14716

The Inhibition of the Succinoxidase System Using Cysteine and Cystine. II. Nature of Inhibiting Substance.*

STANLEY R. AMES AND C. A. ELVEHJEM.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

In a previous study,[1] we investigated the inhibition of the succinoxidase system using cysteine and cystine. At that time we were concerned mainly with the factors influencing the extent of inhibition and it was demonstrated that the concentration of 4-carbon dicarboxylic acids in the tissue homogenate was a determining factor. Although cysteine was added to the enzyme preparations, no inhibition occurred until it was oxidized. The nature of the inhibiting substance was therefore postulated to be cystine, but this was not definitely ascertained.

The experiments of Hopkins and Morgan[2] showed that incubation of a succinoxidase preparation with cystine would cause a loss in activity which could be entirely restored by similar incubation with cysteine. Their conclusions[2,3] were that the oxidized form inhibited and the reduced form restored the activity.

Potter and DuBois[4] discussed the inactivation caused by cysteine and cystine, and reported that both these compounds inhibited the enzyme when they were added to the final reaction mixture which contained cytochrome *c*, in the presence of which sulfhydryl is readily oxidized. However, they reported inhibitions of 51% for cystine and 55% for cysteine at the same inhibitor molarity of M/3000. In view of the fact that the effective concentration of the cysteine when oxidized is only half as great, these values are surprisingly similar. In addition we have shown[1] that in order to obtain reproducible results certain additional factors must be standardized and, therefore, the nature of the inhibiting substance merits reinvestigation.

In this investigation the inhibition caused by the cysteine-cystine system is shown to be due solely to cystine although it cannot be stated whether the dimer or the free radical is the active state.

Methods. White mice of an inbred Swiss strain were used and, after weaning, were maintained on stock ration† and water *ad libitum* plus occasional greens.

A kidney homogenate was prepared by decapitating the mice and allowing the blood to drain for a short time. The tissue was extirpated, rinsed with distilled water, and immediately packed in finely cracked ice. After cooling, bits of fat and connective tissue were removed, the kidney was blotted between moistened filter papers and rapidly weighed. A homogenate of the tissue was prepared by the technic of Potter and Elvehjem[5] in ice-cold redistilled water using a pre-chilled tube and pestle. This homogenate was immediately pipetted into the reaction flasks to which all other reactants had been previously added.

A conventional Warburg apparatus at 37°C was used in all experimental and analytical work.

The activity of succinic dehydrogenase was determined by the method of Potter[6] as modified by Potter and Schneider.[7] A summary

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. These studied were aided by a grant from the Rockefeller Foundation.

1 Ames, S. R., and Elvehjem, C. A., *Arch. Biochem.*, in press.

2 Hopkins, F. G., and Morgan, E. S., *Biochem. J.*, London, 1938, **32**, 611.

3 Hopkins, F. G., Morgan, E. S., and Lutwak-Mann, C., *Biochem. J.*, London, 1939, **32**, 1829.

4 Potter, V. R., and DuBois, K. P., *J. Gen. Physiol.*, 1943, **26**, 391.

† B-B Laboratory Rabbit Diet, Maritime Milling Company, Inc., Buffalo, N.Y.

5 Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114**, 495.

6 Potter, V. R., *J. Biol. Chem.*, 1941, **141**, 775.

7 Potter, V. R., and Schneider, W. C., *J. Biol. Chem.*, 1942, **142**, 543.

of the components of the final reaction mixture is as follows: 0.4 ml of 0.25 M phosphate buffer (pH 7.4), 0.1 ml of 4×10^{-4} M cytochrome *c*, 0.3 ml of 0.5 M sodium succinate (pH 7.4), 0.1 ml of 0.012 M $CaCl_2$, 0.1 ml of 0.012 M $AlCl_3$, the desired amount of homogenate, solution of inhibitor, and redistilled water to make 3.0 ml. The gas phase was air and 0.2 ml of 10% KOH and a small strip of filter paper were placed in the center well to absorb CO_2. The pH of this final reaction mixture was 7.4 as determined electrometrically.

In order to secure reproducible results, the *incubation time, i.e.,* the time the homogenate is incubated at 37°C before the inhibitor is added, and the *contact time, i.e.,* the time the inhibitor is in contact with the enzyme before the substrate is added, were carefully standardized. The numerical inhibitions recorded are valid only for a similar preparation under the identical conditions.

Eastman Kodak Company cysteine hydrochloride was used after being recrystallized from constant boiling hydrochloric acid. Solutions were freshly prepared in redistilled water and neutralized to pH 7.4 just before addition to the reaction flask.

The cystine used was isolated from hair in these laboratories. A suggestion was made in the paper of Hopkins and Morgan[2] that cystine could be brought into solution by means of a small amount of sodium phosphate buffer. In accordance with this information, it was found that cystine could be recrystallized from a sodium phosphate solution. One-half g of cystine was recrystallized in good yield from 120 ml of 0.04 M sodium phosphate buffer of pH 7.4. A stock solution was prepared in 0.05 M sodium phosphate buffer which contained 0.72 mg of cystine per ml of solution. This was supersaturated at 25°C but was stable for 12 hours or more before precipitation occurred.

Experimental. The nature of the cysteine-cystine system must be considered in some detail to see if it is possible to ascertain the nature of the specific inhibiting substance. Previous investigations have not disclosed the fate of the cysteine when it is added to the reaction mixture under consideration. It has been shown by Mathews and Walker[8] that when cysteine is oxidized in a solution of pH about 7.4, cystine is formed. When copper was added to catalyze the reaction, 100% of the theoretical oxygen uptake was achieved assuming the reaction, cysteine → cystine.

It was noted that in the reaction mixture, the addition of cysteine immediately produced a rapid oxygen uptake, but the rate of uptake soon decreased and in 20 min. had ceased entirely. Both cytochrome *c* and the cytochrome oxidase-reductase systems, added as tissue homogenate, were necessary components of the system which oxidized the cysteine. Comparison of the E'_0 values at a pH of about 7 for the couples concerned,

$$\begin{array}{ccccc} \text{cystine} & & \text{cytochrome } c^{+++} & & 1/2\ O_2 \\ \Uparrow\Downarrow & \rightleftharpoons & \Uparrow\Downarrow & \rightleftharpoons & \Uparrow\Downarrow \\ \text{cysteine} & & \text{cytochrome } c^{++} & & H_2O \\ -0.15\ \text{v.}^{\ddagger 9} & & +0.26\ \text{v.}^{10} & & +0.81\ \text{v.}^{11} \end{array} \qquad (1)$$

indicated that if the above mechanism were correct, the amount of cysteine at equilibrium would be extremely small. However, in the reaction mixture, whenever cysteine was oxidized by cytochrome *c*, only about 70% of the theoretical oxygen uptake was observed (Fig. 1). Thus it would appear that there were sufficient hydrogen acceptors in the tissue homogenate to account for 30% of the theoretical oxygen uptake. This postulate may be correct since the uptake decreased from 80 to 60% with a tenfold decrease in cysteine concentration, when the same amount of tissue and cytochrome *c* were present initially. When the oxygen uptake had achieved its maximum value (about 70%), no amount of further incubation would increase the uptake, indicating that no cysteine remained to be oxidized by molecular oxygen through the cytochrome *c* system.

---

8 Mathews, A. P., and Walker, Sidney, *J. Biol. Chem.*, 1909, **6**, 21.

‡ Extrapolated to pH 7 from the E_0 value given by Ryklan and Schmidt.[9]

9 Ryklan, L. R., and Schmidt, C. L. A., *Univ. Calif. Pub. Physiol.*, 1944, **8**, 257.

10 Stotz, E., Sidwell, A. E., and Hogness, T. R., *J. Biol. Chem.*, 1938, **124**, 11.

11 University of Wisconsin Biochemists, *Respiratory Enzymes*, Burgess Publishing Company, 1939, page 179.

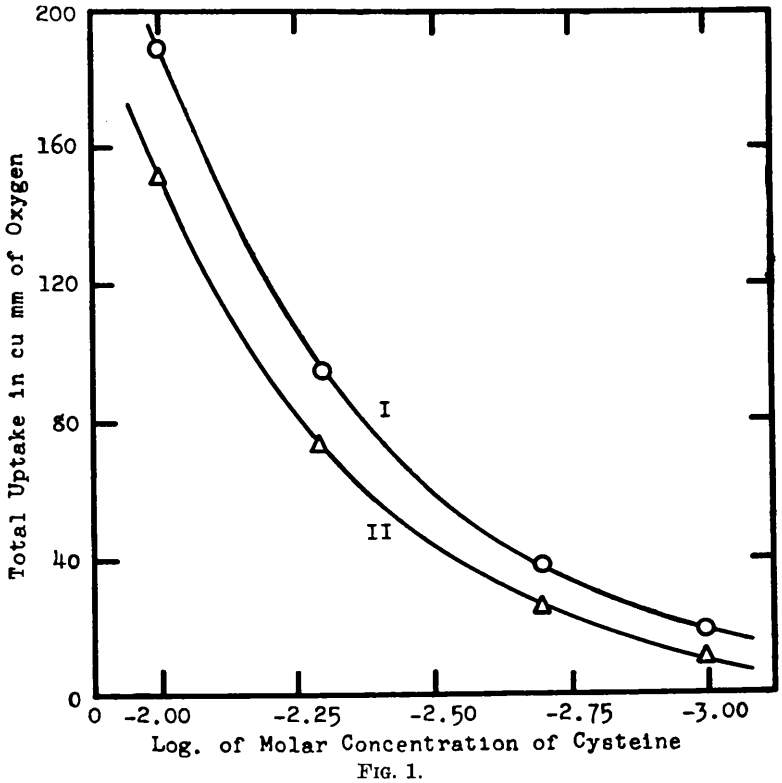

FIG. 1.

Oxidation of cysteine by cytochrome *c* and tissue homogenate. Curve I (O) represents the theoretical uptake which has been corrected to 37°C. Curve II (△) represents the uptake achieved experimentally. Test system used as described in text except that succinate was omitted. Cysteine was added from sidearm after equilibration. Five-hundredths ml of a 10% homogenate of mouse kidney was added. Concentration of cytochrome *c* was 1.3 × 10⁻⁵ M. When either the tissue concentration was reduced by half or the cytochrome *c* doubled, or both, the total uptake remained the same.

TABLE I.
Comparative Inhibitions of Cysteine and Cystine.

Exp.	Description	Q_{O_2}	% inhibition
1	Succinate control, no inhibitor present	190	
2	M/1000 cysteine, cytochrome *c* present initially	125	34
3	M/1000 cysteine, cytochrome *c* added 70 minutes after zero time	170	10
4	M/2000 cystine, cytochrome *c* present initially	113	40
5	M/2000 cystine, cytochrome *c* added 70 minutes after zero time	124	35

Test system used as described in text. Two-tenths ml of a 2% homogenate of mouse kidney was added. The incubation time was 40 min and the contact time was 30 min.

Potter[12] has shown on the basis of spectrophotometric data that in a system where no cytochrome oxidase was present, cytochrome *c* was not 100% reduced by cysteine, even with a tenfold excess. The reaction was slow and never went to 100% of the reduced form of cytochrome *c* while any cysteine was present. This perhaps indicates some discrepancy in Eq. 1, either in the E'_0 values or in the mechanism itself.

[12] Potter, V. R., private communication.

In a previous investigation,[1] we showed that

cysteine alone did not inhibit the enzyme, but only after being oxidized by the addition of cytochrome *c*. It remained to show whether cysteine or some other oxidation product of cysteine was the specific inhibiting substance.

The experiments outlined in Table I were set up to compare the degrees of inhibition of cystine and cysteine in equivalent concentrations, assuming that cysteine is oxidized to the dimer. In one pair, cytochrome *c* was added with the succinate after a 40-minute incubation and a 30-minute contact period. Cysteine without cytochrome *c* being present showed relatively no inhibition. Cysteine with cytochrome *c* present inhibited the enzyme to the extent of 34%. The oxidized dimer at half the monomer concentration and with cytochrome *c* present showed the maximum possible inhibition of 40% at M/2000 concentration (under the specific conditions noted). But in the absence of cytochrome *c* the cystine that was reduced was not reoxidized and the inhibition dropped to 35%. Cysteine with cytochrome *c* and cystine without cytochrome *c* were both lower than the maximum obtainable since the former had a time lag in the oxidation of the cysteine; the latter, nothing to reoxidize the reduced cystine. The results showed that the inhibiting substance in the cysteine-cystine system was cystine, but whether as the dimer or the free radical cannot be stated.

Summary. Cystine, without differentiating between the dimer and the free radical, inhibits the action of the succinoxidase system under the given conditions used in these experiments.

14717

On the Origin of the Fibers in the Pyramid of the Primate Brain.*

FRED. A. METTLER.

From the Department of Neurology, College of Physicians and Surgeons, Columbia University.

That the pyramid (and corticospinal system) contains fibers which do not originate from the precentral gyrus has long been known. Monakow long ago pointed out that the distribution of the pyramidal cells of the cat was more extensive than the homologous region in the primate and published a figure[1] (his 34) showing a very extensive frontal and parietal origin for the human pyramidal system. The present author observed[2], "We have seen in our parietal material that a limited number of cerebrospinal fibers arise from that part of the parietal cortex immediately adjacent to the central fissure. This is by no means a novel observation and is correlated with the occasional finding of a few Betz cells in this region." The fact that corticospinal fibers arise from a location more extensive than the precentral gyrus was subsequently verified by other workers.[3] Few persons at that time questioned the belief that the corticospinal fiber arises from anything but the Betz cell and the extensive origin of the pyramidal system was regarded as due to a widespread distribution of these cells.

The Hoffs, using the degenerated bouton terminaux technic, conjectured that corticospinal fibers arise from cortex other than type 4, and Kennard, using the Marchi method, believed she was able to establish that such fibers arise from area 6. The present writer has not been able to substantiate such an origin for the system and Levin and Bradford,[4] using the cell degeneration technic, gave it as their opinion that few if any such fibers arise

* Aided by the Wm. J. Matheson Commission.

1 Monakow, C. v., *Die Lokalisation im Grosshirn*, Wiesbaden, 1914, *vide* S. 183 *et seq.*

2 Mettler, Fred A., *J. Comp. Neurol.*, 1935, **61**, 536.

3 Levin, P. M., *J. Comp. Neurol.*, 1936, **63**, 369.

4 Levin, P. M., and Bradford, K., *J. Comp. Neurol.*, 1938, **68**, 411.