*PURIFICATION AND ENZYMATIC IDENTITY OF MITOCHONDRIAL CONTRACTION-FACTORS I AND II**

DIETHER NEUBERT,† ANNA B. WOJTCZAK,‡ AND ALBERT L. LEHNINGER

DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE

Communicated July 13, 1962

Contraction-factor (C-factor) is a specific heat-labile mitochondrial substance required in reversal of glutathione-induced swelling of mitochondria by ATP.[1-3] It is detached from mitochondria on exposure to reduced glutathione but it can be recovered again from the suspending medium in active form; it is also found in sonic extracts of untreated mitochondria. On addition to glutathione-swollen mitochondria, C-factor restores ATP-linked contraction in such a manner that the amount of water extruded is approximately proportional to the concentration of added C-factor.[1-4] C-factor activity has been found in mitochondria of all higher animal tissues examined, as well as in human erythrocytes, some microorganisms, and some plant cells.[4] The purification, enzymatic identity, and possible mode of action of C-factor of rat liver mitochondria form the subject of this report.

Purification.—The C-factor activity of sonic extracts of rat liver mitochondria has been resolved into three components by chromatographic procedures, and each has been substantially purified. The first component, C-factor I, which accounts for approximately 80–85 per cent of the total activity, has been purified 136-fold by selective heat denaturation, isoelectric precipitation, alcohol fractionation, and chromatography on DEAE-cellulose columns; the essential details are given in Table 1 and Figure 1. The most highly purified specimen showed a specific activity of about 680 C-factor units per mg protein. One unit of C-factor activity, which is defined as that amount giving one half maximal contraction of glutathione-swollen mitochondria under prescribed conditions,[4] is therefore equivalent to 1.47 μg of the purified preparation. Purified C-factor I is heat-labile and shows other

TABLE 1

PURIFICATION OF MITOCHONDRIAL C-FACTOR I

Procedure: The washed mitochondria from 500 gm rat liver were suspended in 0.02 *M* tris-HCl pH 7.6 (1.0 ml = 2 gm liver) and subjected to sonic oscillation for 60 min at 4° in a Raytheon 9 kc 50 watt apparatus. The suspension was centrifuged at 100,000 × *g* for 60 min and the pellets discarded; the clear supernatant fluid is designated *sonic extract.* The sonic extract was distributed in 5.0 ml portions in 20 ml beakers, shaken for 10 min at 52°, chilled, and centrifuged at 38,000 × *g* for 30 min; the clear supernatant fraction is designated *heated extract.* An equal volume of 0.05 *M* tris-maleate buffer pH 5.1 was added to the heated extract; the mixture, after standing 10 min at 0°, was centrifuged 10 min at 40,000 × *g* and the supernatant fraction quickly brought to pH 7.7. The supernatant was centrifuged again; it is designated *supernatant* pH 5.5. To this fraction was added 0.1 volume of 2 *M* KCl; two volumes of ethanol at −5° were then added slowly with constant stirring. The mixture was kept at 0° for 2 hrs and centrifuged at 0° for 30 min at 40,000 × *g*, and the drained pellets were brought to near-dryness in a vacuum desiccator at 0°. The pellets were extracted with a total of 100 ml 0.01 *M* tris-HCl pH 7.75; the extract after clarification contains the *ethanol-precipitated* C-factor activity.

This extract was applied to a 4.5 × 25 cm column of DEAE cellulose that had been washed thoroughly with 0.01 *M* tris-HCl pH 7.75. The column was again washed with one volume of the buffer. C-factor activity was eluted at room temperature with a logarithmic gradient of tris-buffer pH 7.75 beginning at 0.01 *M* tris and increasing to 0.25 *M* tris. An elution curve is shown in Figure 1.

C-factor recovery and specific activity data are given below for this fractionation.

Step	Total activity (units)	Protein (mg)	Specific activity (units × mg^{-1})	Purification	Yield
1. Sonic extract	48,500	9,800	5	—	(100)
2. Heated extract	47,000	4,600	10	2	98
3. Supernatant pH 5.5	45,000	2,200	20	4	93
4. Ethanol-precipitated	42,000	325	130	26	87
5. DEAE-cellulose	2,800*	4.1	680	136	6*

* Tube with highest specific activity. The total yield from both peaks was about 50% of the starting activity.

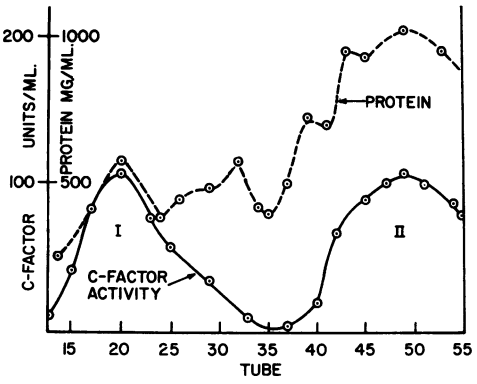

FIG. 1.—Chromatographic separation of C-factors I and II. Details as in Table 1.

properties characteristic of proteins; it gives no light absorption in the visible range. It becomes rather unstable after the chromatographic step and no significant further purification has been achieved.

The second component, C-factor II, accompanies C-factor I in the early stages of the purification but is separated from it during chromatography on DEAE-cellulose; it appears in a second distinct peak as the ionic strength of the eluent is increased (Fig. 1). C-factor II recovered from the peak tubes has a maximum specific activity of 100 units per mg and it is also heat-labile. However, it differs in two significant respects from C-factor I. C-factor II has a spectrum in the visible range characteristic of a heme protein, with a Soret peak between 400 and 405 mμ. Secondly, it is much more stable than C-factor I. C-factor II usually accounts for less than 20 per cent of the total C-factor activity of rat liver mitochondrial extracts.

C-factor III was separated from components I and II by gel filtration on Sephadex G25. It is retained on the gel while components I and II are not; it is probably a relatively small molecule. C-factor III is heat-stable, passes through cellophane membranes, and gives a weak but significant biuret reaction in preparations which have been enriched over 100-fold. It accounts for only a few per cent of the total C-factor of rat liver mitochondrial extracts. C-factor III is somewhat different from C-factors I and II in that it does not support mitochondrial contraction on delayed addition to the standard assay system (cf. ref. 4). It is under further study.

Identification of C-Factor II as Catalase.—Purified C-factors from mitochondria have been found to be highly specific. Assay of over twenty-five different highly purified proteins and enzymes for C-factor activity showed them to be essentially devoid of activity (i.e. < 0.2-factor units per mg), with a single exception. Crystalline beef liver catalase was found to have considerable C-factor activity, approximately 100 units per mg protein (Table 2). This is only about 15 per cent of the specific activity of the most highly purified C-factor I preparation obtained from rat liver mitochondria but is approximately the same magnitude as the specific

TABLE 2

C-FACTOR ACTIVITY OF CATALASE

Enzymes		C-factor activity (units × mg^{-1})
Catalase	Beef liver (commercial)	100
"	" " "	80
"	" " "	100
"	" " "	100
"	Rat liver	120
"	*M. lysodeikticus*	
Cytochrome c, hemoglobin, horseradish peroxidase, milk peroxidase, ferritin, myoglobin, and twenty-two other purified enzymes		≪0.5
Purified C-factor I		680

activity of the most active preparations of C-factor II. The C-factor activity appears to be an inherent property of the catalase molecule, since it was given by all beef and rat liver catalase specimens tested, as well as by crystalline catalase isolated from *M. lysodeikticus* (cf. ref. 5), kindly provided by Roland F. Beers, Jr. Sedimentation of a specimen of crystalline beef liver catalase in an Yphantis-Waugh moving partition cell, carried out by Thomas E. Thompson, showed that the C-factor activity moved with the homogeneous catalase peak. The C-factor activity of crystalline beef liver catalase was found to be destroyed by procedures which inactivated catalatic activity, such as boiling, dissociation of the protein at pH 12,[6] treatment with urea,[7] or irreversible inhibition by 3-amino-1,2,4-triazole and hydrogen peroxide.[8] No other iron-containing protein tested, which included beef hemoglobin, myoglobin, horse heart cytochrome c, horseradish peroxidase, milk peroxidase, and ferritin, showed detectable activity in the C-factor assay.

The sonic extracts of rat liver mitochondria used as starting material for purification of C-factors I and II were found to contain catalatic activity as assayed by the method of Bonnichsen *et al.*,[9] however, the catalase content, when calculated in terms of the C-factor activity of crystalline beef liver catalase (i.e., 100 C-factor units per mg), usually accounted for less than 15 per cent of the total C-factor activity of the mitochondrial extracts. Treatment of such mitochondrial extracts with 3-amino-1,2,4-triazole and hydrogen peroxide[8] to inactivate catalase caused loss of essentially all of the catalatic activity but only 20 per cent or less of the total C-factor activity. It is therefore clear that the presence of catalase can account for some of the C-factor activity of extracts of rat liver mitochondria, but only for a minor fraction.

The eluates from the DEAE-cellulose columns used for separation of mitochondrial C-factors I and II were then assayed for catalatic activity. It was found (Fig. 2) that the early peak, containing C-factor I, was essentially devoid of catalatic

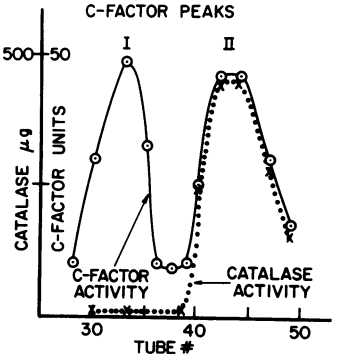

FIG. 2.—Elution of mitochondrial catalase and C-factor activities. The ethanol-precipitated fraction of a rat liver mitochondrial extract (cf. Table 1) was applied to a DEAE-cellulose column (Fig. 1) and eluted with a logarithmic gradient of tris-HCl buffer pH 7.7 starting at 0.01 *M* and proceeding to 0.25 *M*. The eluates were collected in 10.5 ml fractions. The C-factor and catalase activities of tubes indicated were assayed. The catalase activity is expressed in terms of crystalline beef liver catalase.

activity, whereas eluates from the late peak containing C-factor II were rich in catalatic activity. The elution curve for C-factor II activity coincided with that of catalase activity within experimental error. On the basis that 100 units of C-factor activity are equivalent to 1.0 mg pure catalase, the catalase activity present in the late peak accounted for essentially all of its C-factor II activity. The purity of the catalase in peak tubes of C-factor II was at least 50 per cent and in one experiment approached 100 per cent. The visible spectrum of C-factor II and its

response to various reagents was that of liver catalase. From these experiments, it may be concluded that C-factor II is identical with catalase.

Identification of C-Factor I as Glutathione Peroxidase.—The C-factor activity of catalase prompted a careful survey of other enzymes acting on peroxides for C-factor activity. Horseradish and milk peroxidase, which are relatively nonspecific with respect to the electron donor substrate, were found to be devoid of C-factor activity (Table 2). However, it appeared possible that the widely-distributed enzyme glutathione peroxidase, studied in some detail by Mills,[10] might possess C-factor activity. This enzyme, which is apparently not a heme protein, is highly active with reduced glutathione as the electron donor and is only weakly reactive, if at all, with other thiols.[10] This is a point of some significance since glutathione, among a series of thiols tested, is also rather specific in causing detachment of C-factor from mitochondria.[2, 3] Accordingly, the glutathione peroxidase activity of eluates from the chromatographic separation of mitochondrial C-factors I and II was assayed, employing procedure 2 of Mills.[10] Large amounts of activity were present in eluates containing the peak of C-factor I activity (Fig. 3), but essentially

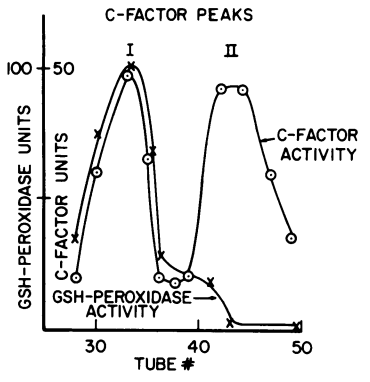

FIG. 3.—Elution of glutathione peroxidase and mitochondrial C-factor activities. Chromatographic details as in Figure 2. The glutathione peroxidase activity was measured by Procedure II of Mills.[10]

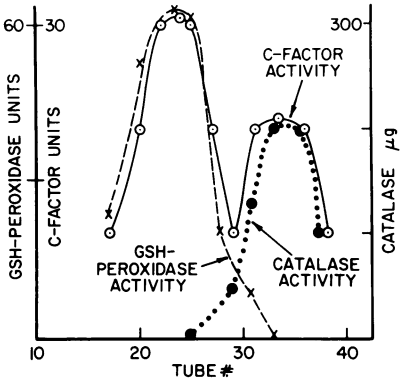

FIG. 4.—Chromatography of GSH peroxidase, catalase, and C-factor activity of beef erythrocytes. The procedure was essentially that described by Mills.[10] Elution of the activities from the DEAE-cellulose column was carried out with a gradient of phosphate buffer pH 7.0 from 0.005 *M* to 0.125 *M* at 21°; each tube contained 10.5 ml of eluate. The eluates were dialyzed prior to assay of C-factor.

none was found in tubes from the late peak containing C-factor II (catalase) activity. The glutathione peroxidase activity was proportional to C-factor I activity within experimental error, demonstrating that the two activities are eluted at essentially identical rates (Fig. 3). In this peak, the ratio of glutathione peroxidase activity to C-factor activity, in terms of the specific unitage of each, was approximately 2.0.

To confirm the tentative identification of C-factor I as glutathione peroxidase, preparations of glutathione peroxidase of beef erythrocytes, purified by chromatography on DEAE-cellulose as described by Mills,[10] were assayed for C-factor activity; earlier experiments had shown that human erythrocytes are rich in C-factor activity.[4] As is shown in Figure 4, the peak of erythrocyte glutathione peroxidase

activity contained C-factor activity; the elution curve of the two activities was found to coincide. In the peak tubes, the ratio of glutathione-peroxidase activity to C-factor activity was again found to be approximately 2.10, in agreement with the results on mitochondrial C-factor I. Neither mitochondrial C-factor I nor erythrocyte glutathione peroxidase showed significant light absorption in the visible range. Catalase activity of beef erythrocytes could be separated from glutathione peroxidase by chromatography on DEAE-cellulose, as was shown by Mills[10] and confirmed in Figure 4. The peak of catalase activity also coincided with the measured C-factor II activity.

It will be recalled that C-factor I is labile after purification; similarly, Mills has reported the erythrocyte glutathione peroxidase to be unstable after chromatography.[10] Direct tests of the rate of inactivation of C-factor activity and of glutathione peroxidase activity of the mitochondrial enzyme, on standing at 20°, showed both activities to be lost at essentially the same rate. From these findings, it is tentatively concluded that C-factor I and glutathione peroxidase are identical. Conclusive identification must await purification to homogeneity.

Earlier experiments have shown that purified C-factor preparations have two other effects on mitochondrial systems: they inhibit glutathione-induced swelling of mitochondria[4] and they increase the P:O ratio of oxidative phosphorylation in pretreated digitonin fragments of mitochondria.[11] These effects are also given by crystalline beef liver catalase. Furthermore, the C-factor activity of crystalline catalase or of erythrocyte glutathione peroxidase preparations is critically dependent on the sequence of addition to the assay system, as is the case with mitochondrial C-factor; i.e., contraction is given only when it is added to the glutathione-swollen mitochondria *prior* to the addition of ATP, but not after addition of ATP.[4] It is therefore concluded that the detachment of catalase and/or glutathione peroxidase from mitochondria by glutathione, as well as the action of these enzymes on mitochondria, is related to the formation or action of ATP in some rather specific manner.

No evidence has been found that both enzymes are required together for maximum contraction of glutathione-swollen mitochondria or that their effects are anything but additive; however, the point is under further investigation.

The specific intracellular location of catalase and of glutathione peroxidase remains to be established conclusively. DeDuve and his colleagues have presented some evidence that catalase is associated with the lysosomes, which can be enriched in the mitochondrial fraction by centrifugation in a sucrose density gradient.[12] However experiments in sucrose media may not be entirely satisfactory since significant amounts of C-factor[4] and also of catalase[13] activity appear in the soluble fraction of rat liver homogenates prepared in 0.25 *M* sucrose. On the other hand relatively little C-factor or catalase appears in the soluble fraction when a sucrose-polyvinylpyrrolidone medium is employed.

Possible Mechanisms of Action of C-Factors.—The identification of glutathione peroxidase and catalase as C-factors I and II suggests that they make possible ATP-linked contraction of glutathione-swollen mitochondria by causing removal of hydrogen peroxide, which may be formed during swelling and which could be expected to be inhibitory to the contraction. Such a mechanism would be analogous to the postulated function of these enzymes and of glutathione and glutathione

reductase in protecting the erythrocyte against damage induced by hydrogen peroxide, i.e., methemoglobin formation and lysis.[10, 14] However, such an explanation does not appear to account for the action of catalase and glutathione peroxidase in restoring the ATP-linked contraction of swollen mitochondria. First, it must be pointed out that reduced glutathione has quite different effects on erythrocytes and on mitochondria; it inhibits lysis of erythrocytes (by serving as reductant of peroxide) but produces rapid swelling and lysis of mitochondria. Secondly, hydrogen peroxide also has quite different effects on these structures. Although hydrogen peroxide causes lysis of erythrocytes, we have found that addition of hydrogen peroxide to intact, swelling, or already fully swollen mitochondria does not cause swelling or lysis nor does it impair ATP-induced contraction of thyroxine-swollen mitochondria, which was found to proceed rapidly without the necessity of adding either catalase or glutathione-peroxidase to destroy the added peroxide. Although hydrogen peroxide is decomposed by mitochondria, it could be added at different points in the swelling-contraction cycle with essentially no effect on the contraction, even at concentrations as high as 0.03 *M*. Furthermore, it was found that mitochondria swollen by 0.02 *M* glutathione, which causes loss of C-factor, readily contracted again in the presence of added hydrogen peroxide when catalase was added, followed by ATP. In such experiments, catalase was required for contraction even in the presence of a demonstrable excess of undecomposed hydrogen peroxide, indicating that the action of catalase in contraction is not merely to remove hydrogen peroxide. It is of course possible that added or "bottle peroxide" does not reach or attack sites in the mitochondrial structure which are sensitive to peroxide formed *in situ*. However, if this is the case, it is difficult to explain why large protein molecules such as catalase and glutathione peroxidase added to the medium can reach such hidden sites of peroxide formation whereas externally added hydrogen peroxide can not. Furthermore, swelling of mitochondria in the presence of ascorbate, which does lead to endogenous formation of peroxides (cf. ref. 15), can be readily reversed again by ATP without the necessity of adding C-factor (cf. ref. 3). From these considerations, it is quite clear that catalase and glutathione peroxidase have a function in ATP-induced contraction of glutathione-swollen mitochondria other than or in addition to decomposition of hydrogen peroxide.

A second possibility is that the role of catalase and glutathione peroxidase in mitochondrial swelling and contraction is related to the formation or removal of organic hydroperoxides, presumably of lipids. Lipid peroxide formation, as detected by the thiobarbituric acid reaction,[16] is known to take place in mitochondria incubated aerobically,[17–22] especially in the presence of Fe^{++} ions, ascorbate, or the combination of Fe^{++} and ascorbate.[20] We have found that glutathione-induced mitochondrial swelling is also accompanied by formation of thiobarbituric acid-reactive substances; however, thyroxine-induced or phosphate-induced swelling is not. Hunter and his colleagues have recently reported peroxide formation in rat liver mitochondria undergoing lysis in the presence of ascorbate as well as glutathione; they have also made the significant finding that cyanide, amytal, and antimycin A, which inhibit respiration and thus glutathione-dependent swelling of rat liver mitochondria, also inhibit formation of the peroxides.[21] In addition, Corwin has observed that organic peroxide formation in mitochondria is greatly stimulated by oxidation of pyridine nucleotide-linked substrates but not by oxidation of succi-

nate.[22] These findings thus indicate that electron transport, mitochondrial swelling, and peroxide formation may have common denominators of mechanism. Formation and removal of fatty acid peroxides in the lipid bilayer of the mitochondrial membrane could be expected to result in changes in the packing arrangement of the lipid layers and thus the mechanochemical properties of the membrane. It appears possible that catalase and glutathione peroxidase may function in contraction by catalyzing reduction of organic hydroperoxides of lipids or fatty acids in the presence of specific electron donors such as reduced glutathione; these relationships are under investigation. Lipids are already known to be involved in mitochondrial swelling and contraction (cf. refs. 23, 24).

A third possible explanation for the action of catalase and glutathione peroxidase is that these enzymes are involved in the action of the coupled respiratory chain, which in turn controls the swelling-contraction cycle of mitochondria (cf. ref. 25). These enzymes are not usually considered as having a function in electron transport, nor is hydrogen peroxide formation considered to be a normal accompaniment of respiration. However, the finding of Yamanaka *et al.*[26] that catalase stimulates the oxidation of ascorbate by cytochrome oxidase of *P. aeruginosa*, and the observation of Chance that peroxide formation and utilization in intact yeast cells proceeds at about the same rate as respiration,[27] are suggestive.

The detachment of these enzymes from the membranes into the medium which is caused by glutathione could interrupt some necessary relationships to the respiratory chain. This detachment could be brought about by the reduction of a specific disulfide linkage between the enzyme molecule and a membrane protein or by a drastic change in permeability of the membrane, permitting escape of large protein molecules. The latter view is supported by the finding that mitochondrial water uptake is greatly stimulated by certain thiols and disulfides, including the disulfide hormones insulin, oxytocin, and vasopressin.[28]

Actually, glutathione is also known to inhibit catalase in the presence of certain electron donors and hydrogen peroxide,[29] presumably by converting it into the inactive complex II.[30] However, this specific effect does not appear to be involved in impairment of mitochondrial contraction in the presence of glutathione, since the C-factor activity which is detached from mitochondria in the presence of glutathione is found in the glutathione-containing medium in a fully active, not an inhibited, form.[2]

In any case, investigation of the action of catalase and glutathione peroxidase in mitochondrial contraction may lead to fuller understanding of the true cellular role of these enzymes.

Summary.—"Contraction factor" of rat liver mitochondria, which is required for ATP-linked reversal of glutathione-induced water uptake, has been resolved into three components by chromatographic procedures, and each has been substantially purified. C-factor I has been identified as the enzyme glutathione peroxidase and C-factor II as catalase; C-factor III, which is heat-stable and is apparently a small molecule, remains unidentified as to its enzymatic activity. Highly purified specimens of glutathione peroxidase from erythrocytes and crystalline beef liver catalase show all the properties of mitochondrial C-factors, including (*a*) activity in supporting ATP-linked reversal of glutathione-swelling, (*b*) inhibition of glutathione-induced mitochondrial swelling, (*c*) mandatory sequence of action on mitochondria

with ATP, and (*d*) ability to increase the P:O ratio of oxidative phosphorylation in digitonin fragments.

The function of glutathione peroxidase and catalase in promoting mitochondrial contraction appears not to be due primarily to the removal of any toxic accumulations of hydrogen peroxide; added hydrogen peroxide does not significantly stimulate swelling or inhibit ATP-linked contraction, even in very high concentrations. It appears more likely that catalase and glutathione peroxidase are concerned either in the formation and removal of lipid peroxides or in the mechanism of electron transport, to which the mechanochemical changes of swelling and contraction are coupled.

The technical assistance of Irene Earling is gratefully acknowledged.

* Supported by grants from the National Institutes of Health, the National Science Foundation, the Nutrition Foundation, Inc., and the Whitehall Foundation.

† Permanent address: Pharmacological Institute, Free University, Berlin, Germany.

‡ Permanent address: Nencki Institute of Experimental Biology, Warsaw, Poland.

[1] Lehninger, A. L., and G. S. Gotterer, *J. Biol. Chem.*, **235**, PC 8 (1960).

[2] Lehninger, A. L., *J. Biol. Chem.*, **237**, 946 (1962).

[3] Neubert, D., and A. L. Lehninger, *J. Biol. Chem.*, **237**, 952 (1962).

[4] Neubert, D., T. H. Rose, and A. L. Lehninger, *J. Biol. Chem.*, **237**, 2025 (1962).

[5] Herbert, D., and J. Pinsent, *Biochem. J.*, **43**, 193 (1948).

[6] Inada, Y., T. Kurozumi, and K. Shibata, *Arch. Biochem. Biophys.*, **93**, 30 (1961).

[7] Samejima, T., and K. Shibata, *Arch. Biochem. Biophys.*, **93**, 407 (1961).

[8] Margoliash, E., and A. Novogradsky, *Biochem. J.*, **68**, 468 (1958).

[9] Bonnichsen, R. K., B. Chance, and H. Theorell, *Acta Chem. Scand.*, **1**, 685 (1947).

[10] Mills, G. C., *J. Biol. Chem.*, **229**, 189 (1957); Mills, G. C., and H. P. Randall, *J. Biol. Chem.*, **232**, 589 (1958); Mills, G. C., *J. Biol. Chem.*, **234**, 502 (1959); Mills, G. C., *Arch. Biochem. Biophys.*, **86**, 1 (1960).

[11] Lehninger, A. L., Symposium Issue, *Fed. Proc.*, **19**, 952 (1960).

[12] De Duve, C., H. Beaufay, P. Jacques, Y. Rahman-Li, O. Z. Sellinger, R. Wattiaux, and S. de Ooninck, *Biochim. et Biophys. Acta*, **60**, 186 (1960); Thomson, J. F., and F. J. Klipfel, *Arch. Biochem. Biophys.*, **70**, 224 (1957).

[13] Greenfield, R. E., and V. E. Price, *J. Biol. Chem.*, **220**, 607 (1956).

[14] Cohen, G., and P. Hochstein, *Fed. Proc.*, **21**, 69 (1962).

[15] Hunter, F. E., Jr., in *Biological Structure and Function*, ed. T. W. Goodwin and O. Lindberg (New York: Academic Press, 1961), vol. 2, p. 52.

[16] Wilbur, K. M., F. Bernheim, and O. W. Shapiro, *Arch. Biochem.*, **24**, 305 (1949).

[17] Ottolenghi, A., F. Bernheim, and K. M. Wilbur, *Arch. Biochem. Biophys.*, **56**, 157 (1955).

[18] Thiele, E. H., and J. W. Huff, *Arch. Biochem. Biophys.*, **88**, 203 (1960).

[19] Tappel, A. L., and H. Zalkin, *Arch. Biochem. Biophys.*, **80**, 326 (1960); **80**, 333 (1959); **88**, 113 (1960).

[20] Ottolenghi, A., *Arch. Biochem. Biophys.*, **79**, 335 (1959).

[21] Hoffsten, P. E., F. E. Hunter, Jr., J. M. Gebicki, and J. Weinstein, *Biochem. Biophys. Research Comm.*, **7**, 276 (1962).

[22] Corwin, L. M., *Arch. Biochem. Biophys.*, **97**, 51 (1962).

[23] Lehninger, A. L., and L. F. Remmert, *J. Biol. Chem.*, **234**, 2459 (1959).

[24] Wojtczak, L., and A. L. Lehninger, *Biochim. et Biophys. Acta*, **51**, 442 (1962).

[25] Lehninger, A. L., *Physiol. Rev.*, in press.

[26] Yamanaka, T., A. Ota, and K. Okunuki, *J. Biochem.*, **49**, 414 (1961).

[27] Chance, B., in *Symposium on the Mechanism of Enzyme Action*, ed. W. D. McElroy and B. Glass (Baltimore: The Johns Hopkins Press, 1954), p. 399.

[28] Lehninger, A. L. and D. Neubert, these PROCEEDINGS, **47**, 1929 (1961).

[29] Marcus, A., and J. Feeley, *J. Biol. Chem.*, **237**, 221 (1962).

[30] Chance, B., *Biochem. J.*, **46**, 387 (1950).