Reports and Letters

Cis-aconitic Decarboxylase

Isotopic tracer studies have shown that the biosynthesis of itaconic acid in fungi is closely related to tricarboxylic acid cycle reactions (*1*, *2*) probably proceeding through decarboxylation of *cis*-aconitic acid (*3*). Previously, Calam, Oxford, and Raistrick had ruled out this possibility because of negligible conversion of citric acid to itaconic acid in replacement experiments (*4*). Furthermore, it has not been possible to demonstrate the presence of citric or *cis*-aconitic acids in culture filtrates of *Aspergillus terreus* (*2*).

Direct evidence for a role of *cis*-aconitic acid in itaconic acid biosynthesis has now been obtained with preparations of a soluble enzyme, *cis*-aconitic acid decarboxylase (*5*). These preparations, which are most active over the range *p*H 5.6 to 5.9, readily decarboxylate *cis*-aconitic acid but not *trans*-aconitic acid, forming stoichiometric amounts of itaconic acid and carbon dioxide. *Trans*-aconitic acid is not a competitive inhibitor. *Cis*-aconitic decarboxylase is a rather unstable enzyme in the present preparations, losing about half of its activity during overnight storage at 0°C. The enzyme is almost fully active after dialysis for 2 hr at 0°C against 0.05*M* phosphate buffer, *p*H 7, but it is completely inactive if this dialysis is continued overnight. The activity of such preparations is not restored by the addition of boiled preparations or by other possible cofactor sources (yeast extract, Mg^{++}, Mn^{++}, and pyridoxal phosphate).

Table 1. Results of a typical experiment. Each flask contained 1 ml of enzyme preparation, 1.6 ml of 0.2*M* phosphate buffer (*p*H 5.6), with *cis*-aconitic acid added as a neutral sodium salt from side arms. The reaction was carried out for 90 min.

Cis-aconitic acid initially* (μ*M*)	Carbon dioxide evolved (μ*M*)	Itaconic acid produced† (μ*M*)	Aconitic acid recovered‡ (μ*M*)
6.6	5.3	5.4	Not determined
13.2	9.8	10.7	1.1

* Calculated from weight of *cis*-aconitic anhydride used.
† These values have been corrected for the itaconic acid originally present in the enzyme preparation.
‡ Mainly as the *trans* form.

The enzyme preparations are obtained from surface cultures of *A. terreus* grown at 28°C on 100-ml portions of the medium described by Lockwood and Ward (*6*). The mycelial pad covers the surface 4 to 5 days after inoculation, at which time the culture medium contains between 7 and 15 mg of itaconic acid per milliliter. The original culture medium is replaced with distilled water (100 ml), and the mycelium is allowed to stand on this water for 1 hr at room temperature. All subsequent operations are carried out in the cold room. The mycelium, which in a typical case has a wet weight of 2.3 g and a dry weight of 0.6 g, is washed with several portions of ice cold water and is then ground in a mortar with 3 ml of 0.2*M* phosphate buffer, *p*H 6.5, in the presence of about 1.5 g of glass beads (*7*). The paste is diluted with more phosphate buffer (7 ml) and centrifuged at 1500 g for 20 min at 0°C. The supernatant is passed through a filter paper, yielding an opalescent solution with a slight orange-tan color. Such solutions have *p*H values between 6.5 and 6.7 and contain some itaconic acid that is released from the cells during the grinding process. The protein content is between 2.5 and 3.0 mg/ml. Similar preparations of the enzyme have been obtained from the vegetative mycelium that is obtained in shake cultures; in these cases, the mycelium is separated and washed by centrifugation prior to grinding.

The decarboxylation reaction is followed manometrically at 37°C. After the reaction is completed, deproteinization is carried out with alcohol, and the organic acids present are separated by partition chromatography on Celite columns (*8*). Solvents are evaporated from the appropriate pooled fractions and the residues are analyzed for aconitic and itaconic acids by a modification of the method of Dickman (*9*). The results in a typical experiment are shown in Table 1.

This appears to be the first description of an enzyme that can bring about the decarboxylation of an α,β-unsaturated acid, producing the methylene group. Similar enzymes may play a role in the biosynthesis of other naturally occurring compounds that contain this group (for example, alliin, penicillic acid, allyl phosphate, and more complex compounds in the terpene series). In particular, it seems possible that the biosynthesis of the recently discovered γ-methylene-α-ketoglutaric acid (*10*) and of the related γ-methylene glutamic acid and γ-methylene glutamine (*11*) may be closely related to that of itaconic acid. It is suggested that the γ-methylene-α-ketoglutaric acid is obtained by the action of a specific decarboxylase on 4-oxo - 1-butene - 1,2,4-tricarboxylic acid. The most likely precursor for this unsaturated acid appears to be 2-hydroxy-4 - oxo - 1,2,4 - butanetricarboxylic acid (oxalocitramalic acid); since this is not a metabolic intermediate (*12*), the closely related 4-phosphate of 2,4-dihydroxy - 1,2,4 - butanetricarboxylic acid may be the actual C_7 compound involved. This phosphate is known to be present in dog liver (*13*,) and has been identified as an intermediate in bacterial metabolism (*14*).

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References and Notes

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Copper in Hair

In a recent paper on the nature of pigments derived from tyrosine and tryptophan in animals (*1*), Kikkawa, Ogita, and Fujito have proposed the idea that color of hair is in some way related to its content of iron, cobalt, nickel, molybdenum, and copper.

Loss of coat color in cattle grazing on pastures high in molybdenum content has been observed in England (*2*) and in California (*3*), and it is now well-known that the feeding of small amounts of copper sulfate to affected animals restores the color to new growth of hair. It has also been shown by Goss (*4*) that the wool of black sheep that are fed an excess of molybdenum as sodium molybdate grows out colorless, but the black pigment quickly reappears when 100 parts of copper per million are added to the high molybdenum ration.

Analyses of the black wool for copper showed 17 ppm, whereas the grey portion of the same fibers after molybdenum-feeding contained only 13 ppm. However, white wool from normal sheep consistently showed more copper than black, contrary to the report of Kikkawa *et al.*

Table 1. Copper content of hair in parts per million of washed, dry hair.

Source	This report	Kikkawa *et al.*
Cat, black	14	
Cat, white	34	
Holstein cow, black area	12	
Holstein cow, white area	13	
Hereford cow, dark red	27	
Hereford cow, light red	30	
Hereford cow, light red	26	
Hereford calf, red area	17	
Hereford calf, white area	23	
Angus bull, red	15	
Dog, black	17	
Dog, white	25	
Guinea pig, black	11	8
Guinea pig, golden	13	5.2
Guinea pig, white (albino)	15	2.3
Hog, black	17	
Hog, white	12	
Horse, black	10	
Horse, white	15	
Man, Caucasian,		
3-yr-old child, red	47	
Same, as adult	15	
Adult, red	18	
Mexican child, red	18	
Man, Mongolian, black	15	19
Man, Negroid	15	31
Rabbit, black	14	3
Rabbit, white (albino)	17	Trace
Rabbit, white (albino)	20	
Rabbit, white (albino)	19	
Rat, black	14	
Rat, white (albino)	14	
Sheep, black	17	
Sheep, black, high Mo feed	13	
Sheep, white	20	

for the guinea pig, rabbit, and man. We therefore collected samples of light and dark shades of hair from a number of species and determined the copper content. The values are given in Table 1, together with the corresponding figure from the paper by Kikkawa *et al.* (*1*), recalculated in parts per million. Apparently their results were obtained by spectroscopic analyses of the ash obtained by combustion of the hair in a furnace at 600°C. Our results were obtained on thoroughly washed and dried hair by a "wet-ashing" method using sulfuric, perchloric, and nitric acids; the copper was estimated colorimetrically in duplicate by use of the diethyldithiocarbamate method described by Clare *et al.* (*5*). Except in the hog, we have found as much, if not more, copper in white or light-colored hair than we have found in black hair of the same species.

As far as the copper content of hair is concerned, our results do not agree with those reported by the Japanese authors, and we find no confirmation of the statements that black hair is associated with a high copper content, and that white hair is low in copper or contains only a trace of copper.

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Enhancement of Radiobiological Effect by Malonic and Maleic Acids

Certain biological actions of radiations are generally believed to involve chemical intermediates, mostly formed from irradiated water, and in some of these actions the intermediates are believed to be peroxides (*1*). These substances may attack various important reaction systems in the cell at various sites, for example, sulfhydryl and other reactive groups, especially in sulfhydryl enzymes (*2*).

In many of our experiments, in which cell death was the criterion of effect, we failed to enhance the radiation effect by inhibiting oxidative phosphorylation, which has recently been reported to be exceptionally sensitive to irradiation (*3*). These negative results may be due to masking effects or to recovery of the system. Only with malonic and maleic acids were positive results obtained. Malonic acid inhibits oxidative phosphorylation and is highly specific as a competitive inhibitor of succinic dehydrogenase. Maleic acid, although not so specific for succinic dehydrogenase, is a universal inhibitor of sulfhydryl enzymes.

Saccharomyces ellipsoideus, the experimental material, was cultured in Nägeli's solution for many generations. After x-irradiation (60 kv, 1000 r/min), the cells were cultured on Nägeli agar at *p*H 7. Photographs were taken and microscopic counts were made at intervals for some 20 hr. Single cells and also pairs of enlarged cells, which are ascribed to death after one division, were scored as nonsurvivors; the counts of these categories do not change in the course of prolonged incubation. Five to 10 cultures were counted in each experiment, and every culture contained 400 to 500 cells.

Malonic acid in concentration $10^{-3}M$ without irradiation produced no detectable injury. Its sensitizing action, when it was added to the Nägeli culture solution before irradiation, is demonstrated by the data in Table 1.

If fumaric or aspartic acid was added to the Nägeli agar on which the cells were cultured after irradiation, the results shown in Table 2 were obtained. It appears that the effect of each of these acids on cells pretreated with malonic acid is essentially to bring the survival back to the value obtained when malonic

Table 1. Sensitization by malonic acid applied before irradiation.

X-ray dose (r)	Percentage survival (calibrated by control)	
	Malonic acid, $10^{-3}M$	No malonic acid
0	100 ± 1.3	100 ± 1.1
10,000	45.1 ± 3.2	77.5 ± 6.5
20,000	32.8 ± 2.1	47.9 ± 3.2
30,000	17.8 ± 2.4	30.0 ± 1.2
50,000	8.6 ± 0.9	10.6 ± 0.4

Table 2. Recovery owing to fumaric and aspartic acids after 20,000 r of x-rays (calibrated by nonirradiated cells).

Treatment before irradiation	Treatment soon after irradiation	Survival (%)
None	None	48.0 ± 2.4
Malonic acid, $10^{-3}M$	Fumaric acid, $2 \times 10^{-3}M$	50.0 ± 1.1
None	Fumaric acid, $2 \times 10^{-3}M$	50.0 ± 1.3
Malonic acid, $10^{-3}M$	Aspartic acid, $10^{-5}M$	51.8 ± 2.8
None	Aspartic acid, $10^{-5}M$	51.8 ± 1.9