heart mitochondria (HBHM). HBHM 14ml. (23mg. protein/ml.) which had been sonicated for 80 sec. were incubated with 14 mg. RNA'ase at 38°C. Aliquots were with down at interval and RNA determined.

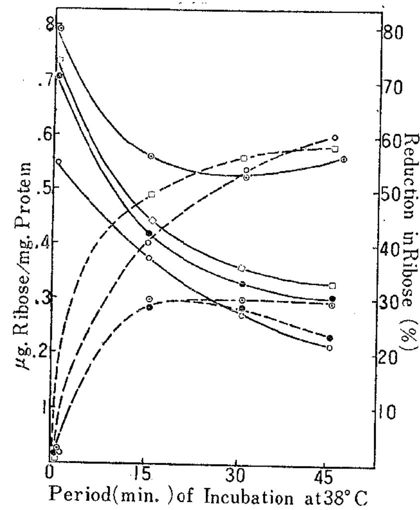


Fig. 7. Heavy beef heart mitochondria (HBHM)3ml. (50mg. protein/ml.) were sonicated for 100sec., diluted and fractionated into particle (40,000rpm for 30min.) and supernatant. The effect of RNA' ase was tested on HBHM, which sonicated of HBHM, particle and supernatant separated from the sonicate. RNA' ase was added a concentration of 0.2mg. /25mg. protein, sample for RNA analysis were with drown after 0, 15, 30 and 45min. at 38°C. Substrates are;- HBHM, ◉—◉, sonicated HBHM ●—●, sonicated particle □—□, sonicated supernatant ◎—◎ Dotted lines are expressed for reduction in ribose (%) for each substrate.

Effect of Sonication of HBHM on RNA'ase Digestion of HBHM and Fraction Therefrom —— These results are difficult to interprect all fractions are attacked to a varying degree by RNA'ase. A fort from the digestion of the HBHM there is no clear distinction of the whole sonicate and the particle and supernatant isolated therefrom. (Fig. 7). In view of the fact that 30% of RNA in BHM suspension can be readily digested the effect of RNA'ase on mitochondrial oxidation and phosphorylation was investigated. The results of this series of experiments and filled. No effect of the rate or extent of the oxidation or phosphorylations were detected.

Table 50. Concentration of RNA and DNA in sonicated HBHM.

SAPMLE	μg. ribose/mg. protein	μg. deoxyribose/mg. protein	Reduction in ribose(%)
A	0.53		—
B	0.32	0.01	38
C	0.27		50

SAMPLE—35ml. HBHM (50 mg./ml.) were sonicated for 30 secs. then diluted with sucrose approx. 1 : 1 and two 31.5 ml. aliquots were incubated as follows:—A, 31.5ml. diluted sonicated 15min. at 38°C;B, 30mg. RNA'ase+31.5ml. dilutedsonicated were incubated at 38°C and 0.27ml. aliquot was with down after 15min. And the rest withdown after 30min. (C)

Dipulicated 2.0ml. 2.0ml. aliquots of the three fractions were estimated for RNA and DNA.

Clearly either concentration of RNA exists which is not attacked by RNA'ase or else the total RNA is being partially digested by RNA'ase. No measurable concentration of DNA contaminates the mitochondria (Table 50).

Summary

The present work is concerned with the linkages by which the pyridino-protein dehydrogenases of citric acid cycle are bound to the electron transport system in phosphorylating beef heart mitochondria. Oxidative phosphorylation is abolished whenever mitochondria are fragmented by any procedure which leads to solubilization of pyridino-protein. The residue material responds aerobically to DPN and oxidation of DPNH is linked to phosphorylation. PETP was not greatly affected by added DPN but the oxidation of DPNH which did not coupled to phosphorylation. The oxidative activity of the Hughes pressed fragmented mitochondria is reduced to zero except in the presence of co-factor suplements, phosphorylating activity was completely destroyed by this procedure. The blending BHM with ballotive beads decreases in the rate of oxidation significantly. It is noteworthy that the observed P/O values are not significantly altered. The salient point appears to be that phosphate brings about extensive changes in the material irrespective of the Hughes press.

The rate of oxidation of pyruvate-malate by ETP is proportional to the concentration of supernatant. When ETP prepared wholly by different centrifugation with the use of the alkalic, phosphate of alcohol procedures, requirement for isocitrate does not, however, suggest any explanation for the results obtained with malate-pyruvate, other than possibility that supernatant contains condencing enzyme, malic, and pyruvic dehydrogenase. Blending of the BHM which producing considerable quantitives of soluble dehydrogenase does not result in the initial complete solublization of these dehydrogenase observed when phosphate buffer 0.15M is added either after blending or before comming to the BHM suspension. No ETP are produced except in the presence of phosphate could perhaps be accounted for in term of splitting on phospholipid. However this does not appear to be the functions.

As a preliminary to a study of conditions recovery to detach the pyridino-protein from the heme chain, using at level of cholate which did not affect phosphorylation *viz.*, 0.2mg. cholate/mg. protein, the effect of varying salt concentrations were examined. Residue material in the presence of 67 μmoles phosphate/15 mg. protein/ 3 mg. cholate is essentially devoided of phosphorylating activity whereas another residue (16μmoles P/15mg. protein/ 3 mg. cholate) contains a considerable amount of phosphorylating activity. The dialyzed supernatant has somehow picked up an inhibitor, the non-dialyzed supernatant appeared to neither inhibite nor stimulate phosphorylative oxidation was increase some 40 percent. If the protein concentration and cholate concentrations are increased 15 times extensive protein solubilization take place with abolition of phosphorylation and some 40 percent reduction in oxidation. The ethanol used in experiment has some effect on presenting the oxidative activity of cholate-phospahte treated mitochondria but it does not inhibit phosphorylation and oxidation at concentration of 330μmoles/manometer cup. The levels of cholate-phosphate required to solubilized some of protein HBHM lead to complete loss of oxidative activity. Possibility was considered that stimulation of phosphorylation by alkaline extract might be due to present of TPNH in the extract. TPN inhibited phosphorylation by some 40 percent. The presence of alkaline supernatant served porticularly

reverse this inhibition.

β-hydrobutyric and pyruvic dehydrogenase of heavy beef heart mitochondria can be centrifuged out at high speed with effective sonication in some and any enzyme left often this treatment can be precipitated with protamine sulphate. Malic and isocitric dehydrogenases were recovered almost 100 percent in the protamine sulphate supernatat.

The estimation of RNA in sonicated HBHM is based on the color reaction given by its ribose moiety on treatment with orcinol in HCl. High concentration of RNA'ase are required to effect of speedy digestion of the total RNA. Either a portion of RNA is inaccessible to the enzyme or that a considerable amount of DNA. Effort from the digestion of HBHM there is no clear distriction on the whole sonicate and the particle and supernatant isolated therefrom. Clearly either a concentration of RNA exists which is not attacked the RNA'ase else the total RNA is being partially digested by RNA'ase.

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