number of patients with cataract formation in this series was 700–1000 m from the hypocenter. No cataracts have been found in the survivors in this report that were in the Fukuya Department Store, a reinforced concrete building 800 m from the hypocenter, where shielding was afforded.

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Manuscript received April 7, 1952.

Histochemical Demonstration of Protein-bound Sulfhydryl Groups[1]

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Protein-bound or fixed sulfhydryl groups are essential for the activity of many enzymes, serve as linkages between proteins and some prosthetic groups, are involved in the contractile phenomena of muscle, and are important in the coagulation of blood, in cell permeability, and in hormone synthesis and activity (*1*). Methods for the detection as well as estimation of sulfhydryl groups of proteins are based on reactions with either oxidizing, reducing, alkylating, or mercaptide-forming agents. Some of these methods are not colorimetric, and most of them lack specificity because functional groups other than sulfhydryl are quite reactive toward these agents (*1*). However, modifications of some colorimetric techniques have been used histochemically to demonstrate sulfhydryls in tissue sections. For this purpose ferricyanide (*2*) and nitroprusside (*3, 4*) have been widely used, although no oxidizing agent other than cystine can be called specific for the oxidation of sulfhydryl groups in proteins (*1*). A method based on a new mercaptide-forming agent, 1-(4-chloromercuriphenylazo)-naphthol-2 (*5*), gives weak color reactions in tissue sections.

In order to improve the sensitivity of sulfhydryl histochemistry by increasing the color value of the final compound and by increasing the specificity of the reaction for sulfhydryls, a reagent was developed which contained a disulfide linkage, the specific oxidative group, and a naphthol moiety for coupling to form an azo dye. As shown in Fig. 1, the reagent,[3]

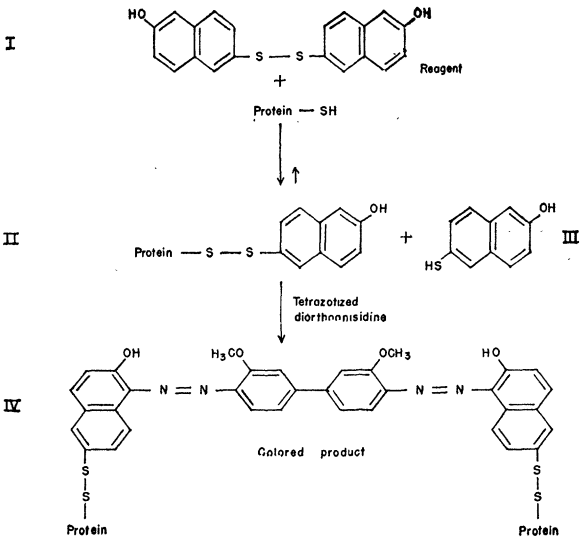


FIG. 1.

2,2′-dihydroxy-6,6′-dinaphthyl disulfide (I), when used in excess at pH 8.5, reacts with active sulfhydryl groups of fixed tissue proteins to form a colorless substance (II), which can be converted into an intensely colored azo dye (IV) by coupling with tetrazotized diorthoanisidine. The colorless oxidation product (II) was insoluble in both water and ether-alcohol, so that the excess of reagent (I) as well as the reduced reaction by-product (III) could be washed out of the tissues with organic solvents. Subsequent treatment of the tissues with tetrazotized diorthoanisidine resulted in the rapid development of a red color (monocoupling) or a blue color (dicoupling) at the sites of protein sulfhydryl groups (IV). Monocoupling (red or pink) was taken to indicate sparse, widely separated sulfhydryl groups, whereas dicoupling (blue) indicated a greater concentration of sulfhydryl groups.

The reagent (I) was prepared from sodium 2-hydroxy-6-naphthalene sulfonate. The hydroxy group was protected by conversion to the carbethoxy derivative, and the sulfonate group was converted to sulfonyl chloride with PCl_5 (*6, 7*). Reduction to a sulfhydryl group was accomplished with zinc dust and hydrochloric acid in alcohol, a method used for other naphthalene derivatives (*8*). Oxidation with ferric chloride gave the disulfide, and the carbethoxy group was hydrolyzed with hot alkali.

The required sodium-2-carbethoxy-6-naphthalene sulfonate (*6, 7*) was prepared by the slow addition of 84 ml ethyl chlorocarbonate to a vigorously stirred solution of 197 g sodium 2-hydroxy-6-naphthalene sulfonate (Eastman technical grade) and 32 g sodium hydroxide in 800 ml water. The stirring was continued for an additional hour, after which the mixture was cooled to complete the precipitation of the carbethoxy derivative. It was collected with the aid of suction and dried by warming on the steam bath. The crude product (177 g) was ground with an equal weight of phosphorus pentachloride and was heated on the steam

[1] This investigation was supported by an institutional grant to Harvard University from the American Cancer Society, by a research grant from the National Cancer Institute, National Institutes of Health, USPHS, and by the Slosberg Fund for Research in Diabetes.

[2] Acknowledgment for technical assistance is due Edith Herman and Ralph Gofstein.

[3] This reagent (DDD) is now available from the Schwartz Laboratories, Inc., New York City.

bath for 1 hr (*6, 7*). The fused melt was poured into 2.5 liters cracked ice and stirred for 20 min. *2-Carbethoxy-6-naphthalene sulfonyl chloride* was filtered with suction, immediately crystallized from 1 liter of hot glacial acetic acid, collected, and used in the next step. In order to determine the yield, one run was dried for 48 hr *in vacuo* at 40° C, mp 116°–118°, 103 g (41% of the starting material).

2-Carbethoxy-6-naphthyl thiol was prepared by heating zinc dust (100 g) and 95% alcohol (400 ml) to 30°, and the undried acid chloride (about 103 g) was gradually added. Concentrated HCl (200 ml) was added drop by drop to the stirred mixture. After the reaction had subsided, the mixture was gradually warmed on the steam bath and then heated for 1 hr. By this time the solution was clear yellow, and a sample diluted with water was completely soluble in base. The solution was rapidly filtered with suction, and the zinc dust on the filter was washed once with hot alcohol. The filtrate was used in the next step below. In one experiment the filtrate was poured into 2 liters of cold 10% HCl, and the pale-yellow product which precipitated was collected and crystallized from dilute alcohol, mp 87°, yield 72 g (90%).

2-Carbethoxy-2′-hydroxy-6,6′-dinaphthyl disulfide was prepared from the sulfhydryl compound (72 g) dissolved in 600 ml alcohol or from the hot alcoholic filtrate (obtained above without isolation of carbethoxynaphthyl thiol) by addition of 300 ml of a saturated aqueous solution of ferric chloride at 50° C. An excess of ferric chloride was assured by persistence of its yellow color. Cold water (600 ml) was added, the mixture was cooled, and the clay-colored disulfide was collected and washed with water. It crystallized from hot acetic acid or dioxane-methanol in fine white crystals, mp 198°–200° (uncorr). The compound proved to be the monocarbethoxy derivative by analysis. This was confirmed by demonstrating coupling with tetrazotized diorthoanisidine at pH 7.2.

Anal: Calcd for $C_{23}H_{18}O_4S_2$: C, 65.38; H, 4.26. Found: C, 65.68; H, 4.28.

2,2′-Dihydroxy-6,6′-dinaphthyl disulfide (*I*) was obtained by heating the crude undried disulfide suspended in 600 ml water and 54 g potassium hydroxide on a steam bath for 2 hr. The hot solution was filtered, cooled, and acidified with 150 ml concentrated HCl in a 4-liter beaker. The clay-colored product was collected, dissolved in hot acetic acid, treated with active charcoal, and crystallized from dilute acetic acid in pale-tan, fine crystals, mp 190°–205° (uncorr); 40.5 g (70% based on the acid chloride). Crude material could be purified by solution in a small amount of acetone, addition of a large volume of toluene and concentration by boiling to one third the volume. The hot toluene solution was filtered rapidly from tarry residue and crystallization took place upon cooling. After recrystallization from hot glacial acetic acid it melted at 220°–223° (uncorr).

Anal: Calcd for $C_{20}H_{14}O_2S_2$: C, 68.54, H, 4.03. Found: C, 68.34; H, 4.26.

For the histochemical detection of protein-bound sulfhydryl groups, tissues taken from rats killed by a blow on the head were fixed for 24 hr in a solution containing 1 g trichloracetic acid dissolved in 100 ml 80% ethyl alcohol. The acid prevented air oxidation of sulfhydryl groups. These tissues were dehydrated, embedded in paraffin in the usual manner, and sectioned at 10 μ. Tissue sections, mounted on slides with a minimal amount of albumin, adhered to the slides during staining procedures better than when albumin was omitted. This procedure was feasible since smears of albumin stained for sulfhydryl did not develop a sufficient amount of color to be detected. Moreover, there was no difference in the degree or localization of staining for sulfhydryl in a series of sections of tissues mounted with and without albumin. After deparaffinization, the mounted sections were passed through a series of alcohols (absolute, 95%, 70%, 50%) to distilled water. They were stained by the following procedures for demonstrating sulfhydryl groups.

1) Incubate up to 9 slides in Coplin jar for 1 hr at 50° C in a solution prepared by mixing 35 ml 0.1 *M* Michaelis barbital buffer, pH 8.5, and 15 ml absolute ethyl alcohol containing 25 mg 2,2′-dihydroxy-6,6′-dinaphthyl disulfide (I). The reagent is almost completely soluble in the above alcohol-buffer mixture.

2) Cool for 10 min at room temperature.

3) Rinse briefly in distilled water.

4) Wash for 10 min in 2 changes of distilled water acidified to pH 4 to 4.5 with acetic acid. This step is necessary to convert the sodium salt of the reagent (I) or of the reaction by-product (III) to free naphthol for extraction with organic solvents.

5) Extract excess reagent (I) and reaction by-product (III) by passage through a graded series of alcohols and wash in absolute ether for 5 min.

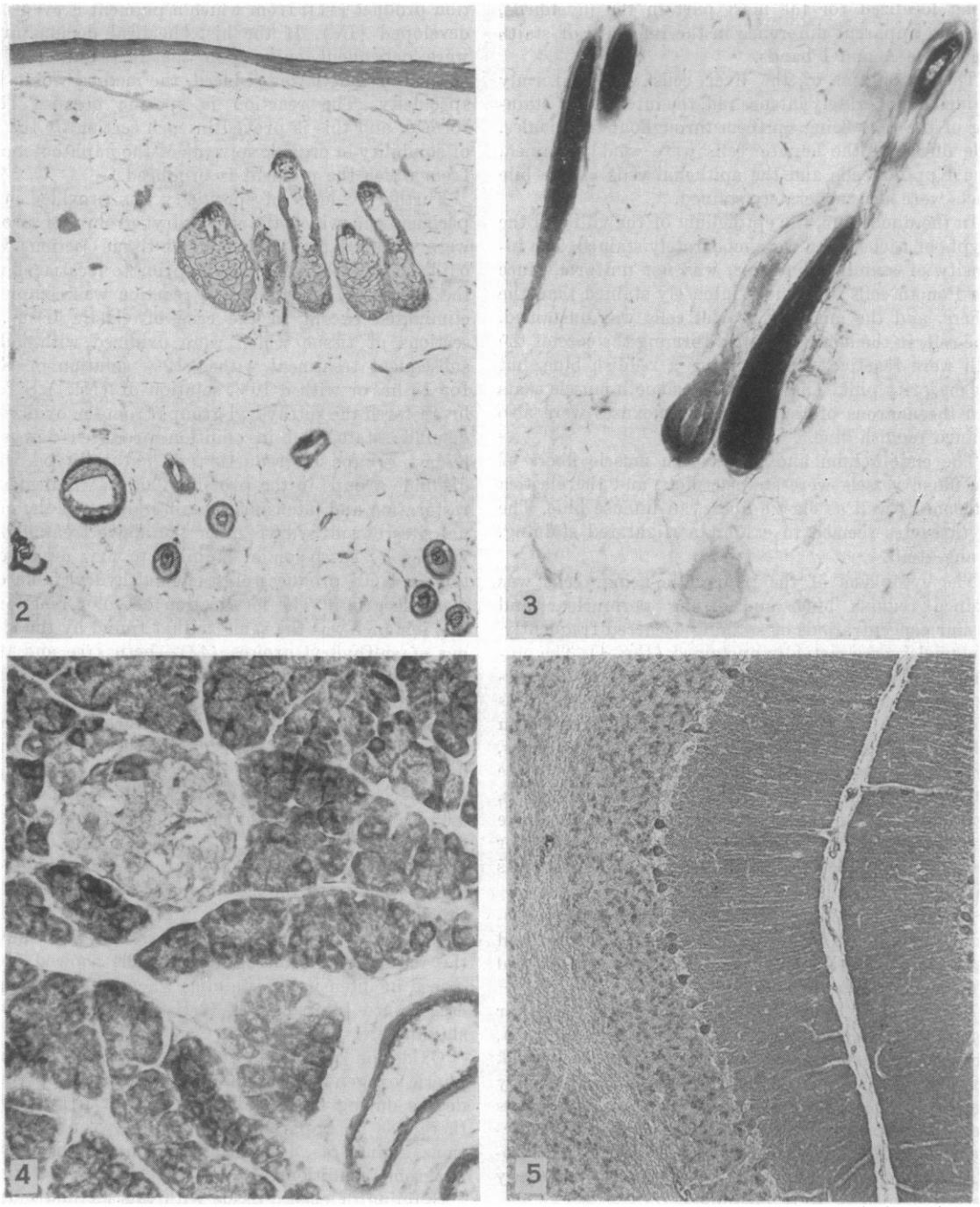
6) Rehydrate and rinse in distilled water.

7) Stain for 2 min at room temperature in the following freshly prepared mixture: 50 mg tetrazotized diorthoanisidine[3] in 50 ml 0.1 *M* Sorenson phosphate buffer, pH 7.4.

8) Wash in running tap water.

9) Mount cover glass with glycerogel, or dehydrate with acetone and mount cover glass with clarite.

Sulfhydryl groups were widespread in distribution in rat tissues. In sections of skin, the epithelial cells of the Malpighian layer were stained a moderately intense red (Fig. 2), whereas those of the stratum corneum were less intensely colored. The epithelial cells of the root sheaths of the hairs were also stained red, as were those of the bulb. The hair papilla, however, was unstained. The cells of the hair cortex stained a moderately intense red in the proximal parts of the hair, a deep blue in the region of horny transformation, and a reddish blue in the intermediate regions (Fig. 3). Distal to this region, the hair cortex was unstained, although the medulla was weakly stained. Connective tissue fibers of the dermis were unstained, but some of the cells of connective tissue were stained light red, as was the cytoplasm of fat cells. The epithelium of sebaceous glands (Fig. 2) and fibers of the arrectores pilorum muscles were stained red.



FIGS 2–5. Ten-micron sections of paraffin-embedded tissues of rats stained for sulfhydryl. 2: The cells of the stratum corneum and stratum Malpighii of the epidermis, the root sheaths of the hairs, and the epithelium of the sebaceous glands are stained red The cortices of the hairs are unstained. Approx × 100. 3: Adjacent deeper part of dermis to that in Fig. 2. The cortices of the hairs in the region of horny transformation are stained blue, whereas the root sheaths and the bulbs are stained red. Approx × 100. 4: The acinar cells of the pancreas are stained reddish blue, and perinuclear and basal localization of staining is common. The cells of the islets of Langerhans are stained light pink. The elastica interna (nonsulfhydryl) and smooth muscle of an artery (lower right-hand corner of the photograph) are stained blue and red, respectively. Approx × 300. 5: The Purkinje cells of the cerebellum are stained reddish blue, and the fibers of the molecular layer and the cells of the granular layer are stained red. The fibers of the white matter in medullary centers are virtually unstained. Approx × 100.

In the deepest part of the dermis, the axis cylinders of nerve fibers were stained a light red, whereas the myelin sheaths were weakly stained or were unstained. Striated muscle was stained reddish blue, the staining

being localized for the most part in the myofibrils, with no apparent difference in the intensity of staining in the A and I bands.

The cytoplasm of the liver cells was uniformly stained a moderately intense red, the intensity of staining of the cells being uniform throughout the lobules. The nuclei of the hepatic cells were weakly stained. The Kupffer cells and the epithelial cells of the bile ducts were also moderately stained.

In the duodenum, the epithelium of the villi and the crypts of Lieberkühn were moderately stained. The intensity of staining, however, was not uniform, since the Paneth cells were more intensely stained than the others, and the mucus of goblet cells was unstained. The cells in the lamina propria forming the core of the villi were reactive, some staining a reddish blue, but the majority pink. The cells of the smooth muscle coats and the neurons of the myenteric plexuses were also stained reddish blue.

The endothelium and the smooth muscle fibers of the blood vessels were stained pink, and the elastica interna, as well as elastic fibers, an intense blue. The erythrocytes seemed to exhibit a slight red staining, if any at all.

The cytoplasm of the pancreatic acinar cells was stained reddish blue, and strong perinuclear and basilar concentrations of staining occurred frequently. The nuclei were virtually unstained (Fig. 4). The epithelial cells of the ducts and of the islets of Langerhans were stained pink. Some of the cells of the islets appeared to be slightly more deeply stained than others, but it should be emphasized that the islet cells, by virtue of their weak staining in comparison to the pancreatic acinar cells and the cells of other tissues, contained only a small amount of alcohol-insoluble protein-bound sulfhydryl (Fig. 4). A method for staining alcohol-soluble disulfide-containing substances will be published later.

The cells forming the cortical and medullary substance of thymic lobules were stained a diffuse and light pink. Hassell's corpuscles, however, were stained a moderately intense blue.

The fibers of the molecular layer of the cerebellar cortex were stained a moderately intense red (Fig. 5). The cells of the granular layer were similarly stained, but the fibers of this layer were much less intensely stained. The Purkinje cells between these two layers were stained most intensely red of all cerebellar elements, and the color was confined mainly to the cytoplasm. Fibers of the medullary centers were very lightly stained or were unstained.

Although derivatives of naphthol have proved useful in developing a variety of histochemical methods (*9–13*), success of the present method is dependent upon the specificity of the reaction of the disulfide group with sulfhydryl groups at alkaline pH (*1, 14–16*). The reaction is reversible and readily influenced by mass action. Furthermore, the reaction is not merely oxidation and reduction with transfer of electrons, but the chromogenic moiety (naphthol) is transferred to the protein and becomes part of the oxidation product (II), from which a pigment is eventually developed (IV). If the histochemical demonstration were dependent only upon the fact that III was formed or that I was reduced, the method would lack specificity. The reaction is specific because II is formed, and this is proved in each section by the loss of solubility in organic solvents of the naphthol moiety from which the pigment is produced.

Further evidence of specificity was provided in experiments in which the sulfhydryl groups of protein were oxidized first. When fixed tissue sections were oxidized with iodine or H_2O_2, prior to treatment with the reagent (I), the staining reaction was completely eliminated except in the case of elastic tissue. In sections of tissue which were oxidized with iodine, subsequent treatment with 1–10% solution of KCN for 18 hr, or with a 10% solution of $(NH_4)_2S$ for 2 hr, restored the sulfhydryl groups from the oxidized or disulfide state, and in addition produced new sulfhydryl groups by reduction of naturally occurring disulfide groups in the protein. This was indicated by restoration and intensification of staining of the original sites of sulfhydryls after the above treatment, as well as by extension of staining to new areas, even though KCN produces only one sulfhydryl from each disulfide group. The localization of color reaction in this instance was the same as that found by the staining of sulfhydryl groups (*17*)—both free and those derived from disulfides—at pH 11 with the oxidizing agent, ditetrazolium chloride (*18*).

Proof of the specificity of the histochemical reaction for sulfhydryl groups was obtained by the following: 0.1 *M* iodoacetate (24 hr) or 0.1 *M* N-ethyl maleimide[3] (4 hr) completely inhibited the staining reaction and 0.03 *M* glutathione (3 hr) completely reversed the reaction prior to coupling.

The sulfhydryl group of glutathione probably did not contribute to the staining of tissues, since glutathione is completely soluble in acidified 80% alcohol. Insulin is also soluble in this fixative. This may be the reason that pancreatic islet cells showed no increase in intensity of staining when sections of pancreas were treated with $(NH_4)_2S$ or KCN before staining.

Oxidation of protein sulfhydryl groups by atmospheric oxygen did not take place to any appreciable degree during the staining reaction at pH 8.5, since there was no apparent difference between tissue sections stained as described and those stained under anaerobic conditions.

Staining of elastic tissue with this method must be attributed to a special affinity for the naphtholic groups of the reagent and not to sulfhydryl groups, since prior oxidation with iodine did not alter the results. Other derivatives of β-naphthol, particularly 6-bromo-1-naphthol, 2-hydroxy-3-naphthoic acid hydrazide (*10*), and 2-hydroxy-6-naphthalene sulfonic acid, 2-hydroxy-8-naphthalene sulfonic acid, and 2-hydroxy-3-naphthoic acid, also have an affinity for elastic tissue (*17*), although after coupling with a diazonium salt, they do not stain structures where large

concentrations of sulfhydryl groups are known to occur.

Further studies in the distribution of sulfhydryl and disulfide groups and their changes in various physiological states will be published elsewhere.

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Manuscript received April 2, 1952.

Role of Coenzyme A and DPN in the Oxidation of α-Ketoglutaric Acid[1]

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From recent work it has become apparent that diphosphopyridine nucleotide (DPN) and coenzyme A (CoA) participate in the oxidation of α-ketoglutarate (*1–4*). Succinyl CoA has been postulated as an intermediate in the reaction. Using α-ketoglutaric oxidase (*5*) of over 90% purity, as shown by electrophoretic and ultracentrifuge analyses, we have found that the primary reaction is as follows:

$$\alpha\text{-Ketoglutaric acid} + \text{CoA} + \text{DPN}^+ \rightarrow \text{Succinyl CoA} + \text{DPNH} + CO_2 + H^+.$$

For the first time an acylated derivative of CoA has been obtained from the enzymatic oxidation of an α-keto acid.

In the presence of CoA[3] and cysteine (or glutathione or borohydride instead of cysteine), the purified α-ketoglutaric oxidase (*5*) catalyzes the reduction of DPN by α-ketoglutaric acid (Fig. 1). The stoichiometry of the reaction with CoA (Table 1) is consistent with the above equation. The disappearance of —SH group and CoA in amounts equivalent to formation of acid anhydride[4] (as shown by the reaction with hydroxylamine) suggests strongly that the product is succinyl CoA. As in the case of acetyl CoA (*10*), the anhydride bond appears to be formed between the —SH of CoA and one of the carboxyls of succinate. Succinyl CoA is obtained from the reaction mixture by adsorption on Nuchar C190 followed by

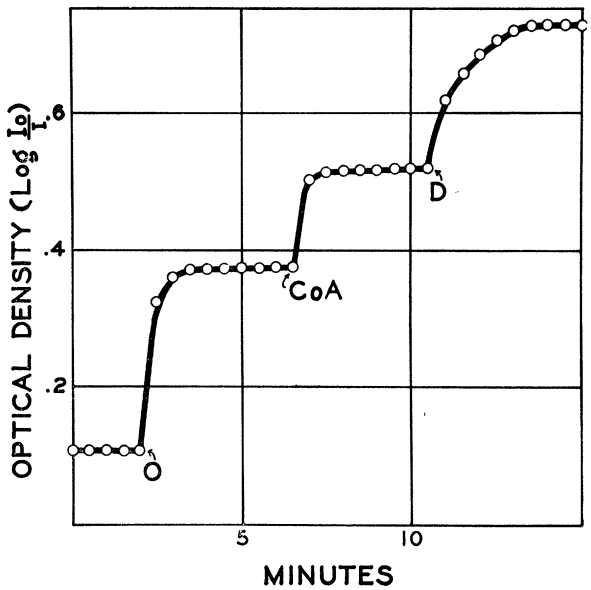

FIG. 1. Reduction of DPN by α-ketoglutarate and CoA. Experimental conditions: cysteine (0.1 ml 0.5 *M* neutralized solution) and CoA (0.1 ml containing 0.3 mg of preparation with an activity of 340 u/mg) were incubated for 3 min at 30°. α-Ketoglutarate (5 μM), DPN (0.3 μM), glycylglycine at pH 7.2 (100 μM), and water to a total vol of 3.0 ml were added to the cysteine-CoA mixture. After 2 min 10 u α-ketoglutaric oxidase in 0.01 ml were added to start the reaction. A mixture of cysteine (0.1 ml) and CoA (0.05 ml) was added at 6.5 min, and purified succinyl CoA deacylase (*4*) at 10.5 min. Optical density ($\log \frac{Io}{I}$) was measured at 340 mμ in a Beckman DU spectrophotometer.

TABLE 1

STOICHIOMETRY OF THE REACTION OF α-KETOGLUTARATE WITH DPN AND CoA*

Expt	Δ α-Ketoglutarate	Δ CoA	Δ —SH	DPN reduced	Hydroxamic acid formed
1	7.0	4.5	—	4.6	4.8
2	2.4	1.5	—	1.4	1.7
3	0.76	—	0.47	—	0.51

* The data are expressed in μM. Three to 7 min after the reaction was started by the addition of oxidase, an aliquot was reacted with hydroxylamine (*6*) for 1 hr. Another aliquot was treated with methanol, and the DPNH determined by the change in optical density at 340 mμ produced by acetaldehyde and crystalline alcohol dehydrogenase. The free sulfhydryl group was determined by the nitroprusside reaction (*7*), CoA by arsenolysis with phosphotransacetylase (*8*), and α-ketoglutarate as the 2,4-dinitrophenylhydrazone (*9*). Cysteine was not present in Expt 3.

[1] Aided by grants from the National Heart Institute, National Institute of Health, USPHS.

[2] Postdoctorate fellow of the National Cancer Institute, National Institutes of Health, USPHS.

[3] We are greatly indebted to H. Beinert and others for generous gifts of CoA (*J. Am. Chem. Soc.*, **74**, 854 [1952]), which made this investigation possible.

[4] Using S-acetyl-β-mercaptoethylamine as a model, Novelli has presented evidence that thioesters behave like acid anhydrides in that the activated carboxyl reacts with hydroxylamine at acid pH (*1*).