AN INVESTIGATION OF THE SEPARATION AND ACTIVITY OF NUCLEAR AND MITOCHONDRIAL FRACTIONS OF PLANT TISSUE

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A Thesis

Submitted to the Faculty of Arts and Science

in Partial Fulfilment of the Requirements

For the Degree

Master of Science

McMaster University

October 1955

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ACKNOWLEDGMENTS

I wish to acknowledge the assistance and interest of Dr. D. M. MacClement throughout the present study. I am grateful to Dr. R. H. Tomlinson of the Chemistry Department for the loan of the Electrometer and Ionisation Chamber, and to Mr. I. B. Stothers for the use of the Vacuum apparatus. I also wish to thank Dr. N. Radforth of the Biology Department for the loan of a Leica Camera and Attachment, and the use of his microfilm.

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INTRODUCTORY

An understanding of the structure and functions of Plant and Animal cells and their particulate and soluble constituents is important not only in the normal functioning and morphogenesis (58) of the cell, but also in the study of abnormalities of metabolism and growth produced by artificial and natural pathogens.

In the investigation of the cell structure, staining techniques based on particular metabolic reactions or on chemical linkage with the stain leads to inconclusive results, since the specificity of the stain is uncertain.

The use of Light microscopy is limited by the size range of the particles while the use of high magnification may lead to the production of artifacts, if no other method of investigation is used in comparison.

Bensley and Claude (45), to solve this difficulty, developed a method of fractionation of the Cell particulates by centrifugation, followed by a chemical analysis of the cell particulates.

Since centrifugation was initially used to separate the cell particulates, the method has been elaborated in order to improve the purity and

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Abbreviations

DNA	Desoxyribose nucleic acid	
RNA	Ribose nucleic acid	
TCA	Trichloroacetic acid	
CoA	Co enzyme A	
DNP	Dinucleopyridine	
TPN	Triphospyridine nucleotide	CoII
DPN	Di " "	CoI
RNace	Ribonuclease	

enzymatic activity of the isolated fractions. This method used in conjunction with isotopically labelled material, such as labelled Amino Acids or Phosphorus, enables one to investigate the activity of the cell particulates and their interdependence in cellular activity.

The uptake of labelled amino acids by cell particulates isolated by differential centrifugation in Plant and Animal tissues varies with the amino acid used (38, 37, 39), and with the tissue (40).

The universal presence of cell particulates has recently been demonstrated. Mitochondria first discovered by Altmann (6) in 1880, have been shown to be present, not only in Vertebrate (74, 120) and in Invertebrate cells (100) but also in Bacteria by the use of straining techniques (166, 167, 111) and the study of the enzymatic activity of the bacterial particulates (24, 108, 142).

The distinction between chloroplasts and mitochondria in Plant tissues noted by Gaston Bonner in 1920 has been confirmed using the technique of differential centrifugation.

Stafford (138) and other workers (66, 84) have been able to separate particulate fractions which have enzymatic activities similar to those found in Animal mitochondria. The oxidation of pyruvic acid and other substrates of the Krebs cycle (85, 97, 14, 96) and the oxidative phosphorylation (98, 22) shown by the isolated fractions, substantiate the presence of mitochondria in Plants, although their size differs from those of Animal mitochondria as can be seen in table VI.

The activities of Plant mitochondria have been recently discussed by Millerd and Bonner (107) and by Goddard and Stafford (66).

Microsomes are present in Mammalian cells (137, 50) and have been

reported in Plant tissues (101, 66, 157); but it is unknown whether they are present in Bacteria.

In Animal tissues labelled amino acids are incorporated in to both the mitochondrial and microsomal fractions (1, 134, 89, 88), the latter having the faster rate of uptake (5). In Plants, Webster's (157) investigation of the uptake of C^{14} glutamate by Bean hypocotyls substantiated this finding.

However, the rate of uptake varies whether the particulates are incubated as tissue slices (36), homogenates (164, 165, 39) or isolated particulate fractions (159) with the labelled amino acid.

The interdependence of the cell particulates has been shown in oxidative reactions, urea synthesis (131), glycolysis (130), in fat (145) and drug (31) metabolism. If the Plant particulates are not maintained in a condition similar to that found in vivo, the uptake of AAs by fractions may not correspond either in distribution or amount to that of the original tissue. In the study of the uptake and breakdown of phosphorus, Potter (123) found both the nuclear and mitochondrial fractions essential for maximum phosphorus breakdown: a mixture of nuclei to mitochondria in ratio of 1:1 having an oxidative activity equal to twice that of the mitochondria alone. Further Johnson and Ackerman (86) noted that the nuclear fraction added to the mitochondrial fraction, increased the oxidative phosphorylation by 280% and increased the amount of phosphate fixed by 200% of that of the isolated mitochondria. Stern has recently (139) confirmed Johnson and Ackerman's findings in vitro, but not in vivo. Siekevitz (135) noted that a fall off of the rate of phosphorylation occurred unless a

factor present in the supernatant was added to the mitochondrial and microsomal fractions. Siekevitz further found that the mitochondrial fraction free of microsomes did not incorporate labelled amino acids, although Borsook (39) found the mitochondria isolated at 10,000 g are capable of incorporating amino acids (160). Plant mitochondria isolated at 10,000 g are capable of incorporating amino acids and can synthesize peptide bonds (161, 158). Webster found in this case that the microsome fraction minus the mitochondrial fraction was unable to take up glutamic acid. Keller (88) noted that incorporation of amino acid required the microsomal fraction together with a heat labile non dialysable component of the soluble fraction.

In view of the faster rate of uptake of labelled amino acids by the microsome fraction and the high concentration of RNA in this fraction in Animal (114, 75) and Plant (138) tissues and the relationship of RNA and protein synthesis in Animal (5 , 80), Plant (158, 59), and in Bacterial cells (62, 64) the participation of the nucleus in either amino acid incorporation or protein synthesis has been questioned. It is unknown whether the uptake and incorporation is solely dependent on the cytoplasmic particulates or directly dependent (103) or indirectly dependent (28, 26) on the nucleus. Even the relationship between RNA concentration and protein synthesis has been questioned by Hokin (79), Chantrenne (43), and Laird (94).

In previous investigations the nuclear fraction has either not been considered (88) in relation to AA uptake or has been found to have a low rate of uptake of amino acids (4) in comparison with other fractions (83).

The isolation of Plant nuclei differs from that of Animal nuclei in

that media, such as citric acid (55) or sucrose solutions (77, 109) used for Animal nuclear isolation do not preserve the structure of the Plant nucleus.

Plant nuclei have been isolated by Brown (32) by grinding in benzene carbon tetrachloride mixture and by pectinase digestion, while Weier and Stocking (156) and others (84, 104) have prepared nuclear fractions of which neither the purity of the fraction nor the integrity is known. Weier and Stocking reported their nuclei were not of uniform structure.

(1) An investigation was therefore undertaken firstly to isolate a structurally intact Plant nuclear fraction by centrifugation in a suitable medium, and secondly to dis cover whether this nuclear fraction showed any uptake of labelled glycine C^{14} and if so, to compare its rate of uptake with that of isolated mitochondrial and supernatant fractions over a period of time. The accuracy of the estimation of the distribution of C^{14} in the fractions was increased by the use of a vibrating reed electrometer instead of an end window Geiger counter (168). Further by applying Zilversmits theoretical product precursor relationship (170) to ascertain if there is any interdependence of the particulates in AA uptake.

EXPERIMENTAL

Pencil Pod wax Beans were sterilized in a 7% calcium hydrochlorite solution for half an hour and then soaked in running water for 18 hours. The Beans were placed on moist filter paper in trays for one to two days. Root tissue was selected when the root was 1.5 cms in length, the first centimetre being used for incubation.

(1) Nuclear isolation

No attempt has been reported on the preparation of pure Plant nuclei consequently the isolation of Nuclei from Bean roots were investigated under varying conditions. The following media for isolation were tried.

0.25 M sucrose

0.25 M " + 0.0018 M $CaCl_2$ (78a)

0.45 M " + phosphate buffer (158)

0.50 M " + 0.001 M $Ca(NO_3)_2$ (156)

0.50 M "

85 and 95% glycerol (58)

Anderson's Solution I, II and III (163)

Table IV shows the results of varying the conditions of isolation on the state of the nucleus. The media such as .5 M sucrose or .45 M sucrose + calcium nitrate or .45 M sucrose + phosphate buffer fail to preserve the nuclei intact.

Centrifuging the Plant nuclei at speeds comparable to those which sediment Animal nuclei (table II) cause fragmentation of Plant nuclei (photo 6).

The final procedure adopted was as follows:

The Bean root tissue was homogenized in a pyrex glass homogenizer similar to that used by Potter and Elvehjem (124), for 15 seconds at 0°C in 4 c.c. of Wilbur and Anderson Solution II. In each case, 0.18 gms wet weight of Bean root tissue were used. The homogenate was filtered through six layers of Bolting silk (no. 20) in a Buchner filter pump. The homogenizer was washed with 5 cc of solution and the washings filtered and added to the homogenate. The homogenate was then centrifuged at 70 g (600 rpm) for six minutes and the supernatant withdrawn by a microsyringe when the nuclear were 'spun' down. The whole procedure was carried out in a cold room at a temperature below 5°C.

Microscopic examination

Smear preparations of the nuclear fraction were allowed to dry overnight and were then stained with methyl green pyronine as follows:

15 minutes in Methyl green pyronine at 63°C.

6 hours in tertary butyl alcohol (two changes)

1 minute in Acetone

Mount in Canada Balsam

DNA estimation

The amount of DNA in the nuclear fraction, together with that of the total filtered homogenate was estimated with a Beckmann quartz spectrophotometer (144, 53).

2.3 ml of ice cold TCA wine added to the nuclear fraction and the whole allowed to stand for two hours. The mixture was centrifuged at 1000 g for 10 minutes and the supernatant withdrawn. 0.05 cc of 5% cysteine

hydrochloride together with 5 cc of 70% H_2SO_4 were added and the mixture allowed to stand for 10 minutes at 25°C. The absorption at 490 mu was noted as % transmission of the solution, after the spectrophotometer had been corrected with a control to read 100% transmission with the aperture of the slit 0.02. The concentration of the DNA corresponding to the % transmission was calculated from a standard curve plotted for known concentrations of DNA against % transmission at 25°C (fig. 1). All estimations of DNA concentration were carried out at 25°C, since the reaction depends on the temperature. Estimations of total DNA in the filtered homogenate were made by centrifuging the homogenate at 2000 g for 15 minutes with the addition of 5 cc of TCA and then the procedure following was as above.

It was found necessary to dilute the total homogenate after treatment with cysteine hydrochloride and sulphuric acid after 10 minutes by 50% and then estimate the DNA concentration, since a linear relationship between DNA concentration and % transmission exists over 25 - 550 gms. DNA concentration.

Similar DNA estimations of the Nuclear fraction were made using Millerd's solution (105).

Nuclear

Results

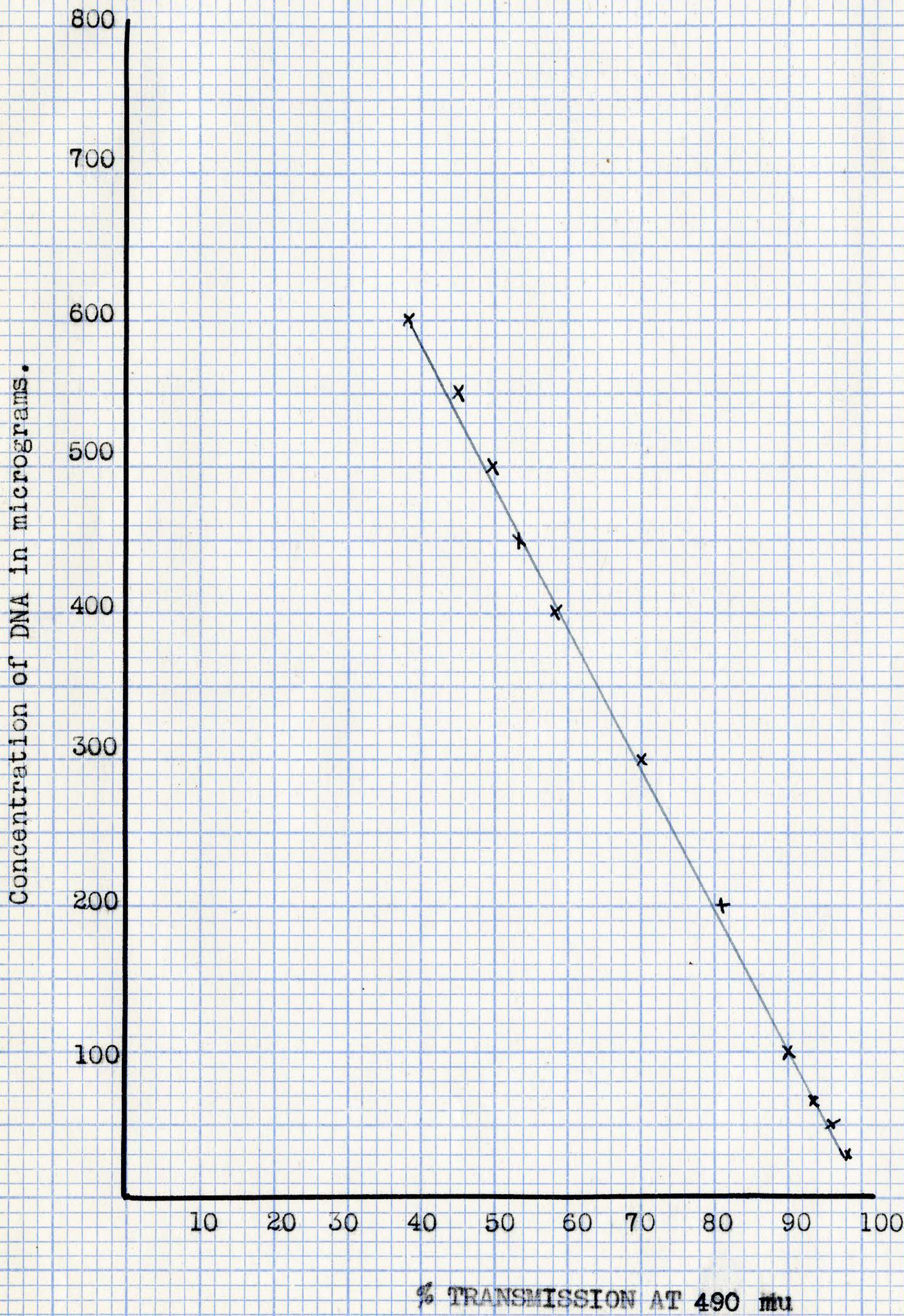
Microscopic examination

Methyl green pyroxine stained nuclei when viewed under the phase contrast microscope showed the nuclei isolated at 70 g for 6 minutes in Anderson II solution to be in good condition, the nucleoli being clearly

FIGURE 1

DNA CONCENTRATION PLOTTED AGAINST % TRANSMISSION

AS NOTED BY A BECKMANN SPECTROPHOTOMETER



visible (82). The nucleus shows a depression on one side, a fact which may be related to a difference in structure of the nuclear membrane in that area. The nucleoli are still present when this depression is present. On Holtfreter's criterion that the nucleolus is present only in an intact cell, the depression may not be correlated with any significant change in the nucleus. Centrifuging out the nuclei at higher speeds or at lower speeds for longer periods of time leads to the formation of cigar shaped nuclei, disruption and formation of bead like fragments (photos 5, 6).

DNA Estimations		% Transmission (average of 7 runs)	Range of Results
Nuclear Fraction in Anderson II		55.81	55.5 - 57.5
Total homogenate in	diluted by	50%	
a) Anderson II solution		54.30	52 - 57.0
b) Millerd solution		55.03	51.6 - 57.0

The nuclear fraction showed no activity of succinic dehydrogenase (54). It should be noted that the DNA has been shown to be present in the cytoplasm (169, 44); Schneider (78a) has estimated this to be approximately 7% of the total DNA in the cell.

From the estimations of DNA, the nuclear fraction contains 50% of the total cell DNA.

Mitochondrial isolation

Mitochondria form Bean roots were isolated as follows: Bean root tissue was homogenized in 4 cc of Millerd's solution (105) (.4 M sucrose + .1 M phosphate buffer) for 15 seconds at 0°C. In each

case 0.18 gms wet weight of Bean root tissue were used. The homogenate was filtered through 6 layers of Bolting silk (no. 20) in a Buchner filter pump. The homogenizer was washed with 5 cc. of solution and this filtered and added to the homogenate. The homogenate was then spun at 1,600 g (2,900 rpm) for 20 minutes to sediment debris and nuclear material. The supernatant was drawn off with a microsyringe and pipetted into a 20 cc. tube in ice water. 0.1% of 10% of 1.5 M KCl (90) was added to the supernatant and allowed to stand for 15 minutes. It was then centrifuged at 2000 g (51) for 20 minutes to sediment the mitochondria which aggregate under potassium chloride treatment. The supernatant was drawn off and retained, while the mitochondria were resuspended in 1 cc of Millerd solution. The procedure was carried out in a cold room at 5°C.

Microscopic examination

Smears of the suspension were prepared and stained by Harman's method (68) and counterstained with safranin O.

Procedure

Slides in 4% solution of F.G.F. in 10%

Aniline water 63°C, and allowed to cool for 8 minutes

Rinse distilled water

10 minutes in saturated solution of Picric acid

Rinse distilled water

2 minutes in a solution of 1% aq. phosphomolybdic acid

Rinse distilled water

10 seconds in safranin O in 50% alcohol

2 minutes in 70% alcohol

1 minute in 90% alcohol

1 minute in absolute alcohol

2 minutes in ethanol/ xylene 1:1

2 minutes in xylene

Mount in Clarite

Mitochondrial smears were also stained with methyl green pyronine to check for DNA impurity.

Succinic dehydrogenase activity

The enzymatic activity of the mitochondria was estimated by the activity of succinic dehydrogenase by a Beckmann spectrophotometer (126). In each experiment the initial wet weight of Bean root tissue was 1.5 gms, which was treated as above. To a Beckmann cell containing the following reactants:

0.3 ml. of .01 M Potassium cyanide

0.3 ml. of .001 M Potassium ferricyanide

0.2 ml. of 0.2 M.Sodium succinate ... was added .2 ml. of the mitochondrial suspension.

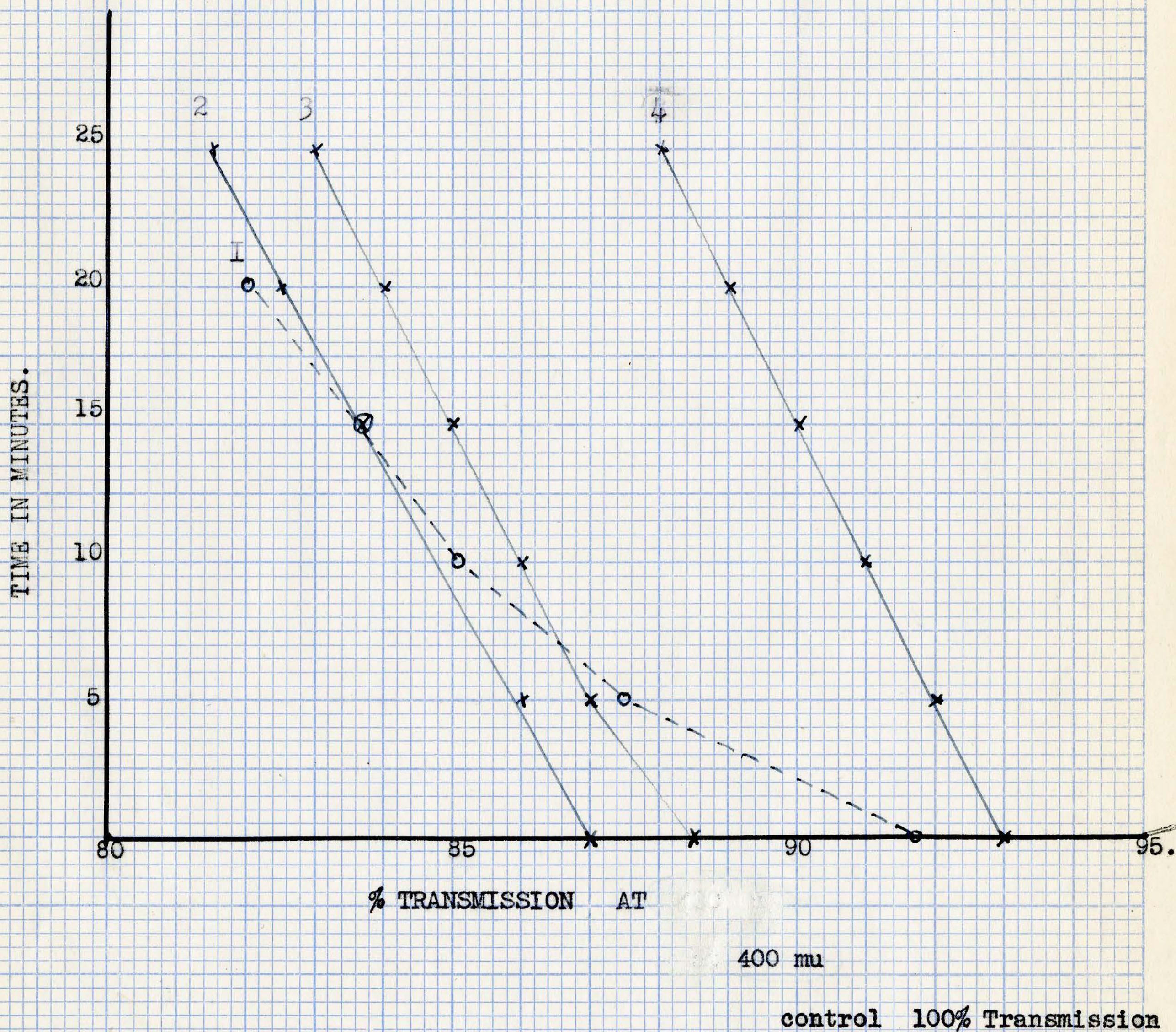
The volume of the cell was made up to 3 cc with phosphate buffer PH 7·2. A control was run minus the mitochondrial suspension. The volume being made up to 3 cc with distilled water. The change in the activity of the succinic dehydrogenase as % transmission at 400 mu at 25°C. over a period of half an hour was recorded (figure II).

DNA impurity of mitochondrial fraction

To estimate the DNA impurity of the Mitochondrial fraction 2.5 cc of ice cold 10% TCA was added to both the mitochondrial and supernatant

FIGURE II

SUCCINIC DEHYDROGENASE ACTIVITY OF THE MITOCHONDRIAL FRACTION OF BEAN ROOT TISSUE



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fractions and allowed to stand for 2 hours. The suspensions were then re-centrifuged at 2000 g for 20 minutes. The DNA were then estimated as described for the DNA estimation for the nuclear fraction.

RESULTS

The mitochondrial smear, stained according to Harman, viewed under phase contrast microscopy showed the mitochondria to be present in aggregated forms. The mitochondria under this treatment become rapidly spherical. If the mitochondria are examined immediately after separation at 2000 g for 20 minutes, the mitochondria appear rod like (photograph I), but when examined after 25 minutes at 20°C, they appear to be spherical and further aggregated (photograph 2). The presence of unknown particles (photographs 1 and 2) at present can not be identified since it is unknown to what extent the potassium chloride treatment affects the other cytoplasmic particles. However, that the mitochondria isolated are enzymatically active, is shown from the graph of the activity of the succinic dehydrogenase (fig. 2). It can be seen from the graph that the results of the four experiments show that the fractions have comparable activities, although it is not known why identical initial weights of Bean tissue show different quantitative activities. In the first curve (I), the temperature of the mitochondrial fraction was allowed to rise from 0°C to 25°C, during the measurement of the succinic dehydrogenase activity. If the sedimented mitochondria are resuspended in Millerd solution at 25°C, the activity shows a linear increase over the time of the investigation. The mitochondrial fraction possess a 7% impurity of DNA, as shown from table 8, which occurs in solution since it does not stain visibly with methyl green pyronine. It

was found that increasing the speed of centrifugation from 1,500 g to 1,600 g, a 10% decrease of DNA in the mitochondrial fraction resulted, although the supernatant fraction increase in DNA concentration by 10%.

To what extent the treatment with potassium chloride affects other cytoplasmic particles is unknown.

TABLE 8

% IMPURITIES OF DNA IN MITOCHONDRIAL AND SUPERNATANT FRACTIONS

Mitochondrial Fraction isolated at 2,000 g	DNA Impurity as average of values	% Transmission range of values	% Impurity of fraction	No. of runs
after initial fraction isolated at 1,500 g	83.8	(82 - 85)	17%	(5)
after initial fraction isolated at 1,600 g	93.1	(94 - 94)	7%	(4)
Supernatant (2,000 g)				
after initial fraction isolated at 1,500 g	69.5	(66 - 75.5)	31%	(3)
after initial fraction isolated	59.8	(57 - 63.5)	40%	(3)

Incubation of Fractions with $2C^{14}$ glycine

In each experiment 0.18 gms wet weight of Bean tissue was sectioned at 30 into .6 cc of .05 M potassium phosphate at PH 6·5 at 25°C (158). Three drops of C^{14} glycine (.1 mc in 8.7 mg of glycine in 1 cc of distilled water) were added and the whole incubated for varying periods of time.

Nuclear Incubation

The medium was withdrawn with a microsyringe and the slices washed twice with Wilbur and Anderson solution II. The slices were then homogenized, etc., as in the procedure for Nuclear isolation. The nuclear sediment was suspended in 3 cc of Anderson solution and transferred to a 25 c.c. combustion flask, and the suspension evaporated to dryness on a water bath.

Mitochondrial Incubation

A similar procedure of washing the slices with Millerd's solution, followed by the mitochondrial isolation method described, was adopted.

Counting Procedure

The combustion flask containing either the nuclear or mitochondrial dry sediment was fitted to a vacuum line through which carbon dioxide was flushed. The ionization chamber was evacuated and flushed twice with carbon dioxide. The final evacuation to a pressure of .1 mm took 15 minutes. The chamber was transferred and C^{14} collected in the chamber according to Burr (34).

The ionization chamber was transferred to a Vibrating Reed electrometer; Under a Potential difference of 300 volts, the number of disintegrations is summated and the time of the sweep of the pointer from zero to 1 millivolt noted (readings set at 1/100 volt).

Results

Counts were carried out on ... a) background
b) nuclear and supernatant fractions
c) fraction sedimented at 1,600 g
d) fraction sedimented at 2,000 g

and plotted on a graph of the time of sweep against the time of incubation. Background count taken initially to check the ionization chamber was 36.20 minutes for one sweep.

It can be seen from Figure 3 that the mitochondrial fraction has the slowest uptake. There is rapid uptake by the supernatant (microsomes and supernatant) in the first 2 hours which increases linearly. It is of interest that the nuclei are initially slow in uptake for the first two hours and then rapidly increase. The rate of uptake being comparable to that of the supernatant fraction. Unfortunately more labelled glycine was not available to enable further investigation of the uptake of nuclear fraction after 3½ hours.

TABLE I

Tissue	Medium & Method of Homogenization	Time & Speed of Centrifugation	Reference	Method Used to Investigate
Animal tissue				
Mitochondrial and Microsomal Fractions				
Mouse liver	homogenized in P.E. 20 sec. in 2 cc of .1 NaOH/litre of .85% NaCl	1,400 g Nuclear 4 min. 23,000 g Mito. 5 min. 23,000 g Mic. 90 min.	Omachie et al (115)	Enzyme estimation
Rat liver	homogenized in P.E. in .25 M sucrose	800 g Nuclear 10 min. 5,000 g Mito. 10 min. 110,000 g Mic. 25 min.	Strittmather & Ball (143)	Enzyme distribution
Heart muscle	homogenized for 75 sec. in .28 M sucrose + 8 ml of .04 M $NaHCO_3$pH 7·0 and filtered through gauze	150 g Nuclear 10 min. 2,700 g Mito. 20 min. (Total)	Harman & Feigelson (69)	Preparation of enzymatic active mitochondria
Rat liver	homogenized in .25 M or .88 M sucrose passed through hypodermic needle at 0°C.	700 g Nuclear 20 min. 5000 g Mito. 20 min. 148,000 g Mic. 30 min. etc (data 0.25 M sucrose)	Hogeboom & Schneider (76)	Enzymic distribution
Pigeon	homogenized in glass homogenizer in (Tris KCl) medium	600 g Nuclear 5 min. 8,000 g Mito. 30 min.	Perry (121)	Bioproperties of mitochondria
Rat liver	homogenized in P.E. in .88 M sucrose for 2.3 min. at 0°C.	211 g Nuclear 10 min. 114,500 g Mic. 69 min.	Novikoff (114)	Fractionation of cell particulates
Rat liver	homogenized pulled through steel in .25 M sucrose and homogenized in	Centrifuged as in J.B.C. 183 but mito spun with density gradient 2.22 - 6.36 Ms. at 108,000 g	Kuff and Schneider (92)	Enzyme distribution in fractions of mitochondria

TABLE II

Tissue	Medium & Method of Homogenization	Time & Speed of Centrifugation	Reference	Comment
Animal tissue				
Nuclear Isolation				
Rat liver	homogenized in .25M sucrose at 0°C.	300 g Nuclear 7 min.	Johnson & Ackermann	of nucleus in cell
Rat liver	homogenized in waving blender in ice water and then .1 M citric acid pH 6 - filtered through cheesecloth	less than 600 g	Dounce A.NYASSo (54)	Nuclear isolation
Rat liver	homogenized in Ball type homogenizer in .44 M sucrose pH 6.0 - 6.2		Dounce (57)	"
Rat liver	homogenized in Potter Elvehjen homogenizer. 2 min. in .25 M s + .0018 M $CaCl_2$ & with .24 M sucrose + .00018 g $CaCl_2$	2,000 rpm N. 40 min. total	Hogeboom & Schneider (78a)	"
Rat liver	homogenized with hand homogenizer in sucrose bicarbonate solution and layering method	475 g 8 min. initial speed etc.	Wilbur & Anderson (163)	"
Lyophilised	tissue frozen and dry powder suspended in petroleum ether	centrifuge & separate using cyclopropane carbon tetra-chloride mixture	Mirsly (109)	
Rat liver	frozen liver in 70% glycerol, washed, stained and homogen-ized at high speed		Schneider (129)	Nuclei good condition
Rat and Rabbit liver	homogenized in Potter Elvehjen homogenizer in 75% glycerol at 4°C and filtered through 4 layers of cheesecloth & rehomogenized in 95% glycerol	900 g Nuclear 10 min.	Dullam (58)	

TABLE III

Tissue	Medium & Method of Homogenization	Time & Speed of Centrifugation	Reference	Comment
Plant				
Nuclear isolation				
Rye embryo & root tips	Grind in mill & spin in benzene, carbon tetrachloride mixture or peptic digestion of root tips for 3 hrs. at 5°C. grind and filter	3 hrs. at 5°C.	Brown (32)	State of nucleus unknown
Tobacco leaves	leaves ground in cold m/15 phosphate buffer pH 7.0 in .4 M sucrose	76 - 600 g sediments nuclei	Jagendorf & Wildman (84)	State of nucleus unknown
Tobacco leaves	homogenized in Waring blender for 30 seconds in M/2 sucrose + .01 M phosphate buffer pH 6.8 at 0°C.	60 g 20 min. green sediment containing nuclei 20,000 g 20 min. fragment chloroplasts	McClendon A.J., (104)	Impure separation of nucleus

TABLE IV

Medium	Speed of Centrifugation	Time of Centrifugation in minutes	Comments
0.5 M sucrose	700 g	5	nucleus fragments
	30 g	15	forming bead like fragments nuclei fragmented
0.45 M sucrose in phosphate buffer	90 g	3	nuclei fragmented
	65 g	5	nuclei fragmented
	60 g	5	and not complete sedimented
0.5 M sucrose in 0.001 M calcium nitrate	70 g	8	nuclei fragmented
	50 g	6	nuclei not completely sedimented
0.25 M sucrose	70 g	4	nuclei granular &
	60 g	10	badly fragmented
Anderson solution I	70 g	4	few nuclei present
	60 g	10	nuclei not completely sediment
Anderson solution III	50 g	7	nuclei present but
	60 g	5	majority fragmented
	70 g	10	
Anderson Solution II	80 g	4	few nuclei - good condi
	70 g	10	nuclei present in " "
	70 g	6	50% nuclei " " " "
	70 g	5	nuclei present " " "
	60 g	5	nuclei not completely sedimented

N.B. Where the method of preparation yielded nuclei in good condition the time of the speed of centrifugation were changed until a maximum amount of DNA was found in the fraction. The fraction was checked to see the state of the nuclei under the conditions of the separation.

TABLE V

Tissue	Medium & Method of Homogenization	Time & Speed of Centrifugation	Reference
Plant **Mitochondrial Isolation**			
Bean Tissue	homogenized in 6 sec. in .1 M sucrose + .1 M potassium phosphate pH 7.0 at 2°C.	3,000 g 10 min. 14,000 g 15 min. Mito	Beaudreau and Remmert (14)
Mung Bean and Pea Hypocotyls	grind with quartz sand in .4 M sucrose + .1 M KH_2PO_4 + .1 M Na_2HPO_4 pH 7.1 and filter through cheesecloth	500 g 5 min. 10,000 g 15 min. Mito	Bonner and Millerd (22)
Pea Tissue	using Kennedy's KCl preparation (JBC, 179)	1,700 g Nuclei & 7,000 g Mito	Davies (51)
Cauliflower Inflorescence	homogenized in sand and water at 0°C. pH 6.6	500 g 5 min.	Laties (98)
Pea Stems	chilled tissue ground in P.E. in .2 M sucrose + .03 M PO_9 pH 7.0	500 - 1,000 g starch & nuclei 8,000 g Mito	Price & Thimann (125)
Lupin Seedlings	grind in mortar with sand or .5 M sucrose + phosphate buffer pH 7.0 - 7.4	16,000 g Mito	Conn & Young (48)
Chloroplast Isolation			
Beta Vulgaris	homogenized in a Waring blender for 3 mins. in M/15 potassium phosphate at 4°C. and filtered through cheesecloth	3,000 rpm. 1 min. large granules 25,000 g 20 min. chloroplasts	Arnon (8)
Algae and Liverworts	homogenized in Waring blender in .5 M S for 5 min. & filtered through nylon	800 g starch granules 15,000 g 20 min. chloroplasts	Clendenning and Gorham, P. (46)
Spinach	homogenized in Waring blender in .05 M $KHPO_4$ + .5% KCl pH 7.0 & filtered through muslin	2,000 g 10 min. 20,000 g 20 min.	Vischnac and Ochoa (154)
Tobacco Leaves	homogenized in Omni mixer in cold M/15 phosphate buffer pH 7.0 in .4 M sucrose	650 g 12 min. whole chloroplasts	Jagendorf & Wildman
" "	homogenized in Waring blender in .5 M glucose or .05 M PO_4 pH 6.5 at 5°C.	200 g 5 min. chloroplasts	Granick & Potter (67) Granick & Potter A med. J. Bot

TABLE VI

Tissue	Medium of Isolation	Diameter of Mitochondria in	Author
Rat liver	.25 M sucrose	.55 - .94	Appelman and DeDuve (6a)
" "	.25 M "	.41 - .86	Davidson and Smellie (50)
" "	isotopic saline .88 M sucrose	.5 - 2 .3 - .5	Hogeboom and Schneider (74)
Animal tissue	E. M. study of fixed tissue	.2 - 1. varies with tissue	Palade (118)
Myoberteria	E. M. " "	.5 - .75	Mudd (111)
Flight nuclei of Drosophila and Phormia	phosphate buffer & bovine plasma albumen	.25	Williams and Watanabe (155)
	"	1. - 7.5	Levenbrook (100)
Peas	in water	.1 - 6.0	Stafford and Goddard (66)
Tobacco leaves	.4 M sucrose + M/15 phosphate buffer	.2 - 2 (length)	Jagendorf and Wildman (84)
Bean seedlings	.4 M sucrose + .1 M phosphate	.5 - 2 (dia)	Millerd (105)

TABLE VII

Microsomes

Tissue	Medium of Isolation and Time of Centrifugation	Diameter of Microsome	Author
Rat liver	.25 M sucrose 25,000 g 1 hr.	10-200 mu	Davidson and Smellie (50)
Rat liver	Adopted Hogeboom and Schneider's technique (6)	20-150 mu	Abood and Romanchek (12)
Rat liver	.88 M sucrose 100,000 g 1 hr.	100-150°A	Palade and Siekevitz (119)
Rat pancreas	.25 M sucrose + phosphate buffer. 40,000 rpm.	10 mu	Allfrey and Mirsky (5)
Rat liver	.88 M sucrose - 41,000 g	greater than 50 mu	Hogeboom and Schneider (73)
Rat liver	.44 M sucrose at 114,500 g	33.0 mu 177.0 mu 351.0 mu	Novikoff (114)
Rat liver	.88 M sucrose at 137,000 g	129 mu 22 mu 78.7 mu } means	Slautterback (137)

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DISCUSSION

The initial separation of cell particulates by Bensley (16) and Claude (45) using differential centrifugation was based on the size differences of the particles which possessed specific chemical and enzymic constituents.

Further investigation of the enzymatic activity of fractions has shown that a number of enzymes are localized in more than one fraction (133, 71, 125) (Table I), the percentage of the enzyme, for example in the case of alkaline phosphatase, varying with the rate of sedimentation of the fraction (117, 114). Similar non specific localizations of enzymes were found in the microsomal fraction by Glick (115) and Schneider (92).

The fractions initially defined as mitochondrial and microsomal which were analyzed chemically by Schneider and Hogeboom (128) and Swanseon (147) were found to contain particles of different size (Table VI). Ada (1) divided the mitochondrial fraction into large and small granules on the basis of different chemical constitutions of the granules.

Investigation of the mitochondrial fraction, using labelled Phosphorus, ultra violet absorption (116) and chemical analysis (95) has lead to further subdivision of the mitochondrial fraction (92, 114, 33).

Similar subdivision of the microsomal fraction into distinct and separate fractions has been demonstrated by Slautterback (137) and others (33, 114) (viz Table VII).

Whether this heterogeneity of Animal mitochondrial fractions is the result of Biochemical differences (92, 114) or of distinct classes of

particles (6a) is uncertain, although Buchner and McGurrahan (33) noted that in rat liver tissue, cholestrol synthesis occurred in the large microsome fraction. The heterogeneity is also complicated by the fact, in the case of the mitochondria, that mitochondria vary with the type of tissue concerned (118), different genetical strains appearing to have chemically different mitochondria (42). This has also been found in Bacteria (112). It is unknown whether Plant mitochondria show either biochemical or genetical differences.

Apart from the heterogeneity of the fractions, it is of interest to consider the boundaries which separate the fraction. When one considers the fraction denoted as microsomes, the separation between microsomes and supernatant shows clearly the lack of a distinct boundary. (Table VII) Hogeboom and Schneider (73) sedimented particles in the microsomal fraction down to a particle size of 50 mu, while Novikoff's(114) minimum size of microsomes was 41 mu diameter. Recently Allfrey and Mirsley isolating microsomes in .25 M sucrose included particles to a minimum of 10 mu in the microsomal fraction. It seems likely that Novikoff's and Slautterback's small microsomes were included in Schneider and Hogeboom's supernatant. How reliable the distinction between particles of these sizes under the methods of isolation can be seen from the effects of the media and centrifugation on the structure (Table VI - compare diameters of mitochondria) and enzymatic activity of the particles (70, 76).

Two methods of fractionation of cell particulates by centrifugation are used:

(1) Density gradient separation

(2) Differential speed separation

(1) Holter (81) and others (109, 148) have investigated the separation of particulates by gradients of sucrose solutions, based on the density difference of the particulates (29).

Although this method separates the particulates it gives only an average range of size of particulate per fraction and does not take into account possible osmotic changes produced by different concentrations of sucrose.

(2) Novikoff (114) using differential speed separation, applying Pickel's Equation (122) to calculate the centrifugal forces required to sediment particles of assumed density, found nine fractions to be present in mammalian liver. It should be noted that separations based in Pickel's Equation are subject to two assumptions: (1) The particles are spherical. (2) That the density of the particles is known. Kuff and Schneider (92) similarly demonstrated subdivisions of their mitochondrial fractions, and showed clearly the different results obtained by the two methods. The enzymic distribution varied according to the method of separation. Berthet and DeDuve (19) have recently used a new method for separation. The efficiency of separation of fractions can only be judged therefore if both the homogeneity and the percentage receovery of enzymatic activity are considered.

The medium of isolation affects the particulates structure and enzymatic activities. In considering the effects of the isolation medium on nuclei, the importance of the preparation of enzymatically active nuclei, is seen in the fact that Schneider and Hogeboom (78a) claimed the nucleus to be enzymatically intact, although Dounce (55) critizes this claim.

Since Behrens (15) published his aqueous extraction procedure for

nuclear isolation, a number of modifications of this method have been used (91). Nuclei resulting from these procedures are subject to the extraction of water soluble enzymes (78) while Tyler and LePage (151) have critized Dounce's modification of Behren's Technique, which they claim, leads to distorted nuclei and cytoplasms contamination. Many isolations of Plant particulates have been carried out in aqueous media (138, 98).

Media containing citric acid (102, 56) or sucrose (78a, 57) used for nuclear isolation, extract the protein (63, 109) and can not be used for the determination of intracellular distribution of soluble enzymes (140). Animal nuclei have been prepared in good condition in glycerol (129, 58) and in Wilbur and Anderson's solution.

In the isolation procedure used, it was found that Anderson's solution II (specific gravity 1.031) gave plant nuclei in good condition as shown by microscopic examination of methyl green pyronine stained nuclei. Kurnick (93) has shown that the reaction is specific for DNA.

With Animal mitochondria the effects of different concentrations of sucrose solutions (71,69) and salts (126,33) on the enzymatic activity have been found, while in Plant mitochondria similar effects have been noted (135, 125). The structure of the mitochondria is affected by the Tonicity of the medium (74). As a result of this, versene (120) or bovine serum albumen (125,155) have been added to the medium.

In isolating the mitochondrial fraction in Plants, it is necessary to remove possible contamination by chloroplasts or grana. As shown from Table V, the speeds of centrifugation necessary to sediment mitochondria and grana overlap. To remove this difficulty non photosynthetic tissue or etiolated tissue (125) are used.

In the procedure adopted the isolated Plant mitochondria are stained according to Harman (68) technique in preference to Janus Green B, although Janus Green B has been widely used as a specific stain to demonstrate the presence of mitochondria (124, 111). Bensley (17) cautions against this specificity, while Lazarow and Cooperstein (99) showed that any enzyme system capable of reducing diphosphopyridine nucleotide and flavoprotein reduced Janus Green B. It is known that potassium chloride apart from aggregating the mitochondria, inhibits enzymatic activity (126). However, in this investigation it is unknown to what extent this treatment affects the microsomes, or chloroplasts. From the activity of the succinic dehydrogenase of the isolated mitochondrial fraction, it appears the mitochondria isolated were enzymatically active.

From the heterogeneity of the fractions, the effect of the medium and centrifugation, the reasons for the lack of agreement in the uptake of labelled amino acids by fractions of cell particulates is apparent. In view of the interdependence of cell particulates, accurate separation of fractions, with known concentrations of impurities, is essential.

A comparison of Plant and Animal cell particulates shows similarity in properties. Mitochondria in both Animals and Plants possess enzymes of the Krebe cycle (51, 124, 131) and cytochrome oxidase (62, 127), and will oxidise substrates of the Krebs cycle (106). Millerd (105) found that plant mitochondria unlike Animal mitochondria were independent of enzymes and ATP for normal activity, although Stafford's (138) findings are contrary to this. From the results of James and Elliott's (85) investigation on the effects of Potassium cyanide on the respiration of mitochondria, a second oxidative system may be present. Fatty acid oxidation takes

place in Animal mitochondria (131) while in Plants, Stumpf (146) observed that in hypocotyls of peanuts, the microsome fraction participated in oxidation of fatty acids. Stumpf also noted that no addition of cofactors, such as CoA, ATP or DNP were required. No explanation of the independence of cofactors of plant mitochondria and microsomes has been found. Glycolytic enzymes occurring in the soluble fraction in Animal liver tissue (131) have been claimed to be present in the nucleus of
in Plants (146).

Plant cells are complicated by photosynthesis and starch metabolism. Chloroplasts, or their constituents, grana, apart from the specific reaction of photo reduction (72, 162) and the coupling of this with TPN, DPN (153, 154) and nitrate reduction (60) are capable of synthesis of ATP (152, 9). The fixation of carbon dioxide takes place in the chloroplasts (7) and in other cell fractions (47), although Tschen and Vennesland (149) find different methods of fixation in Plants. The activity of the chloroplasts overlaps that found in Animal mitochondria. In the mitochondrial fraction isolated no chloroplasts were found, although unknown particles were present as seen from the photographs (Figure 5). DNA estimation and staining proved that the particles were not of nuclear material.

The incorporation of labelled glycine by cell particulates and the general metabolism of glycine has been discussed by Tyler (151) and Arstein (10). Incorporation of glycine into cytoplasmic RNA (61) and nuclear RNA and DNA (150) has been observed.

Tyler et al (151) investigating the biosynthesis of nuclear acids in normal and tumour tissues, with P^{32} and $2C^{14}$ glycine, demonstrated

that the nuclear RNA had an higher activity than the cytoplasmic RNA in the ratio of 6:1. Tyler and LePage observed the maximum activity of the nucleus appeared two hours after injection of the labelled material. Similar results were obtained by Davidson and Smellie and others (12, 23). The differences in the time of maximum activity of the nucleus probably depends on the state of the tissue and the conditions of the medium (52).

Gale and Folkes (63) and Allfrey and Mirsky (5) have shown a correlation exists between the rate of protein synthesis and the concentration of the RNA. Chantrenne (43) claimed that RNA was not the limiting factor for the incorporation of Amino acids, while Laird (94) found that doubling of nuclear volume caused by an increase of nucleoprotein, was not associated with a change of RNA concentration. This was also observed by Alfert and Bern (3) when nuclei were treated with estrogen. In Plants, Bonner (21) was unable to note any effect of Auxins in the uptake of labelled amino acids. Gale (62, 64) has further shown that the uptake of Amino acids not only depends on the RNA concentration, but also on the specific nucleotide fraction present. Treatment with RNase leads to the fall off of Amino acid (28, 159). Danielli (49) has recently discussed the relation of RNA and protein synthesis.

That Plant and Animal microsomal fractions have a high concentration of RNA, to which protein is bound (5, 59) and show the highest rate of uptake of Amino acids (87, 159) substantiates the above finding.

Ada, in an attempt to elucidate the relationship of the cell particulates, applied Zilversmit's (170) hypothetical relationship of precursor product to the particulate fractions. On comparison of the

specific activities of the particulate fractions, no relationship was apparent, the fractions behaving independently of each other. Allfrey and Mirsky applying the precursor product relationship demonstrated a relationship existed between the microsome pellet and the mixed tissue protein.

From the Figure 3, the rapid uptake shown by the supernatant (microsome and supernatant combined) confirms previous findings, although no relationship between the particulates is apparent.

The Nuclear fraction shows a slow initial uptake followed by a more rapid uptake after 2 hours, which is considerably greater than appears from the graph, since only 50% of the nuclei are isolated in the fraction. In Animal tissues, Tyler and LePage (151) observed a similarly slow initial uptake which increased after 3½ hours, while Barton (13) found a correspondingly high nuclear uptake after 3 hours.

The microsomal fraction has been shown to be associated with Amino acid uptake and protein synthesis. The role of the nucleus in protein synthesis, if any, is however unknown. Brachet (24) has suggested that the nucleus is concerned in oxidative phosphorylation, while Hogeboom and Schneider claim it is associated with synthesis of DPN. It should be noted that little agreement is found on the enzymes actually present (109, 11, 41, 139).

Whether the uptake of labelled glycine after two hours by the nuclear fraction is related to protein synthesis or nucleic acid synthesis is unknown.

In the future, the role of the nucleus might be elucidated by the use of Briggs and King's (30) nuclear transfer technique, using

labelled nuclei, followed by isolation of the cytoplasmic particulates by differential centrifugation. The isolated particulates could then be tested to see if any transfer of labelled material had taken place.

The role of the nucleus in relation to the cell particulates remains unexplained.

CONCLUSIONS AND SUMMARY

A Nuclear fraction containing 50% of the total nuclei of the initial wet weight of tissue can be isolated in a pure state, using Wilbur and Andersons' solution.

Mitochondria, isolated in Millerd's solution by Kennedy and Lehninger's Potassium Chloride procedure, although aggregated and varying in appearance from rod-shaped to spherical are enzymatically active.

The most rapid uptake of I.C.14 glycine by the nuclear fraction, a rapid increase in the rate of uptake takes place after 2 hours. The reason for this increase is unknown.

The Mitochondrial fraction shows a slower rate of uptake than either the Supernatant or the Nuclear fraction after 2 hours of incubation.

In the comparison of the uptake of Glycine by the above three fractions, it should be noted that the Mitochondrial fraction possesses a 7% impurity of DNA, while the Supernatant has a high DNA impurity of 40%. In centrifuging the initial homogenate, the DNA passes into the soluble fraction, the concentration increasing with increasing speed of centrifugation.

It is interesting to note that Bean root tissue fractions show comparable activities to those of Animal cell fractions.

No relation between the activities of the different Plant fractions can be seen when Zilversmits relationship is applied.

It appears therefore that the Microsome/Supernatant fractions in

Plants are associated with A.A. uptake and protein synthesis, although the role of the Nucleus in relation to the uptake of the uptake of A.A.s and protein synthesis is unknown.

APPENDIX

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Precursor A

Product B

If p = rate of conversion of A to B (const. assumed)

R = amount of B present

x = amount of B present in radioactive state

$f(T)$ = s.a. of immediate precursor A

Amount of radioactivity converted into B per unit Time is $p\,f(T)$

and amount of radioactivity lost from B is $p\,\frac{x}{R}$

Therefore, rate of change of amount of radioactivity in B in the tissue

$$\frac{dx}{dt} = p\left[f(T) - \frac{x}{R}\right]$$

Divide by x

$$1/x\,\frac{dx}{dt} = p\,\frac{f(T)}{x} - \frac{p}{R}$$

Therefore plot $\frac{1}{x}\,\frac{dx}{dt}$ against $\frac{f(T)}{x}$ P and R consts.

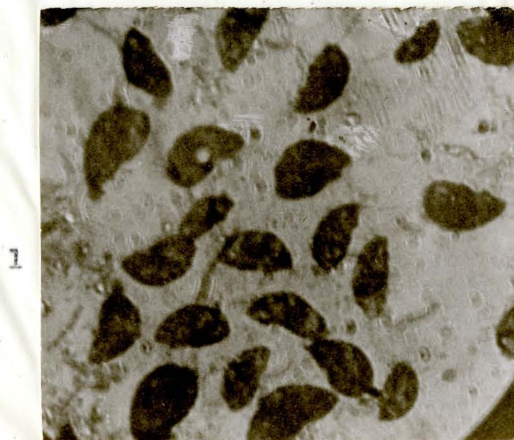
A precursor product relation if straight line obtained.

A — a precursor product.

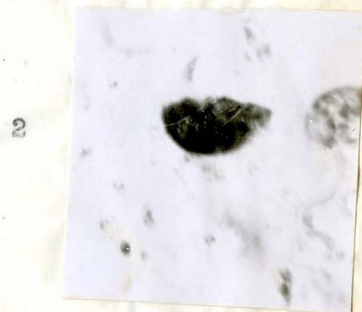
FIGURE 3

NUCLEAR PHOTOGRAPHS

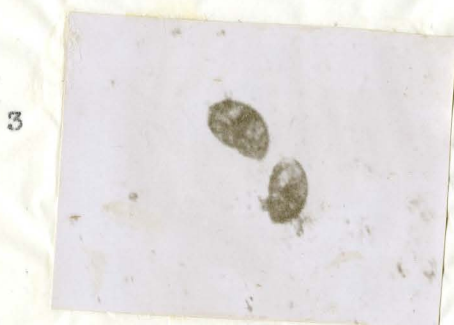
1



2

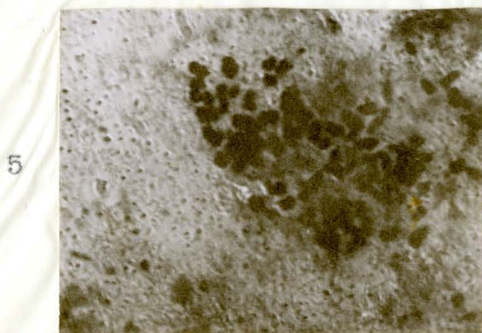


3



4

5



6

Phase Contrast 40x
Magnification 400x
Taken with microfilm. 5-15 sec. exposure.

FIGURE 4

Photographs taken with a Leica Camera attached to a Phase contrast microscope with an Ibsol attachment.

1) Photograph of the Nuclear fraction isolated at 70 g for 6 mins showing nuclei with nucleoli.

2) Photograph of Nuclei showing the presence of nucleolus and lateral depression.

3 & 4) Photographs of Nuclei showing the presence of nucleolus without a lateral depression, which is not shown by allthe nuclei of the fraction.

5) Photograph of the Nuclear fraction forming bead like fragments caused by excessive speed of centrifuging (100 g for 10 min.).

6) Photograph of the Nuclear fraction drawn out into cigar-shaped nuclei by centrifugation at 70 g for 15 minutes. The nuclei were not all broken up to the same degree. Apart from the cigar-shaped nuclei, some fragments staining more densely, similar to those in the previous photograph, are also present.

FIGURE 5

1) Photograph showing the Mitochondrial fraction isolated at 2000 g for 20 minutes. Fraction photographed after resuspension in Millerds solution before the enzyme activity was estimated.

2) Photograph showing the Mitochondrial fraction similarily isolated and resuspended in Millerds solution, but photographed after 25 minutes of Enzyme activity estimation.

Comparison of the two photographs before and after the enzyme estimation shows that the rod shaped mitochondria decrease in number, while the spherical mitochondria increase in number. The fragments also present, are not nuclear fragments since they did not stain with methyl green pyronine.

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