THE POSITION OF THE CELL NUCLEUS IN PATHWAYS OF HYDROGEN TRANSFER: CYTOCHROME C, FLAVOPROTEINS, GLUTATHIONE, AND ASCORBIC ACID

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The fact that isolated cell nuclei are capable of metabolizing carbohydrate at least to the stage of pyruvate or of ribose formation (1, 2) may be taken to mean that in the course of their intracellular metabolism nuclei will reduce either the glycolytic coenzyme DPN, or the hexosemonophosphate dehydrogenase coenzyme TPN. The intensity of such metabolism being similar in nucleus and cytoplasm, the proportion of total cell coenzyme reduced by the nucleus will vary with its size—some 10 per cent in a liver cell, about 50 per cent in a thymus cell. Since the coenzymes in question are not terminal acceptors of hydrogen but serve only to mediate its transfer, it is a requirement of nuclear metabolism that mechanisms exist whereby reduced nuclear coenzymes become reoxidized. Little, however, is known about the possibilities of hydrogen transfer in nuclei, and the present communication is therefore addressed to this problem.

It is almost certain that nuclei cannot transfer hydrogen to molecular oxygen, a relation unequivocally demonstrated for cytochrome oxidase in rat liver by Hogeboom, Schneider, and Striebich (3). Thus, the ultimate problem of nuclear coenzyme reoxidation—at least for aerobic cells—resolves itself into a search for pathways of hydrogen transfer between nucleus and cytoplasm. No better situation illustrative of this point is to be found than in calf thymus where the nucleus accounts for 60 per cent of total cell mass. Although the nuclei of this tissue are capable of accounting for half the glucose-6-phosphate metabolized (2), it is clear from the data of Table II that the capacity to react with molecular oxygen is entirely restricted to the particles of the peripheral cytoplasm.

The restriction of terminal oxidation to autonomous mitochondrial bodies means in effect that the reoxidation of coenzymes, reduced in the course of intermediary carbohydrate metabolism, is a problem common to cytoplasm and nucleus. It will be seen, however, that cytoplasm and nucleus do not share the same mechanisms in response to this common need, even though it is im-

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probable that a study of this sort would at present exhaust all the possibilities of oxidized coenzyme regeneration. The proposition that nuclear coenzymes are reoxidized at the expense of substrate reduction, as in the glycolytic formation of lactic acid, has not been put to test. It had been considered by Stern and Mirsky (1) in the case of wheat germ nuclei, but ruled out on the ground that evidence was lacking for an accumulation of lactic acid during germination, a result to be expected from the high proportion of nuclear glycolysis. Temporary accumulations of lactic acid in the course of germination have been noted however (4); in germinating seeds therefore, nuclear DPN·H may transfer its hydrogen to pyruvate, the resultant lactic acid being ultimately reoxidized by the cytoplasm. Proof of this as a general phenomenon is nevertheless lacking, and alternate pathways must be considered. The mobility of the pyridine nucleotide coenzymes, or at least the easy interchange between free and enzyme-bound forms (5), makes reasonable their diffusion across the

TABLE I

Cytochrome c Content of Calf Tissues

(Milligrams/gram dry weight (fat-free))

Fraction	Heart	Kidney cortex	Liver
Whole tissue	2360	904	600
Nuclei	192	72	0
$\frac{\text{Nuclei}}{\text{Whole tissue}} \times 100$	8	8	0

nuclear membrane, but evidence for this type of behavior is clearly wanting and experimentally difficult to obtain. The oxidation of reduced nuclear coenzymes *via* the flavoproteins to the cytochrome system, in view of its established operation in the cell as a whole, suggests itself as a possible pathway. The more so, since cytochrome *c* is a protein of relatively low molecular weight associated with both mitochondria and the soluble portion of the cell (6) it could act as a mobile hydrogen acceptor in nucleus and cytoplasm.

The oxidation of coenzymes by the cell nucleus at the expense of cytochrome *c* reduction presupposes the presence of some intranuclear cytochrome. Beinert (28), studying the artificial redistribution of cytochrome *c* in rat liver homogenates by means of adding labelled compound, concluded that all the cytochrome *c* present in sucrose-isolated nuclei was due to adsorption. However, since nuclei thus isolated are freely permeable to protein (2), only adsorbed cytochrome would be expected to remain. The conclusion, because of its importance to nuclear physiology, begged further testing. For this, nuclei from several tissues of calf were isolated in non-aqueous media and analyzed for their cytochrome *c* content. Results of this study are summarized in Table I. In liver

nuclei none could be detected, and in this connection it might be pointed out that identical results were obtained with two fractions of nuclei, the one sedimenting at a higher, the other at a lower specific gravity of suspension medium. The low concentrations in kidney and heart nuclei are almost certainly due to contamination.

This absence of cytochrome *c* furnishes a pointer. One should expect to find that the flavoprotein enzymes mediating the reduction of cytochrome *c* are also lacking in nuclei. This aspect of nuclear composition has already been explored by a number of investigators, but with results that allow for equivocation. Dounce (7), for example, concluded from studies of nuclei isolated at pH 6.0 that flavoproteins are "probably" lacking in nuclei. Hogeboom and Schneider (8) would regard with skepticism their own values for DPN cytochrome *c* reductase activities in nuclear fractions of sucrose homogenates because of the

TABLE II

Cytochrome c Oxidase and Reductase Activities of Calf Thymus Fractions Isolated in 0.25 M Sucrose

Fraction	Whole tissue	Nuclei	Sediment 8,500 *g*	Sediment 18,000 *g*	Soluble
Cytochrome oxidase $-\Delta E_{550}$/mg. N/1 minute	2.76	0	49.0	2.3	0
DPN cytochrome *c* reductase ΔE_{550}/mg. N/1 minute	3.95	0.910	23.0	8.1	0

Designation of non-nuclear sedimentable fractions according to force required for sedimentation is used because of lack of any positive identification respecting purity of fractions.

probability of contamination. Indeed, the presence in nuclear preparations of cytoplasmic particles concentrated with respect to this reductase activity has been the principal difficulty in resolving the question. From this standpoint, wheat germ nuclei, so active in reducing DPN by glycolysis, could not be used. Undefined contamination was evident microscopically (1) and mitochondrial contamination was inferred from the cytochrome oxidase activity of the nuclear preparations. Experiments here performed with calf thymus nuclei did, however, provide an unequivocal answer because of the high degree of purity obtainable with such nuclear preparations.

First, fresh thymus tissue was fractionated in 0.25 M sucrose according to the procedure of Hogeboom and Schneider (14), and, as expected, it was found that the distribution of DPN-cytochrome *c* reductase was restricted to the sedimentable portion of the tissue (Table II). The activity of the nuclear fraction obtained by the procedure of Schneider and Petermann (29) was found to be much less than that reported for mouse liver (8). Non-aqueous fractionation of nuclei provided an even more definitive answer (Table III). Nuclear activity

was about 11 per cent that of the whole tissue and about 5 per cent of that of the cytoplasm. So low a value in absence of evidence to the contrary must be attributed to contamination. Samples retained at various stages of nuclear purification for assay revealed a decrease in activity with increasing DNA concentration. The possibility that a qualitative difference in properties might distinguish the nuclear from the cytoplasmic enzyme was unsuccessfully sought: pH activity curves of cytoplasmic and "nuclear" reductase activities showed the same broad optimum extending from pH 7.0 to 8.6; centrifugation of nuclear and whole tissue suspensions at 40,000 R.P.M. in a Spinco centrifuge sedimented all the activity in both preparations.

The absence of DPN·H cytochrome *c* reductase activity in thymus nuclei suggested the possibility of a more general situation with respect to other flavoproteins. It is, in fact, observed as routine that extracts of liver of thymus nuclei obtained by non-aqueous fractionation are colorless in appearance as

TABLE III

DPN Cytochrome c Reductase Activity in Non-Aqueous Fractions of Calf Thymus

(ΔE_{550}/milligram tissue/minute)

Fraction	Activity
Whole tissue	0.220
Nuclei	0.025
Cytoplasm (calculated)	0.510

against the yellowness of whole tissue extracts. That this difference includes the flavoproteins could be readily substantiated by fluorimetric analyses for flavin nucleotides, a procedure in which the hazards of enzyme assay are by-passed. An analysis of calf liver yielded the following results (expressed as equivalents of riboflavin): whole tissue—16 μg./gm.; nuclei—0.67 μg./gm. A nuclear concentration equal to about 4 per cent that of the cytoplasm clearly bespeaks contamination.

While evidence thus points against the transport of hydrogen beyond the pyridine nucleotide level in nuclei, the conclusion, except for ruling out a flavoprotein mechanism, remains uncertain. It can readily be shown that aqueous or acidic extracts of thymus nuclei contain solutes of sufficient potential to reduce cytochrome *c*, a property which has been followed spectrophotometrically (Fig. 1). Since this property is unstable to heating at or above neutrality, but stable in the acid extract, ascorbic acid and—by association—glutathione were sought as the agents responsible.

Whole tissue and nuclear extracts of wheat germ, calf thymus, and liver, give positive nitroprusside reactions thus showing the presence of soluble thiols. Tests with thymus for the stability of the colored complex in absence of

added cyanide further indicated that the thiol in question was largely glutathione since the quick fading observed with cysteine was not encountered in the extracts. Electrolytic reduction of extracts gave variable readings for the proportion of oxidized glutathione present (5 to 15 per cent) depending upon the particular preparation used; little oxidation of SH occurs therefore in the non-aqueous procedure for preparing nuclei. In general, nuclei showed less

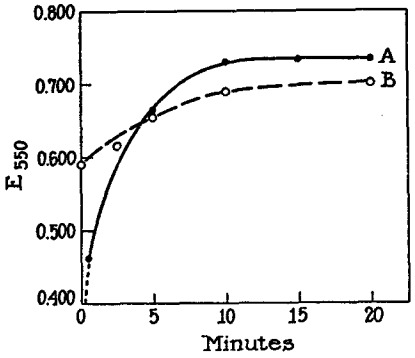

FIG. 1. Reduction of cytochrome *c* by extracts of thymus nuclei. Curve *A*: neutralized acid extract from 6 mg. nuclei; Curve *B*: aqueous extract from 0.35 mg. nuclei. Mixtures were buffered with Tris (pH 7.5), and contained 0.05 ml. of cytochrome *c* (6.7×10^{-4} M). Total volume = 1 ml.

TABLE IV

Ascorbic Acid and Glutathione Content of Calf Tissues

(Values in micromoles/milligram tissue)

Fraction	Ascorbic acid		Glutathione	
	Thymus	Liver	Thymus	Liver
Whole tissue	0.0095	0.0083	0.0116	0.0180
Nuclei	0.0085	0.0083	0.0103	0.0190
Cytoplasm (calculated)	0.010	0.0083	0.0135	0.0180

oxidized glutathione than whole tissue, although the significance of this, if any, must be by-passed for the present. The principal result of these assays (Table IV) has been to demonstrate a substantial concentration of soluble thiol, most probably glutathione, in the thymus and liver nucleus. The immediate point of interest, from the standpoint of hydrogen transfer, relates to the possibility that nuclear glutathione might serve as hydrogen acceptor from reduced TPN *via* the enzyme, glutathione reductase. This, however, does not appear to be the case, at least in so far as the ratio of distribution between enzyme and substrate acceptor have any significance. Whereas the concentra-

tion of nuclear glutathione approaches that of the cytoplasm, nuclear glutathione reductase has an activity equal to 16 per cent of the cytoplasm in thymus and about 20 per cent of that in liver (Table V). These values for the nuclei are probably real and not due to contamination; but even so, they point to the oxidation of nuclear TPNH as being a lesser function of nuclear glutathione.

The presence of ascorbic acid in both nuclei and cytoplasm has also been demonstrated. That the factor in tissue extracts responsible for the rapid reduction of 2,6-dichlorophenolindophenol was ascorbic acid could be shown by paper chromatography. Extracts of thymus tissue were run first with *n*-butanol and then with phenol to differentiate if necessary, substances closely related to ascorbic acid. In both solvents, the positions of the bleached spots after spraying with the dichlorophenolindophenol reagent corresponded to that for *l*-ascorbic acid or for the acid plus tissue extract. These results do not support the conclusion of Chayen (25) that histochemical procedures which demonstrate the

TABLE V

Glutathione Reductase Activities of Calf Tissues

(Micromoles GSH formed/milligram tissue/10 minutes at 37°C.)

Fraction	Thymus	Liver
Whole tissue	0.109	0.093
Nuclei	0.033	0.018
Cytoplasm (calculated)	0.210	0.105

presence of ascorbic acid in cytoplasmic granules are more reliable than those which show a distribution between nucleus and cytoplasm. Neither do they support the data of Hagen (26) who found 87 to 100 per cent of the total tissue ascorbic acid in the non-sedimentable fraction of ox liver and dog adrenal sucrose homogenates. To the extent that the results here obtained have a general application it must be assumed that in the histochemical procedures favoured by Chayen, as in sucrose homogenization, ascorbic acid is washed out of the nuclei.

The finding of ascorbic acid in the thymus and liver nucleus has already a setting pointing to its possible importance in nuclear function. Chayen (25) has reviewed the evidence for its concentration in plant meristematic tissues where cell divisions are frequent and has also referred to evidence suggestive of its pertinence to mitosis. It has been possible to pursue this matter further by virtue of a collection of lily anthers at various stages of the mitotic cycle made in the spring of 1951 according to procedure described by Erickson (27). These anthers had been lyophilized in the frozen state and extracted with 2:1:1 petrol ether: cyclohexane: carbon tetrachloride. The dry powders thus obtained were stored at −26°C. While such a procedure must allow for a variable de-

struction of ascorbic acid, the analytical results are so bold that some special relation of ascorbic acid concentration to the process of nuclear division cannot be doubted (Fig. 2). It is, of course, by a purely speculative extension of the implication of ascorbic acid occurrence in thymus and liver nuclei—and presumably in others—that a connection is suggested with the behavior just noted in respect of mitosis. Nevertheless, it is not unreasonable to assume that in regard to such fundamental and ubiquitous properties as energetic metabolism, the process of chromosome duplication represents a more intensive instance of what constitutes the normal pattern of behavior in absence of division.

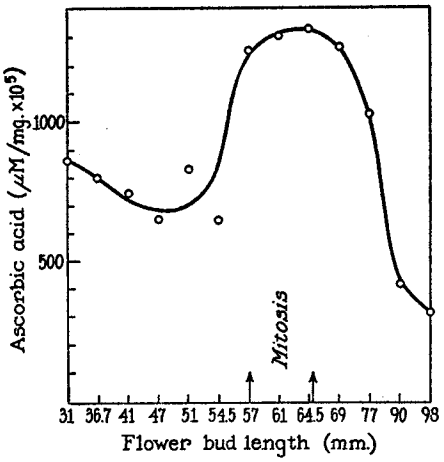

FIG. 2. Ascorbic acid content of lily anthers plotted according to length of flower buds. Arrows indicate approximately the occurrence of microspore mitosis.

That ascorbic acid can be functional in accepting hydrogen from reduced pyridine nucleotides would appear to be established by a variety of studies (9, 10). Such a system has, nevertheless, not been demonstrated in the nuclei. Furthermore, if this pathway of hydrogen transfer proves to be mediated by a flavoprotein (10), then the problem of ascorbic acid function in the nucleus would be as puzzling as that of glutathione.

DISCUSSION

The evidence here adduced for mechanisms of hydrogen transport in nuclei compels the conclusion that the metabolic organization of the nucleus is anaerobic in character. Not only is the most developed route of aerobic metabolism—the cytochrome oxidase system—lacking, but also the principal connecting link, the system of flavoproteins. Added significance must be attached to the fact that the flavoproteins can directly reduce atmospheric oxygen under a variety of natural conditions; the nucleus, it would appear, is thus doubly

insulated from aerobic reactions. The only contrary evidence known to the writers is that of Rubinstein and Denstedt (30) who claimed the succinoxidase system of chicken erythrocytes to be localized in the nuclei. Their experimental data are strongly suspect, however, since apart from disrupting cells by repeated freezing and thawing—one method of choice for solubilization of particulate enzymes—a variety of sedimentable material was collected in one fraction, the presumption being that the metabolic activity of that fraction was due chiefly to nuclear material. Quite the contrary; not only are cell oxidases localized in the cytoplasm, but they function independently of the nucleus; this may be inferred from the fact that enucleated protozoa consume oxygen for a very long time at a rate unaffected by the physiological trauma of nuclear removal (11). To be sure—and to emphasize a point—the characteristic of autonomy here attached to cytoplasmic oxidation in no sense implies the absence of electron transfers between nucleus and cytoplasm. The opposite is true, for in aerobic organisms the mitochrondria must exercise a strong influence on the redox potential of the nucleus simply because the nucleus must somewhow dispose of its reduced metabolites. Such a relation is probably implied in the streaming of mitochrondria about cell nuclei quite frequently observed in plant tissues. It is significant, nevertheless, that chromsome metabolism does not directly require aerobic reactions; if it did, the indifference of the genetic machinery of facultative anaerobes to extreme variations in oxygen availability would be the more difficult to explain.

It might be supposed that the absence of aerobic mechanisms in the nucleus is associated with a correspondingly low metabolic activity relative to the rest of the cell. The latter point of view has been favored by some (31), though criticized by others (7). So far as the facts go, however, the argument is unsupportable. The special activities of the mitochondria apart, on a per milligram N basis the nucleus functions at a metabolic level not radically different from the cytoplasm. The sources of evidence for this are varied. Administration of labelled metabolites to cells readily shows the activities of chromosome residual protein and cytoplasmic protein to be comparable (12), and the activity of nuclear ribonucleic acid to exceed that of the cytoplasm (13). Studies of nuclear composition—though neither comprehensive nor exhaustive—which include metabolites such as DPN (1), ascorbic acid, and glutathione, or enzyme activities of the glycolytic and oxidative cycles in hexose utilization (1, 2), point, like tracer studies, to an active metabolism within the nucleus. It would certainly be difficult, in view of the evidence listed, to explain the absence of oxidative mechanisms in the nucleus by attributing to it a paucity of metabolic activity.

The organization of the nucleus with respect to hydrogen transfer suggests rather that this pattern is a requirement of chromosome metabolism. Indeed, one might well ask why in the course of evolution, with several enzymatic mechanisms for aerobic oxidation available in the cell, none of these have

become incorporated to any significant extent into nuclear organization. It might be argued that all aerobic elements of the cell have, like the mitochondria appear to have, an autonomous origin. The argument, however, begs the question as to why more than one variety of cytoplasmic element should have incorporated flavoproteins, but not so the nuclei. The speculation which most suggests itself is that the evolutionary strength of the chromosome mechanism lay not in its capacity to utilize metabolites more efficiently at the molecular level, but in its ability to mediate and transmit formative influences of cell organization. One might suppose that the energies required for the mediation of such influences were adequately served by an anaerobic mechanism, and that the stability required for their transmission was best served by exclusion of the aerobic one. It is perhaps significant, therefore, that the anaerobic character of the nucleus is matched by a temporary anaerobiosis of the dividing cell at the time of nuclear membrane breakdown (24).

There remains, nevertheless, a deep gap in the evidence here presented respecting the position of the nucleus in hydrogen transfer. On the one hand, reduction of DPN and TPN has been demonstrated previously for nuclei (1, 2); on the other, the presence in nuclei of the labile redox elements glutathione and ascorbic acid has now been established. Although it is difficult at present to attach a specific point of importance to either of the latter metabolites, the fact of their presence in the nucleus against the absence of other soluble cytoplasmic components such as cytochrome *c*, suggests their special role in nuclear metabolism. The cyclic behavior of ascorbic acid in relation to the mitotic cycle adds substance to this conclusion. Nevertheless, there has been no direct demonstration of nuclear mechanisms capable of transferring hydrogen from the pyridine nucleotides to ascorbic acid and glutathione, although it is attractive to suppose that the chromosomes themselves mediate such a transfer. It is not clear, however, that both glutathione and ascorbic acid act principally as hydrogen acceptors and donors. While it is highly probable that ascorbic acid acts so, glutathione may well be implicated in a number of ways other than hydrogen transport.

Methods

Fractionation of Tissues.—Sucrose fractionation of calf thymus followed the procedure of Hogeboom and Schneider (14). Since clumping of tissue (due to the high DNA content) prior to straining through gauze, prevented a quantitative separation of fractions, sediments were collected at 8,500 and 18,000 *g* respectively after removal of nuclei (2) and all activity measurements compared in terms of the acid insoluble nitrogen of the fractions. The non-aqueous separation of nuclei followed the modified procedure of Behrens (15).

Cytochrome Oxidase.—Cytochrome *c* was reduced with ascorbic acid according to Hogeboom and Schneider (16). Tissue was suspended in 0.95 ml. 0.25 M sucrose containing 0.08 M potassium phosphate buffer (pH = 7.5); 0.05 ml. reduced cyto-

chrome *c* (6.7×10^{-4} M) was added at zero time, and the fall in extinction at 550 mμ. recorded every 30 seconds for 3 minutes at room temperature.

DPN-Cytochrome c Reductase.—The reaction mixture contained the following components: 0.1 ml. nicotinamide (0.27 M); 0.1 ml. KCN (0.001 M); 0.1 ml. versene (0.01 M); 0.1 ml. reduced DPN (0.73 mg./ml. of a commercial sample obtained from Sigma); 0.05 ml. Tris buffer (pH 7.5, 2.0 M); tissue plus water to 0.95 ml.; 0.05 ml. cytochrome *c* (6.7×10^{-4} M) was added at zero time and the extinction followed at 550 mμ. for 15 minutes at room temperature. In thymus tissue the addition of versene was prompted by a fall in extinction at 550 mμ. which could be observed in the absence of reduced DPN and versene, but in the presence of KCN and reduced cytochrome *c*. Versene was therefore added as routine, but the nature of the effect was not pursued.

Glutathione Reductase.—Zwischenferment was prepared according to the procedure of Kornberg (17). The product used had an activity of 1.34 units/mg. of protein. 0.03 units were added to the test system, and the trace of glutathione reductase present in it accounted for in blank runs. Components of the reaction mixture were as follows: Tris buffer (pH = 7.5), 50 μ moles; versene, 0.25 μ moles; TPN (Sigma "80"), 12.5 μg.; glucose-6-phosphate, 1.25 μ moles; oxidized glutathione, 0.5 μ moles; Zwischenferment; tissue and water 0.5 ml. The mixture was incubated at 37°C. for 5 to 20 minutes and the reaction stopped with 0.5 ml. 6 per cent metaphosphoric acid saturated with respect to NaCl. Tissue incubated with reduced glutathione showed inappreciable loss of glutathione either through oxidation or destruction.

Glutathione.—The method of Grunert and Phillips (18) was used as follows: all reagents and Beckmann cells were kept at 0°C. in Dewar flasks. 0.2 ml. of sample in 3 per cent metaphosphoric acid half-saturated with respect to NaCl was pipetted into a 1 ml. Beckmann cuvette, followed by 0.6 ml. saturated NaCl, 0.1 ml. sodium nitroprusside (20 mg./ml.), and 0.1 ml. of 1.5 M Na_2CO_3 containing 0.067 M NaCN. The contents were mixed by inversion of the cuvette, and the extinction read at 520 mμ after 30 seconds. (We are indebted to Dr. E. S. G. Barron for kindly supplying us with a sample of iodosobenzoic acid with which to check our titers of glutathione).

Reduction of glutathione followed the electrolytic method of Dohan and Woodward (19). It was found necessary to maintain the mercury-containing beaker at zero by surrounding it with ice in order to obtain complete recoveries of known amounts of oxidized glutathione. Substituting metaphosphoric for sulfosalicylic acid did not appear to affect the reduction of pure samples of oxidized glutathione. Tissue extracts, however, particularly those of nuclei, showed an accelerated fading of the colored nitroprusside complex following such treatment.

Ascorbic Acid.—The method of Hochberg, Melnick, and Oser (20) was reduced in scale so that final volumes were 2 ml. and were read directly in Coleman microcuvettes. For reduction, 5 ml. volumes in place of 25 ml. were used. Although measurements were made before and after reduction of tissue extracts, the results reported are those obtained after reduction since the prolonged procedure for tissue preparation in non-aqueous media makes questionable the significance of the proportion of oxidized ascorbic acid found to be present. It is to be noted, nevertheless, that the amount of oxidized ascorbic acid found to be present was in the order of 10 per cent.

Identification of ascorbic acid by means of paper chromatography was according to the procedure of Chen, Isherwood, and Mapson (21). 100 mg. samples of tissue were extracted with 2 ml. saturated oxalic acid and about 1 ml. of each extract was applied in a series of superimposed streaks, 30 cm. long on Whatman No. 3 paper. The remainder of the procedure followed exactly as described.

Flavin Nucleotides.—The fluorimetric method of Bessey, Lowry, and Love (22) was used. The entire extract was heated before measurement to obtain total flavin nucleotides.

Cytochrome *c* was determined by the method of Rosenthal and Drabkin (23).

SUMMARY

A study has been made of calf thymus and liver tissue to ascertain the position of the nucleus with respect to mechanisms capable of hydrogen transfer. Although previous work had shown that reduced pyridine nucleotide coenzymes are produced in the course of nuclear metabolism, it has now been established that the flavoprotein system of cytochrome *c* reductase, cytochrome *c*, and most, if not all, of other flavoproteins are absent from nuclei. Metabolites capable of cytochrome *c* reduction, notably ascorbic acid and glutathione, have been demonstrated in the nuclei. Glutathione reductase has been found present in nuclei only to a minor extent, suggesting that nuclear glutathione might function largely in a capacity other than that of hydrogen carrier in the nucleus. Although no enzymatic relation could be established between ascorbic acid and hydrogen transfer in nuclei, it was possible to demonstrate a close association between ascorbic acid concentration and the mitotic process in lily anthers. The significance of the anaerobic character of nuclear metabolism to chromosome function is discussed.

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