$$A_1 = \left[2 + 2 \int_0^\infty \eta^{1/3} d\psi_2 \int_0^\infty \eta^{-1/3} d\psi_2 \right] \quad \text{(Eq. 12)}$$

when $\alpha = 1$ and $A_1 = 4.0$, the rate constant of Eq. 11 reduces to that of Eq. 6 for the homogeneous case. Thus, A_1 is a significant parameter accounting for the effect of heterogeneity on the rate of aggregation. By comparing Eqs. 3 and 11,

$$\alpha = \frac{3K\mu}{A_1 kT} \quad \text{(Eq. 13)}$$

For the protein system, α was approximately 1, and A_1 was 4.148 ± 0.022. Then heterogeneity accounted for about 4% increase in the rate. From Eq. 11 the rate constant found was 5.81×10^{-12} cm.^{3} sec.$^{-1}$. The comparison of methods of determining the rate constant is summarized in Table III.

TABLE III.—COMPARISON OF METHODS OF DETERMINING RATE CONSTANTS OF AGGREGATION

Method of Treatment	Rate Constant, cm.^{3} sec.$^{-1}$
Smoluchowski's theory for monodispersed distribution taking N_∞ from	
(*a*) $\geq v = 0.6289 \times 10^{-12}$ cm.^{3}	6.19×10^{-12}
(*b*) $\geq v = 0.02 \times 10^{-12}$ cm.^{3}	5.91×10^{-12}
Self-preservation theory	5.81×10^{-12}

It should be noted that the approach of self-preservation theory offers a method of determining the aggregation rate constant independent of that approach which assumed a monodispersed system at initial periods of the aggregation process. Since the effective collision between particles was unity and the particle size distribution was essentially monodispersed, the rate constants determined by both methods were in good agreement.

Studies are continuing on the mechanism of the pH influence on the aggregation behavior. Experiments on the effects of electrolytes, temperature, and other additives are also planned.

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Some Effects of Chloroquine on Oxidative Processes in Rat Heart

By GLORIA A. ARDUESER* and HAROLD C. HEIM

Chloroquine at a concentration of 5.4×10^{-4} *M* enhanced the oxygen consumption of rat ventricle homogenates respiring in the presence of succinate. This effect was not noted with aged homogenates. Total α-keto acids present after incubation of homogenates with succinate was observed to be diminished when chloroquine was present. The oxidation of malate and β-hydroxybutyrate was inhibited by the addition of chloroquine. Chronic poisoning by this drug did not affect the ability of heart homogenates to utilize β-hydroxybutyrate, but the oxidation of malate was impaired. A purified malic dehydrogenase was not inhibited by chloroquine but, at a concentration of 5.4×10^{-4} *M*, the drug markedly inhibited the oxidation of NADH by a fragmented mitochondrial suspension prepared from rat myocardium.

CHLOROQUINE has been shown to have an unexpectedly wide spectrum of therapeutic uses since its introduction into medicine as an antimalarial. The increased utilization of this drug at fairly high dose levels for extended periods has prompted reconsideration of its potential toxicity.

Visual disturbances, headache, bleaching of hair, electrocardiographic changes, and weight loss have been reported during short-term administration of chloroquine to healthy subjects (1). Electrocardiographic changes, consisting of lowered T wave, ST segment depression, and prolongation of QTc interval have occurred when

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the drug was used in the treatment of conditions such as extraintestinal amebiasis (2). Similar changes have been noted during the course of acute, fatal, chloroquine poisoning (3). Muscle weakness and bilateral loss of knee and ankle reflexes have been demonstrated during extended chloroquine therapy in rheumatoid arthritis (4, 5).

Upon examination of several cases of acute chloroquine poisoning in children, a striking similarity to poisoning by quinidine was noted (6). It was suggested that the cardiac arrest which occurred might be due to a direct myocardial action, to anoxia from respiratory depression, or to both.

It has been reported that substantial differences exist in the toxicity of chloroquine for various laboratory animals, the compound being most toxic to the dog and least toxic to the mouse. The rat appeared to be more susceptible to the drug than the mouse. The study further indicated that chloroquine had a distinctly steeper dose-toxic response curve than other 4-aminoquinolines (7). This suggests that the drug has a narrow margin of safety, a factor which may be of great significance in therapeutic applications where high dose levels are necessary.

Long-term, chronic administration of chloroquine to rats indicated slight gross pathological changes (8). However, microscopic examination of tissues revealed a slowly developing necrosis of cardiac and skeletal muscle.

Because some of the cardiac effects of chloroquine might be due to the interference with metabolic processes of the myocardium, it appeared to be of interest to investigate the effect of the drug on some of the respiratory processes of heart homogenates.

METHODS

Conventional manometric techniques were used to study the effect of chloroquine on the oxidation of succinate, malate, and β-hydroxybutyrate by myocardial homogenates prepared from male Sprague-Dawley rats (9).

The animals were stunned by a blow to the head, exsanguinated, and the hearts removed immediately. Each heart was allowed to beat a few seconds in cold 0.1 *M* phosphate buffer, pH 7.35, after which the ventricular tissue was dissected away, cut open, washed in cold buffer, blotted dry on filter paper, and weighed on a torsion balance.

The homogenate was prepared by placing the tissue in a glass homogenizer containing sufficient cold buffer so that 0.5 ml. of homogenate contained 10 mg. of fresh tissue for the succinate studies or 40 mg. for the malate and β-hydroxybutyrate studies. Not more than 15 min. elapsed from the time the animals were sacrificed until the addition of the homogenate to the Warburg flasks. In some experiments the homogenates were aged before use by allowing them to remain in the refrigerator at 5° overnight followed by 5 hr. at room temperature.

The mitochondrial preparations utilized in the study of the effect of chloroquine on the oxidation of reduced nicotinamide adenine dinucleotide[1] were obtained by centrifuging homogenates, prepared in cold 0.25 *M* sucrose, at 700 × g for 12 min. at 2° in a Servall RC-2 refrigerated centrifuge equipped with a SS-34 rotor to remove cell fragments. The supernatant was then centrifuged at 10,000 × g for 12 min. The resulting pellet was dispersed in 0.25 *M* sucrose, and the suspension added to the flasks so that each contained mitochondria representing 100 mg. of ventricle. The suspension was found to oxidize NADH quite rapidly.

In all of the studies utilizing manometric techniques the center well of the Warburg flasks contained 0.2 ml. 10% KOH and a pleated strip of filter paper. All of these experiments employed air as the gas phase. A temperature of 30° was maintained during respiration determinations utilizing malate, β-hydroxybutyrate, and NADH as substrates. The effects of glutamate and chloroquine[2] on the oxidation of succinate were studied at 37°.

The buffer used for control of pH in the Warburg vessels during the succinate studies was 0.1 *M* potassium phosphate, pH 7.35, containing 0.01 *M* disodium ethylenediaminetetraacetate (EDTA) per liter as recommended by several investigators (10, 11). The main compartments of the flasks in which the oxidation of succinate by aged homogenates was studied contained 0.5 ml. of homogenate; 0.3 ml. of 5×10^{-2} *M* chloroquine; 0.3 ml. of 5×10^{-4} *M* cytochrome c; 1.3 ml. of phosphate buffer. Distilled water was used to replace the chloroquine in the control flasks. The sidearm contained 0.4 ml. of 0.1 *M* succinate which was tipped into the main compartment at zero time. In some of the experiments with aged homogenates, 0.2 ml. of 10^{-3} *M* NAD, dissolved in phosphate buffer, replaced 0.2 ml. of the buffer.

When the effect of glutamate and chloroquine on the oxidation of succinate was studied, Warburg vessels with two sidearms were employed. One sidearm of each vessel contained 0.4 ml. of 0.1 *M* succinate and was tipped at zero time. The second sidearm contained 0.3 ml. of either distilled water or 5×10^{-3} *M* of chloroquine or 0.1 *M* glutamate and was tipped after 45 min. The main compartment contained 0.5 ml. of homogenate; 0.3 ml. of 5×10^{-4} *M* cytochrome c; 1.0 ml. of phosphate buffer, and sufficient distilled water to give a final volume of 3.0 ml. In some experiments 0.3 ml. of 0.1 *M* glutamate or 0.3 ml. of 5×10^{-3} *M* chloroquine was placed in the main compartment instead of in the second sidearm.

In the study of the oxidation of malate and β-hydroxybutyrate, the main compartment of each flask contained 0.5 ml. of homogenate; 0.3 ml. of 5×10^{-3} *M* chloroquine or distilled water; 0.1 ml.

[1] Henceforth designated as NADH. The oxidized form will be designated as NAD.

[2] The chloroquine used in this study was graciously supplied by the Winthrop Laboratories, New York, N. Y., as Aralen Diphosphate.

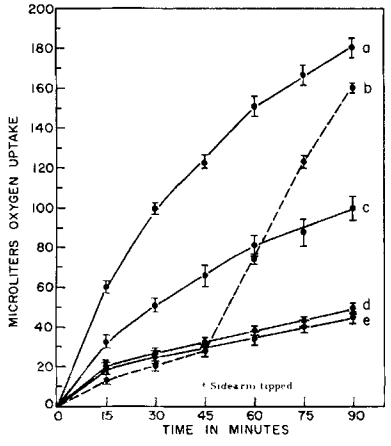

Fig. 1.—The effect of glutamate and chloroquine on succinate in heart homogenates. Key: curve a, glutamate in main compartment; curve b, glutamate tipped from sidearm; curve c, chloroquine in main compartment; curve d, control (no additions); curve e, chloroquine tipped from sidearm.

of 5×10^{-4} *M* cytochrome c; 0.1 ml. of 1.5×10^{-2} *M* $MgCl_2$; 0.1 ml. of 1.5×10^{-2} *M* nicotinamide; 0.1 ml. of 2×10^{-2} *M* adenosine triphosphate; 1.1 ml. of 0.1 *M* potassium phosphate buffer with no EDTA added, pH 7.35; and 0.4 ml. of 0.1 *M* substrate. When malate was employed as the substrate, 0.3 ml. of 1.5×10^{-2} *M* glutamate was included, and the 0.1 ml. of 1.5×10^{-2} *M* malate which was used in the β-hydroxybutyrate studies was excluded from the reaction mixture. Distilled water was added to make the final volume 3 ml.

A reaction mixture similar to that which has been used by other investigators (12) was employed to study the effect of chloroquine on oxidation of NADH by a suspension of swollen and/or fragmented mitochondria. The main compartment of each flask contained 0.75 ml. of a buffer, pH 7.4, consisting of 0.04 *M* potassium phosphate, 0.04 *M* tris (hydroxymethyl) aminomethane, and 0.4 *M* KCl; 0.15 ml. of 0.4 *M* KF; 0.15 ml. of 0.12 *M* $MgCl_2$; 0.5 ml. mitochondrial suspension, and 0.3 ml. 5×10^{-3} *M* chloroquine or distilled water. The sidearm, containing 0.4 ml. of 0.04 *M* NADH, was tipped at zero time. Distilled water was added to make the final volume 3 ml. Readings were taken every 5 min. during a 45-min. period.

The method described by Friedemann (13) was used for the determination of total α-keto acids present in the flasks after a 90-min. incubation of fresh homogenates with succinate as the substrate.

The effect of chloroquine on malic dehydrogenase was determined by following the rate of oxidation of NADH at 340 mμ according to the method of Ochoa (14). In these experiments a purified malic dehydrogenase, obtained from commercial sources,[3] was used, and the reaction was followed for 4 min. at 25° with a Beckman DU spectrophotometer equipped with a Guilford automatic recorder.

The possibility of complex formation occurring between chloroquine and NAD was studied spectrophotometrically using a Beckman DB and recording the spectra between 220 and 400 mμ. In these experiments 0.5 ml. of 5×10^{-5} *M* NAD, 0.5 ml. of 5×10^{-5} *M* chloroquine, 1.0 ml. of H_2O, and 1.0 ml. of 0.1 *M* phosphate buffer (pH 7.35) were incubated for 10 min. at 25° prior to the determination. The reference cell contained the same reagents, except that NAD was replaced by buffer.

In order to study the chronic effects of chloroquine administration on the respiratory processes of the heart, young male rats weighing approximately 100 Gm. were separated into 2 groups of six animals each, further designated as groups A and B. Each rat in group A received daily intraperitoneal injections of 5 mg. of chloroquine phosphate in a volume of 0.5 ml. of distilled water for 7 days and 9 mg. of the drug in the same volume thereafter. Group B received 1.5 ml. of distilled water daily by the same method of administration. On the 14th day the animals were sacrificed, and heart homogenates prepared from each animal. The ability of heart homogenates prepared from animals in group A to utilize malate and β-hydroxybutyrate was compared to that of group B.

RESULTS AND DISCUSSION

Figure 1 shows that chloroquine enhanced the oxygen uptake of freshly prepared homogenates in the presence of added succinate. This effect was previously noted by Datta and Basu (15) with mouse liver homogenates, but not by Hess and Haugaard (16) who used rat heart slices and homog-

Table I.—Effect of Chloroquine on α-Keto Acid Accumulation

	Chloroquine Absent	Chloroquine Present
α-Keto acid/10 mg. tissue/90 min., mcg.	15.5	3.25
	15.0	5.0
	20.0	4.4
	19.0	5.5

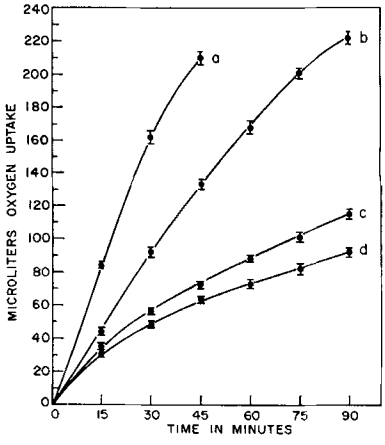

Fig. 2.—Oxidation of succinate by aged heart homogenates. Key: curve a, control (no drug added); curve b, chloroquine (final concentration 5.4×10^{-3} *M*); curve c, NAD (final concentration 7×10^{-5} *M*); curve d, NAD (final concentration 7×10^{-5} *M*) and chloroquine (final concentration 5.4×10^{-3} *M*).

[3] Sigma Chemical Co., St. Louis ,Mo.

enates. The oxidation of succinate proceeds *via* the following pathway: succinate → fumarate → malate $\xrightarrow{NAD}$ oxaloacetate.

It has been reported that oxaloacetate is an inhibitor of succinate oxidation, *in vitro*, (10) and it follows that an accumulation of oxaloacetate in the Warburg vessels might cause a curtailment of oxygen consumption. On the other hand, chloroquine, by preventing the accumulation of oxaloacetate, could conceivably increase oxygen uptake. The data reported in Table I support this concept and show that after 90 min., the α-keto acid content of flasks to which chloroquine was added at the beginning of the experiment was much lower than that of the control flasks. Heim *et al.* (17) have reported that emetine prevents the accumulation of oxaloacetate and augments the oxygen consumption of heart homogenates respiring in the presence of added succinate. Wolff and Ball (10) showed that thyroxine evokes the same effect.

When chloroquine was added to the flasks 45 min. after the beginning of the experiments an enhancement of oxygen uptake was not observed, possibly because sufficient oxaloacetate had accumulated during the 45-min. period to exert an inhibitory effect. Heart tissue is rich in glutamate-oxaloacetate transaminase and, if the lowered oxygen consumption were due to an accumulation of oxaloacetate, the addition of glutamate would be expected to reverse the inhibitory effect of the oxaloacetate. As shown in Fig. 1, when glutamate was added to the flasks, either at the beginning of the experiment or after 45 min. had elapsed, the oxygen consumption was greatly increased. Therefore, it seems probable that glutamate, *via* transamination, removes the oxaloacetate while chloroquine, when added at the beginning of the experiment, appears to prevent the accumulation of the inhibitory compound. This suggests that the drug may inhibit at some point on the pathway between succinate and oxaloacetate.

With aged homogenates it was found that chloroquine evoked an appreciable inhibition of oxygen uptake in the presence of added succinate. When NAD was added at the beginning of the experiments, the oxygen consumption was markedly inhibited and further reduced, but only to a slight extent, by chloroquine. These results are shown in Fig. 2.

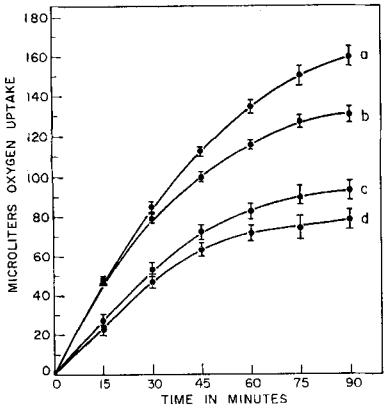

Fig. 3.—Respiration of rat heart homogenates in presence of malate. Key: curve a, control (no drug added); curve b, chloroquine (final concentration 5.4×10^{-4} *M*); curve c, endogenous respiration; curve d, endogenous respiration with added chloroquine.

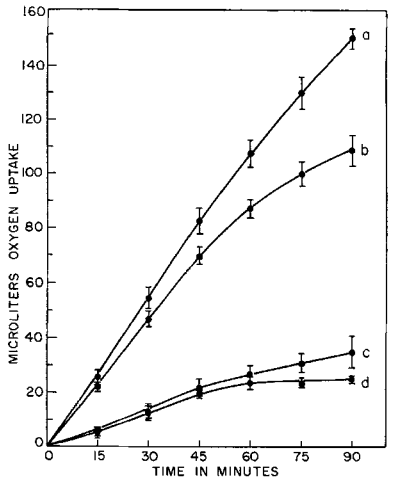

Fig. 4.—Oxidation of β-hydroxybutyrate by heart homogenates. Key: curve a, control (no drug added); curve b, chloroquine (final concentration 5.4×10^{-4} *M*); curve c, endogenous respiration; curve d, endogenous respiration with chloroquine added.

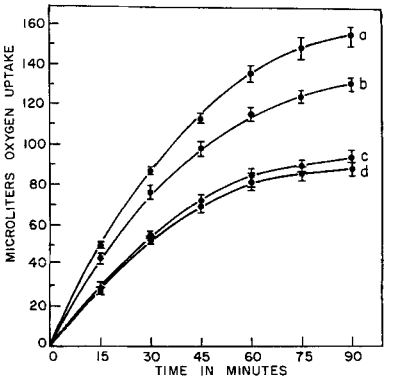

Fig. 5.—Oxidation of malate by heart homogenates from sham-injected and chloroquine-treated rats. Key: curve a, sham-injected animals; curve b, chloroquine-treated animals; curve c, endogenous respiration of chloroquine-treated animals; curve d, endogenous respiration of sham-injected animals.

The oxidation of malate, unlike that of succinate, is an NAD-linked process and contributes substantially to the production of high energy phosphate compounds. Figure 3 shows that chloroquine evokes an inhibitory effect on the oxidation of added malate by homogenates. Datta and Basu (15), utilizing considerably higher concentrations of chloroquine than those employed in this study, demonstrated a rather marked depression of malate oxidation by the drug with mouse liver homogenates.

The oxidation of β-hydroxybutyrate was inhibited by chloroquine, as shown in Fig. 4. It has

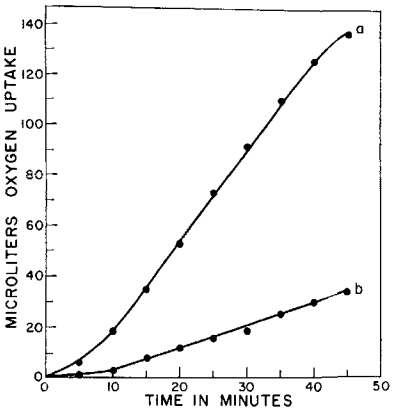

Fig. 6.—Effect of chloroquine on respiration of fragmented mitochondrial suspensions in presence of added NADH. Each point represents determination in duplicate from four animals. Key: curve a, control (no drug added); curve b, chloroquine (final concentration 5.4×10^{-4} *M*).

also been reported by Datta and Basu (15) that certain other NAD-linked reactions are inhibited by chloroquine.

In the animals chronically poisoned by chloroquine a significant loss of weight, similar to that reported by Fitzhugh *et al.* (1), was noted. The ability of heart homogenates, prepared from poisoned animals, to utilize β-hydroxybutyrate was not impaired, but the ability to oxidize malate was somewhat inhibited as illustrated in Fig. 5.

Studies of a preliminary nature were conducted to learn if a complex formation occurs between NAD and chloroquine. No alteration in the absorption spectra could be observed when solutions of the two compounds were mixed. The conclusions which may be drawn from such an observation are limited but present an indication that a complex formation does not occur.

Malic dehydrogenase activity was found to be not significantly affected by chloroquine, in confirmation of the work of Gerlack (18), who observed that the drug elicited no appreciable effect on the activity of either lactic or malic dehydrogenases.

Mushinski *et al.* (19) reported that chloroquine was a potent inhibitor of NADH-cytochrome c reductase in rat skeletal muscle preparations. If this mechanism is operative in heart tissue, it could explain the depression of malate oxidation without a direct effect on malic dehydrogenase. Malate is oxidized in the presence of malic dehydrogenase and NAD to oxaloacetate with the concomitant formation of NADH. NADH is then oxidized to NAD as hydrogen atoms are transferred to flavoproteins, a step which is followed by a series of oxidation–reduction reactions involving the cytochromes. It has been reported that the impermeability to extra-mitochondrial NADH is a characteristic of intact mitochondria (12). The oxidation of added NADH will occur, however, if the mitochondria are exposed to conditions, such as hypotonic media or rigorous isolation procedures, which cause the swelling and/or disruption of mitochondrial membranes. The mitochondrial preparation used in this study displayed a nearly linear oxidation of added NADH over a period of 30 min., and it was found that chloroquine, at a concentration of 5.4×10^{-4} *M*, elicited a marked inhibitory effect on the oxidative process, as shown in Fig. 6. This finding, together with the observed inhibitory effect of the drug on the oxidation of succinate by aged homogenates to which NAD had been added, and the insignificant effect evoked on a purified malic dehydrogenase, suggests that chloroquine may exert effects at or beyond the NAD–NADH step of terminal respiration.

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