Biochemical Pharmacology, 1965, Vol. 14, pp. 245–254. Pergamon Press Ltd., Printed in Great Britain.

THE EFFECTS OF CANTHARIDIN UPON SUBCELLULAR PARTICLES*

FILLMORE K. BAGATELL and KSENIJA DIMITROV

Section of Dermatology, Western Reserve University School of Medicine, Cleveland, Ohio, U.S.A.

(*Received* 11 *August* 1964; *accepted* 6 *October* 1964)

Abstract—The effects of cantharidin on subcellular particles of rat liver were investigated. Some mitochondrial processes were affected. In the poisoned animal oxidative phosphorylation was found to be uncoupled. *In vitro* studies showed a variable effect, uncoupling when succinate or D,L-β-hydroxybutyrate was used as substrate and a stimulation when L-glutamate, α-ketoglutarate, or pyruvate was utilized. With the latter substrates the amount of inorganic phosphate uptake and oxygen consumption increased about twofold, and the P/O ratio was maintained. The effect was sensitive to 2,4-dinitrophenol.

Cantharidin was shown to be capable of causing swelling in rat liver mitochondria. This action was not reversed by 2,4-dinitrophenol or carbonyl cyanide phenylhydrazine.

Amino acid incorporation into microsomal protein was not impaired in the cantharidin-poisoned animal. Lysosomal hydrolases were found not to be increased in these animals.

CANTHARIDIN ($C_{10}H_{12}O_4$) is a toxic compound that has been studied in recent years because of its vesicant properties on skin. The histological changes in skin produced by cantharidin (CAN), acantholysis or separation of cells, results in an intraepidermal vesicle[1] resembling the microscopic changes seen in pemphigus vulgaris.[2] We have been investigating the biochemical effects of CAN in skin and liver. Stoughton and Bagatell[3] have presented evidence that CAN induces an enzymatic acantholysis in skin.

In recent years, accumulated data have suggested an effect of CAN upon terminal oxidation of carbohydrate. Haas *et al.*[4] showed that CAN inhibits respiration in mouse liver slices. Allison and Williamson[5] found that respiration and anaerobic glycolysis of mammalian tissue and yeast were inhibited by CAN. They also noted a decrease in intracellular level of ATP in the yeast cells respiring in the presence of CAN. On the other hand, oxygen uptake of yeast cells respiring in the absence of glucose was increasedin the presence of CAN. Weakley and Einbinder[6] have shown that 0·1 M malonate and 0·1 M glucose prevent induction of acantholysis by CAN in skin slices. Decker[7] has shown that both oxidation and phosphorylation in mitochondria aged after isolation from rat livers utilizing glutamate or succinate are inhibited by CAN. Methylene blue and 2,4-dinitrophenol completely or partially reversed the inhibition of oxygen uptake. Decker concluded that acantholysis requires energy supplied by oxidative phosphorylation.

* Supported in part by Research Grants AM05808 from the National Institutes of Health and DA-49-193-MD-2335 from the Armed Forces Epidemiology Board.

In the present investigation, the effect of CAN *in vivo* and *in vitro* was investigated. The effect of cantharidin upon known biochemical processes associated with isolated cell particles of rat liver was determined. We found an effect of CAN upon the mitochondria.

MATERIALS AND METHODS

A. Biological preparations

Mitochondria were isolated from rat liver and kidney according to the procedure described by Schneider.[8] Versene was not used in these studies. The final pellet was suspended in 0·25 M sucrose to give a suspension containing 20 mg protein/ml. Chicken liver mitochondria were prepared by an unpublished procedure of M. F. Utter.

Microsomes were prepared from 24-hr fasted rats by the method of Sachs.[9] Fresh preparations were used in all experiments. The supernatant fraction from the 105,000-*g* centrifugation was used as the source of pH 5 enzyme and soluble RNA. Lysosomes were prepared exactly as described by Rademaker and Soons.[10] In the mitochondrial swelling experiments, the mitochondria were prepared in 0·44 sucrose without Versene. The last wash and final suspension were made in 0·15 M KCl plus 0·02 M Tris, pH 7·4.

In preparation of the CAN-poisoned animal, male albino rats (250 g) of the Holtzman strain were used. About 5 mg cantharidin in 0·1 M Tris buffer, pH 7·4, was injected into the left lower quadrant of the peritoneal cavity. This dose is about ten times the amount required to give acantholysis. The animals were sacrificed 60 min after injections. The usual time of death is 75 min post injection.

B. Analytical methods

Manometric technique. The conventional direct Warburg method was used. Each 14 to 16 ml flask contained in a final volume of 3·0 ml: 40 μmoles K_2HPO_4-KH_2PO_4 buffer, pH 7·4; 15 μmoles Tris-HCl buffer, pH 7·4; 7·5 μmoles ADP; 10 μmoles $MgCl_2$; 60 μmoles glucose; 30 μmoles NaF; 300 Kunitz-MacDonald units hexokinase; 50 μmoles substrate; and 10 mg mitochondrial protein. Cantharidin was used as the Tris salt; 0·25 M sucrose was used to make the final volume 3·0 ml. Readings were taken every 10 min for 30 min and the reaction was stopped by adding 0·2 ml 65% tricholorocetic acid. The mixture was centrifuged, and the centrifugate was used for inorganic phosphate determination by the method of Gomori.[11]

Protein incorporation studies. The reaction mixture consisted of 0·5 ml of a microsomal suspension containing 1·5 ml of the supernatant from the 105,000-*g* centrifugation, 8 to 10 mg of protein, and 0·4 ml of the ATP-generating system described by Smuckler and Benditt.[12] Valine-1-^{14}C was the amino acid whose incorporation into microsomal protein was determined. The incubation and isolation procedures were similar to those of Smuckler and Benditt. The protein residue was dissolved in 1·3 ml of concentrated formic acid, and 1·0-ml aliquots were counted in a liquid scintillation counter. Protein was determined either by the biuret method of Gornall *et al.*[13] or the Folin-phenol method of Lowry *et al.*[14]

Enzyme assays. Acid phosphatase, cathepsin, and glucose-6-phosphatase activities were assayed as described by Gianatto and deDuve.[15] Rhodanese activity was assayed by the method of Rosenthal *et al.*[16] Succinoxidase activity was determined by the manometric procedure of Schneider and Potter.[17]

Mitochondrial swelling measurements. Mitochondrial swelling was followed by changes in turbidity (D_{520}) in a Beckman DU spectrophotometer modified by a Gilford Instruments model 2000 multiple absorbance recorder. Recordings were made at 5-sec intervals. The mitochondrial suspension was added in appropriate amounts to 0·125 M KCl + 0·02 M Tris buffer, pH 7·4. The total content of the cuvet was 4·0 ml.

Lipid peroxide determination. The thiobarbituric acid color reaction was determined by the method of Ottolenghi.[18]

C. Materials

All common chemicals were of analytical reagent grade. Valine-1-^{14}C with a radioactivity of 1 μc/μmole was obtained from New England Nuclear Corp. Sodium pyruvate, 2-phosphoenolpyruvic acid, and pyruvate kinase were obtained from the California Corp. for Biochemical Research. Hexokinase type III or IV, GTP, L-glutamate, and α-ketoglutarate were obtained from the Sigma Chemical Co.; ATP and ADP were products of the Pabst Brewing Co.; CAN was purchase from Inland Alkaloid Co. D,L-β-Hydroxybutyric acid sodium salt was obtained from Mann Research Labs.; carbonyl cyanide phenylhydrazone was a gift from Dr. P. G. Heytler of the Dupont Co. Distilled water was redistilled from an all-glass still.

RESULTS

Effects upon mitochondria. Our initial attempts to determine which subcellular biochemical events are affected by cantharidin led us first to study the mitochondria. Succinoxidase activity of the liver mitochondria of rats poisoned with CAN was decreased when compared to control preparations. The coupled oxidation of succinate by the mitochondria in the presence of inorganic phosphate and ADP was then studies. These investigations showed that there was an uncoupling of the liver and kidney mitochondria of poisoned animals with a marked decrease in P/O ratio (Table 1). Other substrates tested, such as L-glutamate and D,L-β-hydroxybutyrate, also followed this pattern.

In-vitro studies were performed by incubation of CAN with rat liver mitochondria. We found as did Decker[7] that relatively high concentrations of CAN (5 mM) were necessary to produce the uncoupling effect. We did find that the effect of CAN upon the rat liver mitochondria *in vitro* varied with the substrate tested (Table 2). Succinate and D,L-β-hydroxybutyrate followed the pattern found in the *in vivo* study—namely, an uncoupling of oxidation from phosphorylation. However, when L-glutamate, α-ketoglutarate, or pyruvate was the substrate, the P/O ratios were maintained and, in fact, a near two-fold stimulation of inorganic phosphate uptake and oxygen consumption was noted. The stimulatory effect increased with the concentration of CAN in the vessel. We have determined that a concentration of 2,4-dinitrophenol as low as 6 μM in the presence of 8 Mm Can will abolish the stimulatory effect noted. The phosphate uptake is almost abolished, whereas oxygen consumption contines at greater than normal levels. This uncoupling of oxidation from phosphorylation serves to indicate that the stimulatory effect is not related to substrate phosphorylation.[19]

TABLE 1. P/O RATIOS OF MITOCHONDRIA FROM CANTHARIDIN-POISONED RAT COMPARED WITH CONTROL PREPARATIONS

Substrate	Type	Liver				Kidney			
		N	O_2 Consumption (μ atoms/30 min)	PO_4 Uptake (μ moles/30 min)	P/O	N	O_2 Consumption (μ atoms/30 min)	PO_4 Uptake (μ moles/30 min)	P/O
Succinate	Control	9	8·0	15·3	1·91	6	11·15	20·65	1·85
Succinate	Treated	9	8·56	6·45	0·75	6	8·8	3·87	0·44
L-Glutamate	Control	24	7·64	23·2	3·03	9	3·98	10·6	2·67
L-Glutamate	Treated	24	3·46	4·82	1·40	9	2·52	1·29	0·51
D,L-β-Hydroxybutyrate	Control	9	4·39	11·8	2·69				
D,L-β-Hydroxybutyrate	Treated	9	2·41	2·58	1·08				

Additions as described in the text; 5 mg cantharidin was injected into the peritoneum of the treated animals and they were sacrificed 60 min after injection. N = number of determinations.

TABLE 2. EFFECT OF CANTHARIDIN UPON RAT LIVER MITOCHONDRIA *IN VITRO*

Substrate	Control				Treated			
	N	O_2 Consumption (μatoms/30 min)	PO_4 Uptake (μmoles/30 min)	P/O	N	O_2 Consumption (μatoms/30 min)	PO_4 Uptake (μmoles/30 min)	P/O
Succinate	6	17·0	28·4	1·66	6	11·5	9·12	0·79
L-Glutamate	24	5·16	16·75	3·24	24	13·2	39·2	2·99
D,L-β-hydroxybutyrate	6	6·70	19·0	2·83	6	5·3	9·34	1·77
Pyruvate	6	2·16	6·2	2·87	6	3·58	10·0	2·79
α-Ketoglutarate	6	2·17	5·23	2·41	6	5·77	17·74	3·07

Each cup contained CAN concentration of 8·5 mM. Additions as described in the text. N = number of determinations.

TABLE 3. EFFECT OF CANTHARIDIN UPON RAT KIDNEY AND CHICKEN LIVER MITOCHONDRIA *IN VITRO*

Substrate	Type	Control				Treated			
		N	O_2 Consumption (μ atoms/30 min)	PO_4 Uptake (μ moles/30 min)	P/O	N	O_2 Consumption (μ atoms/30 min)	PO_4 Uptake (μ moles/30 min)	P/O
Succinate	Chicken liver	9	20·2	36·77	1·82	9	17·55	25·80	1·47
Succinate	Rat kidney	6	20·5	28·71	1·40	6	25·5	7·74	0·30
L-Glutamate	Chicken liver	9	4·46	12·40	2·77	9	6·66	11·29	1·90
L-Glutamate	Rat kidney	6	1·88	6·45	3·44	6	3·38	5·81	1·73

Each cup contained CAN concentration of 8·5 ,mM. Additions as described in text. N=number of determinations.

We have extended our studies to the *in vitro* effect of CAN upon rat kidney and chicken liver mitochondria. We found that with these preparations, CAN shows an uncoupling effect with succinate as well as L-glutamate as substrate (Table 3).

Mitochondrial swelling. As a result of our experiments on oxidative phosphorylation, we tested the ability of CAN to cause swelling of rat liver mitochondria. This compound is indeed a potent swelling agent with some unique properties. The ability to cause swelling is not strictly proportional to concentration (Fig. 1). The lowest

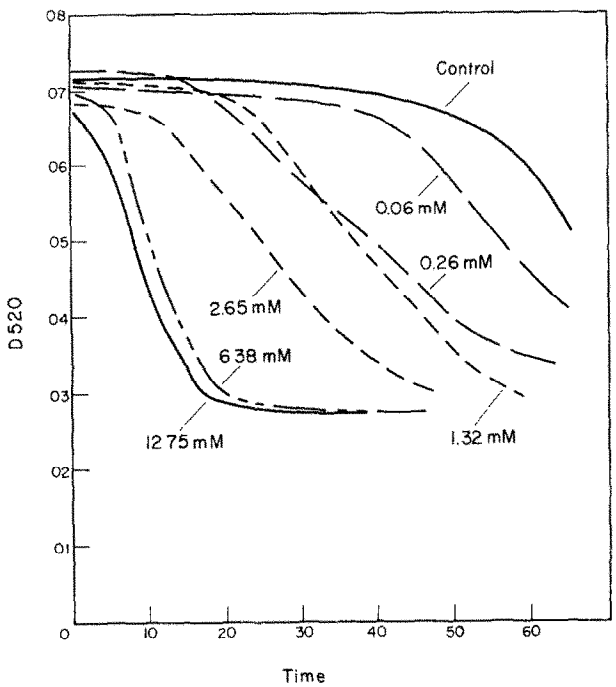

FIG. 1. Swelling of rat liver mitochondria related to cantharidin concentration. Suspension medium was 0·125 M KCl + 0·02 M Tris buffer, pH 7·4. Results shown are the average of at least three close determinations.

concentration of CAN noted to cause swelling is 63·8 μM. The CAN-induced swelling is inhibited by cyanide but is not affected by 2,4-dinitrophenol and carbonyl cyanide phenylhydrazone (Fig. 2) in concentrations known to inhibit oxidative phosphorylation. High concentrations of cantharidin can overcome the inhibition by cyanide. The swelling is readily reversed by ATP. Addition of ATP + Mg^{2+} + bovine serum albumin had a slightly better reversal effect than ATP alone (Fig. 3); Mg^{2+} or albumin alone showed no ability to cause reversal. Chicken liver mitochondria also undergo swelling upon CAN addition.

The ability to produce lipid peroxides as determined by the thiobarbituric acid reaction was tested. We were unable to detect any difference in the cantharidin-treated mitochondria as compared to their controls. Concentrations of CAN up to 25·5 mM were used in these studies.

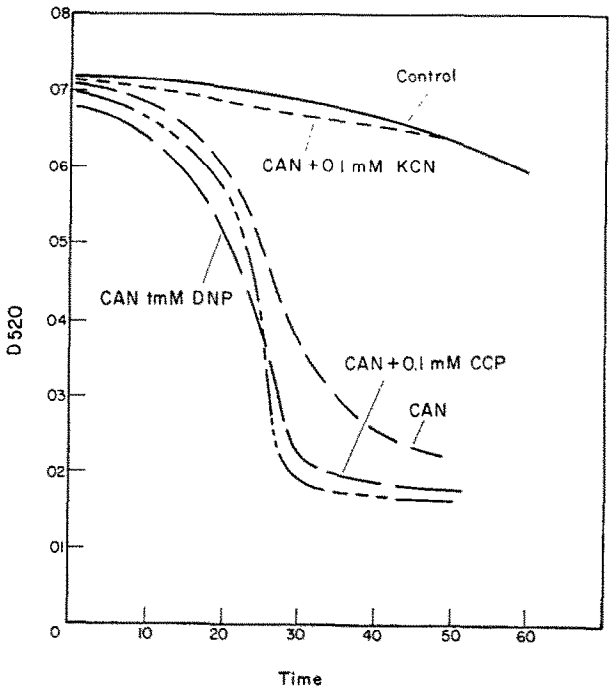

FIG. 2. Effect of known inhibitors of oxidative phosphorylation on swelling of rat liver mitochondrai induced by 0·26 mM cantharidin. Results shown represent a minimum of six determinations.

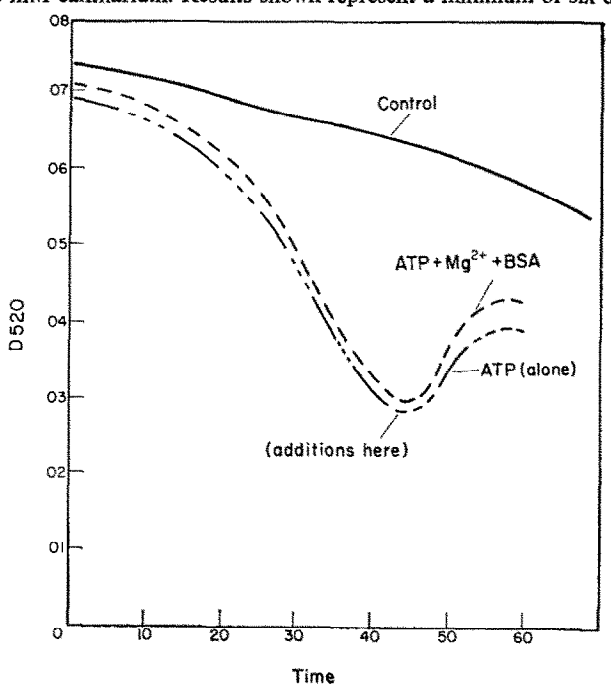

FIG. 3. Reversal of cantharidin-induced swelling by ATP. One hundreth of the volume in the cuvet (0·04 ml) was added. The final concentration of ATP was 1 mM in each instance. The final concentration of Mg^{2+} was 5 mM and of albumin was 2 mg/ml. Results shown are the average of five close determinations.

Effect upon lyosomes. Lysosomal particles have been associated with cellular death.[20] We reasoned that the acantholytic liver and skin cells may represent dying cells and therefore examined the lysosomal particles from the livers of cantharidin-poisoned rats. DeDuve and collaborators[21] have shown that acid phosphatases and cathepsin activities in rat liver are associated with the lysosomal particles. We examined these enzymatic activities in the CAN-poisoned rat plus rhodanese as a representative enzyme associated with mitochondria and glucose-6-phosphatase associated with microsomal particles. We found, as seen in Table 4, that the activity of enzymes

TABLE 4. ENZYMATIC ACTIVITIES ASSOCIATED WITH SUBCELLULAR PARTICLES IN THE CANTHARIDIN-POISONED RAT AS COMPARED TO CONTROL ANIMALS

Enzyme	Fraction examined	Control	CAN-Treated
Acid phosphatase	Lysosomes	2·43	2·65
Cathepsin	Lysosomes	0·43	0·38
Rhodanese	Mitochondria	103	112
Glucose-6-phosphatase	Microsomes	12·7	11·5

Activities are expressed as decomposition of one μmole substrate per min/g liver tissue.

associated with the lysosomal particles from treated animals does not vary from the control preparations. We did not examine the activities of these enzymes in the soluble phase.

Effects upon amino acid incorporation into microsomal protein. Recent studies by Smuckler and Benditt[12] and Recknagel and Lombardi[22] have indicated that in the carbon tetrachloride-poisoned rat, the primary action is upon the microsomal fraction. The ability of microsomes isolated from such poisoned animals to incorporate amino acids into protein is impared. In our experiments, microsomes isolated from control and treated rat livers were incubated with 5μc of valine-1-^{14}C. Microsomes from three control

TABLE 5. INCORPORATION OF VALINE 1^{14}-C INTO MICROSOMAL PROTEIN

Microsomes	105,000-*g* Supernatant	dpm
Control	Control	544
Control	Treated	374
Treated	Control	487
Treated	Treated	368

Tracer was 4 μc of valine-1-^{14}C. The media used is as described in text. Microsomal protein was adjusted to 8 mg in each experiment. Activity measurements were converted to disintegrations per minute (dpm) by addition of an internal standard.

animals incorporated the amino acid on the average of 1,525 $\pm$ 45 dpm, and those from three treated animals incorporated on the average 1,545 $\pm$ 80 dpm. In another set of experiments (Table 5), liver microsomes from control animals were incubated with liver supernatant fraction from control or experimental animals, and microsomes from experimental animals were incubated with supernatant fractions from control

or experimental animals. Our data indicate that there is some impairment of amino acid incorporation by the 105,000-*g* supernatant solution obtained from the liver of the cantharidin-poisoned rat.

DISCUSSION

We have used rat liver as a model system to study the effects of the vesicating agent upon cellular particles. Opie[23] in 1912 first showed that animals poisoned by cantharidin exhibited the same histological changes in liver portal cells and kidney tubule cells as that seen in the skin epidermis. The dosage employed in this study is a lethal one, and the cellular changes in liver and kidney occur in 15 min or less.[24] Correlation of the biochemical events reported here with cellular acantholysis requires further investigation.

The *in vitro* studies reported here show that a concentration of 5 mM CAN is required as an uncoupling agent when succinate is used as substrate. Other substances such as salicylate[25] are also known to uncouple oxidative phosphorylation at this concentration. The stimulatory effect noted with L-glutamate, α-ketoglutarate, or pyruvate as substrate would indicate that possibly some process related to, or as a result, of the oxidative decarboxylation that these compounds undergo may be the site of action.

The investigation of the ability of CAN to cause swelling of rat liver mitochondria may be of importance in determining the primary pathologic event. We have found that the smallest concentration of CAN necessary to produce acantholysis in skin epidermal cells *in vitro* is 20 μM. This compares favorably with the lowest concentration of CAN, 63·8 μM, found to cause swelling of mitochondria *in vitro*. The results reported here make it difficult to classify CAN with other known swelling agents, since compounds such as phosphate and thyroxine are inhibited by 2,4-dinitrophenol and CAN is not. The reversal of cyanide inhibition of CAN-induced swelling may indicate the possibility that oxidative phosphorylation plays no active role in this process. The lack of inhibition by the potent inhibitors 2,4-dinitrophenol and carbonyl cyanide phenylhydrazone also supports this supposition. The disruptive influence of CAN on the mitochondrial membrane does not result in lipid peroxide formation. The ability of CAN-induced swollen mitochondria to "contract" in the presence of ATP would indicate that they are not completely distorted. It is possible that the compound, being lipid soluble, acts generally to disrupt lipoprotein membranes in some manner. Electron microscopy of liver cells from poisoned animals indicates that the outer cell membrane, the mitochondrial membrane, and the endoplasmic reticulum are distorted.[26]

REFERENCES

1. G. Miescher, *Arch. Derm. Syph. (Berlin)* **173**, 142 (1936).
2. W. F. Lever, *Medicine (Baltimore)* **32**, 1 (1953).
3. R. B. Stoughton and F. K. Bagatell, *J. invest. Derm.* **33**, 287 (1959).
4. H. Haas, A. Kraushaar and C. Cordua, *Naunyn-Schmiedeberg's Arch. ex. Path. Pharmak.* **209**, 138 (1950).
5. J. H. Allison and D. H. Williamson, *Brit. J. Derm.* **72**, 383 (1960).
6. D. E. Weakley and J. Einbinder, *J. invest. Derm.* **84**, 459 (1961).
7. R. Decker, *J. invest. Derm.* **42**, 465 (1964).
8. W. C. Schneider, *J. biol. Chem.* **176**, 259 (1948).
9. H. Sachs, *J. biol. Chem.* **228**, 23 (1957).

10. W. RADEMAKER and J. B. SOONS, *Biochim. biophys. Acta* **24**, 209 (1957).
11. G. GOMORI, *J. Lab. clin. Med.* **27**, 955 (1941).
12. E. A. SMUCKLER and E. P. BENDITT, *Science* **140**, 308 (1963).
13. A. G. GORNALL, D. J. BARDAWELL and M. DAVID; *J. biol. Chem.* **177**, 751 (1949).
14. O. LOWRY, N. ROSEBROUGH, A. FARR and R. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
15. R. GIANETTO and C. DEDUVE, *Biochem. J.* **49**, 433 (1955).
16. O. ROSENTHAL, C. ROGERS, H. VARS and C. FERGUSON, *J. biol. Chem.* **185**, 669 (1950).
17. W. C. SCHNEIDER and V. R. POTTER, *J. biol. Chem.* **149**, 217 (1943).
18. A. OTTOLENGHI, *Arch. Biochem.* **79**, 355 (1959).
19. F. E. HUNTER, JR., *Phosphorus Metabolism* I, p. 297.
20. C. DEDUVE, in *Subcellular Particles*, T. Hayashi, Ed., 128. New York, Ronald Press (1959).
21. C. DEDUVE, B. C. PRESSMAN, R. GIANETTO, R. WATTAUX and F. APPELMANS, *Biochem. J.* **60**, 604 (1955).
22. R. O. RECKNAGEL and B. LOMBARDI, *J. biol. Chem.* **236**, 569 (1961).
23. E. L. OPIE, *J. exp. Med.* **16**, 331 (1912).
24. F. K. BAGATELL. Unpublished experiments.
25. J. T. MIYAHARA and R. KARLER, *Fed. Proc.* **23**, 178 (1964).
26. G. WILGRAM. Unpublished experiments.