Reduction-like Effect of Carbohydrates on Cytochrome c

Abstract. *Certain carbohydrates cause changes in the absorption spectrum of cytochrome c; these changes are the same as those accompanying conversion from the oxidized to the reduced form. Various carbohydrates are compared by measuring changes in absorbance at a characteristic wavelength.*

A variety of carbohydrates convert oxidized cytochrome *c* into the reduced form as indicated by the absorption spectrum. The rate and completeness of the conversion are dependent upon the carbohydrate. The effect occurs at physiological *p*H, and is fully reversible.

Beef-heart cytochrome *c* (Sigma type V, 90 to 100 percent) was purified by boiling for 5 minutes to precipitate any denatured material, cooling, and then centrifuging to remove any sediment that might have formed. Similar results were obtained with horse-heart cytochrome *c* (Sigma type III). The cytochrome *c* was dissolved in 0.05*M* phosphate buffer, *p*H 7.5, at a final concentration of 0.5 mg/ml. Carbohydrate samples were added in solid form to achieve a final concentration of 5.0 mg/ml. The difference spectrum was recorded (Cary 15 spectrophotometer) for each cytochrome *c* solution, a blank containing the buffer and the carbohydrate being used. Changes in the spectrum, as a function of time, were observed as the solutions were incubated at room temperature. The presence or exclusion of oxygen made no appreciable difference, and controls showed that autoreduction under these conditions was negligible. Reoxidation with $K_3Fe(CN)_6$ fully reversed the changes in the spectrum. Figure 1 illustrates that these effects, in this case produced by D-ribose, are accompanied throughout the entire spectrum by changes characteristic of conversion from the oxidized to the reduced form.

The peak of ferricytochrome *c* at 550 mμ is the most convenient indication of conversion from the oxidized to the reduced form (Fig. 2). The increase in absorbance at 550 mμ (hyperchromicity) was recorded (Beckman DU spectrophotometer) with time, and the rate of hyperchromicity was used to compare the effects of various carbohydrates (Fig. 3). The greatest hyperchromicity was produced by D-galactosamine HCl, D-glucosamine HCl, and D-ribose. The overall increase in optical density caused by D-ribose was comparable to that caused by D-glucosamine HCl, although the initial rate was slower (Fig. 3). Considerable effects were also observed with D-galacturonic acid, D-arabinose, D-fructose, D-fructose-1, 6-diphosphate (Mg salt), and D-glucosaminic acid. Smaller, but similar, changes occurred in the presence of 2-deoxy-D-ribose, L-arabinose, L-rhamnose, and D-mannose. However, synthetic polyglucose and polyribose (*1*), glycogen, dextran, D-glucose, D-glucose-1-phosphate, methyl α-D-mannoside, D-xylose, L-fucose, sucrose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, and sulfated chitosans gave negligible hyperchromicity. Samples of each carbohydrate tested were obtained from at least two different sources; in the case of D-ribose, four different preparations were tried. Some samples were obtained from the stocks of carbohydrate chemists, and some we recrystallized ourselves. All results were reproducible, but we observed somewhat smaller effects when carrying out similar experiments in 0.05*M* tris buffer, *p*H 7.3. We also observed a rapid reversion of the reduction-like effects of the carbohydrates when adding alcohol (40 percent by weight) to the mixture.

It was necessary to determine whether traces of dehydrogenase were present in the cytochrome *c* preparation, which might have been responsible for the observed effects with carbohydrates. The reactions were therefore carried out in the presence of $5 \times 10^{-5}M$ sodium *p*-chloromercuribenzoate, which deactivates dehydrogenases by reacting with their sulfhydryl groups; there was no change in the rate of hyperchromicity caused by the carbohydrates. Also, the effects were similar with either beef-heart or horse-heart cytochrome *c*.

In an effort to interpret all the carbohydrate effects, we have eliminated a number of possibilities, but have established no general scheme. That the polysaccharides tested were inactive suggested the possibility that the potential aldehyde groups present in most of the active monomers were responsible for the apparent reduction of cytochrome *c* and that the activity was lost when this group was in glycosidic linkage. However, the rate and magnitude of changes observed in this spectrum did not parallel the relative reducing powers of the monosaccharides, for example, as determined iodometrically at 100°C (*2*). D-Glucose has the greatest relative reducing power of all monosaccharides so tested, while it has virtually no effect on cytochrome *c*. On the other hand D-ribose, which has 15 percent less reducing power than D-glucose or D-mannose (*2*), produced large changes in the spectrum of cytochrome *c*.

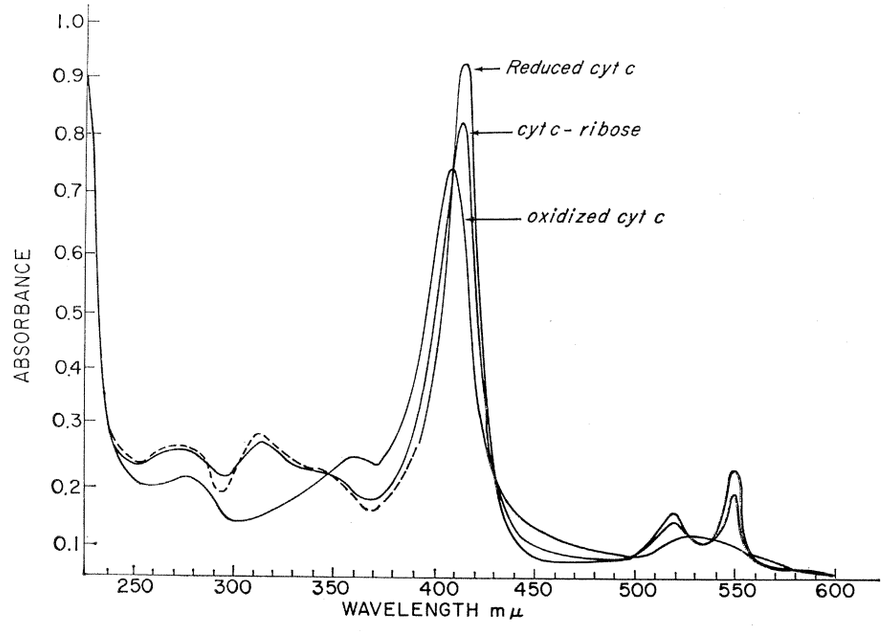

Fig. 1. Cytochrome *c* (0.5 mg/ml) and D-ribose (5.0 mg/ml) were incubated for 25 hours at room temperature in 0.05*M* phosphate buffer, *p*H 7.5, and the spectrum was recorded after fivefold dilution. Also included are spectra of the original, oxidized cytochrome *c* preparation and the reduced cytochrome *c* [solid line, $Na_2S_2O_4$ reduction; broken line, H_2 reduction, as taken from Margoliash and Frohwirt (*6*)].

Similarly, glucosaminic acid would be expected to be less reducing than glucose, if the "reducing" groups in the sugars were responsible for the observed spectral changes. The fact that glucosaminic acid produced great hyperchromicity in cytochrome *c* seems to rule out the general explanation that, in a balanced redox equation, the carbohydrates are oxidized to aldonic acids. While the potential furanose form is common to almost all active samples, glucosaminic acid is once more an exception.

The question of carbohydrate purity has been carefully considered. We can conceive of no impurity which could be present in all the active sugars. The sugars employed in these experiments were isolated from different sources (some natural, some synthetic) and were prepared and purified by different procedures. Several of the samples obtained from carbohydrate chemists were most carefully purified. Samples of the three most effective carbohydrates (D-galactosamine HCl, D-glucosamine HCl, and D-ribose) are considered to be of the highest purity (*3*). Furthermore, results are reproducible when using sugars from different sources.

Since many polyhydroxy compounds form coordination complexes with iron, several known chelating agents were tested under the same conditions as the carbohydrates. Sorbitol, sodium D-glycero-D-guloheptonate, and disodium ethylenediaminetetraacetate (EDTA) did not change the spectrum of ferricytochrome *c*.

Attempts to ascertain the redox state of the iron by measurements of electron-spin resonance were unsuccessful, since the signal of ferricytochrome *c* was too weak to detect at the temperature of liquid nitrogen and neutral *p*H.

We should note here that we have observed (*4*) similar changes in the spectrum of cytochrome *c* in the presence of polycations, which would not be expected to cause reduction. Equally puzzling was the ability of polyanions to "induce" the oxidized spectrum when added to reduced cytochrome *c* (*4*). These were also rate processes, like the carbohydrate effects; in contrast, reduction or oxidation of cytochrome *c* by conventional reducing or oxidizing agents is typically instantaneous and complete (*5*).

We are reporting the apparent reduction of cytochrome *c* with carbohydrates in spite of our present lack of an explanation. The variety of carbohydrates which give this effect, the magnitude and varying rates of the changes in spectrum, and the low concentration and physiological *p*H all suggest biological significance. These findings may imply that various hitherto-unsuspected factors control the oxidizability or reducibility of cytochrome *c*, perhaps

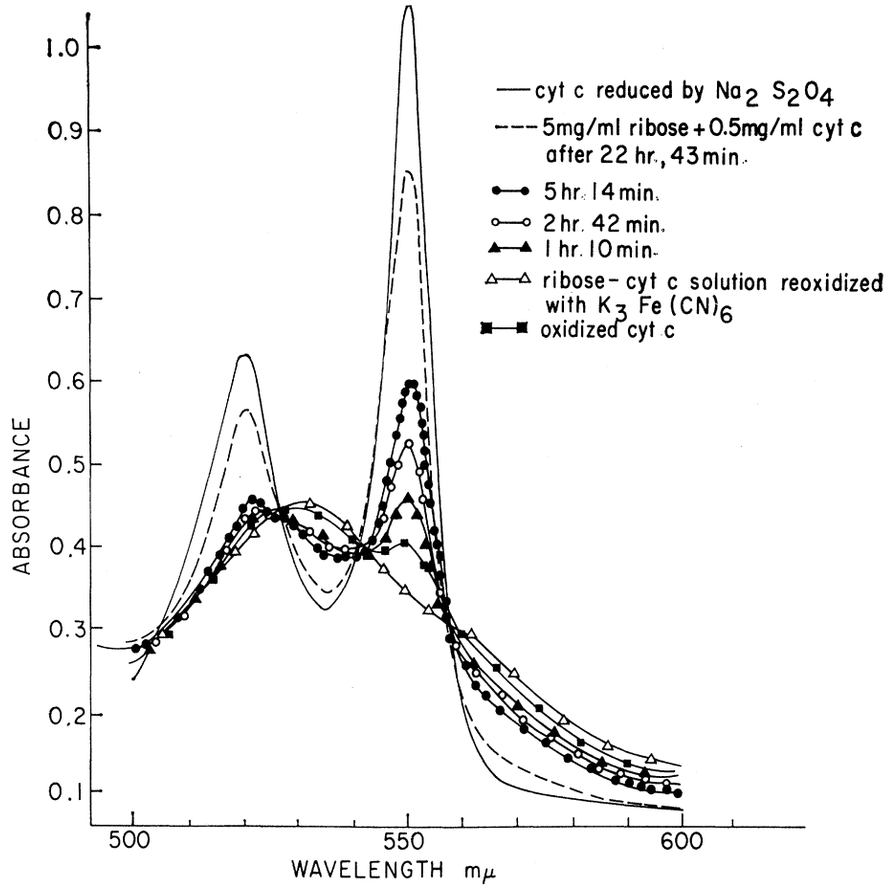

Fig. 2. Cytochrome *c* (0.5 mg/ml) and D-ribose (5.0 mg/ml) after incubation for specified time intervals, under conditions specified in Fig. 1. The $K_3Fe(CN)_6$ reoxidized preparation shows no maximum at 550 mμ. The spectrum of cytochrome *c* reduced by $Na_2S_2O_4$ is also included.

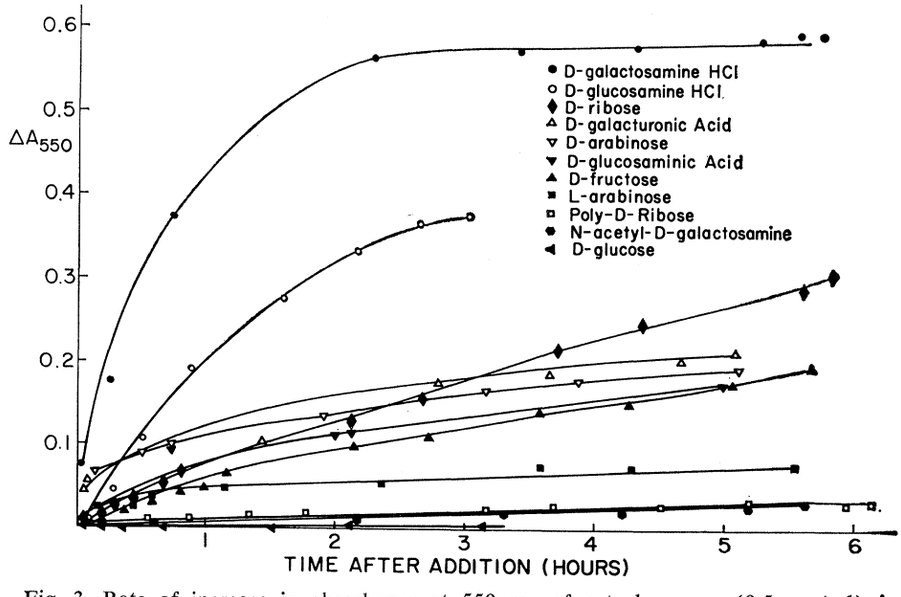

Fig. 3. Rate of increase in absorbance at 550 mμ of cytochrome *c* (0.5 mg/ml) in the presence of various carbohydrates (5.0 mg/ml).

through induced changes in the (tertiary) protein structure, or by "polymerization," or by alteration of the "water structure," or by a combination of these factors.

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References and Notes

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3. As defined by "Specification and Criteria for Biochemical Compounds," Nat. Res. Council–Nat. Acad. Sci. Publ. No. 719 (June 1960).
4. P. Person, A. Fine, P. Mora, H. Zipper, *J. Biol. Chem.*, in press.
5. For example, we found that the well-known reduction of cytochrome *c* by ascorbic acid [E. C. Slater, *Biochem. J.* **44**, 305 (1949)], when carried out under the same conditions as we use for the carbohydrates, was instantaneous and complete.
6. E. Margoliash and N. Frohwirt, *Biochem. J.* **71**, 570 (1959).
7. We thank Dr. H. G. Fletcher, Jr., for carbohydrate samples and advice.

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Adenosylmethionine Elevation in Leukemic White Blood Cells

Abstract. *White blood cell preparations from patients with chronic myelocytic leukemia have more* S-*adenosylmethionine, an important metabolic intermediate, than normal peripheral white cells or thoracic-duct lymphocytes. The previously reported elevation of cyanocobalamine (vitamin* B_{12}*) in the serum of patients with this disease is corroborated. The possible usefulness of methylation antimetabolites in cancer chemotherapy is suggested.*

Although many biochemical and metabolic studies have been reported (*1, 2*), biological transmethylation has not been evaluated extensively in relation to leukemia. The chemistry of methyl transfers in relation to cyanocobalamine (B_{12}) and folic acid metabolism has been reviewed (*3, 4*). Folic acid is increased in leukemic cells (*5*), and antifolate agents have been used for chemotherapy in leukemia (*6*). Patients with chronic myelocytic leukemia have increased amounts of B_{12} in their serums (*7*).

In our study we assayed *S*-adenosylmethionine (SAMe), an important donor of methyl groups in transmethylation reactions. We studied white cell preparations from blood of chronic myelocytic leukemia patients and normal (control) patients. In several instances the B_{12} in serum of patients and controls was also determined.

A total of eight determinations in seven untreated patients with chronic myelocytic leukemia were made at the National Cancer Institute. The diagnosis was established by hematologic and cytogenetic examinations of peripheral blood and marrow. Total peripheral white counts ranged from 67,100 to 195,000 cells/mm³ (Table 1).

All the patients had a much higher proportion of immature cells (younger than myelocytes) (6 to 31 percent, median 20 percent) in their peripheral blood than normal individuals, who have none, as determined by differential white cell counts of peripheral blood smears (Table 1). The concentration of B_{12} in the serum of four patients (475, 560, 690, 1080 $\mu\mu$g/ml) was much higher than normal (100 to 300 $\mu\mu$g/ml). The B_{12} was determined by the method of Mendelsohn and Watkin (*7*).

Whole blood (50 ml) or white-cell-rich plasma (20 ml) obtained by plasmapheresis was subjected to dextran centrifugal sedimentation and osmotic stress at 4°C by the method of Fallon *et al.* (*8*). The concentrated white cells were stored at −20°C until assayed for SAMe, usually within 24 hours. Microscopic study of the cell preparations confirmed that they contain white blood cells, platelets, and a minimum of debris (*8*).

Similar white cell preparations from four normal blood donors were obtained by plasmapheresis and the same sedimentation technique. These samples served as controls in the assay of leukemic blood cells. Comparisons were made with lymphocytes prepared by dextran sedimentation of lymph, obtained at thoracic duct cannulation, from patients with isolated, solid, nonhematogenous visceral tumors. These preparations were found, by microscopy, to consist entirely of small (98 percent) and large (2 percent) lymphocytes.

The fractionation of leukemic blood by differential centrifugation allowed us to obtain a preparation rich in immature cells. In this preparation more than 50 percent of the cells were "blast" cells and more than 60 percent of the cells were younger than myelocytes (Table 1), as shown by microscopy.

The white-blood-cell preparations were weighed, homogenized, and assayed by a method (*9*) in which melatonin-H^3-methoxy-C^{14} is synthetized enzymatically from *N*-acetylserotonin-H^3, SAMe-methyl-C^{14}, and hydroxyindole-*O*-methyl transferase (from bovine pineal gland) in the presence of tissue extracts containing unlabeled endogenous SAMe. The ratio, H^3 to C^{14}, is determined in melatonin-H^3-C^{14}, the only radioactive product extracted and counted. Tissue SAMe reduces the specific activity (radioactivity per mole) of the SAMe-C^{14} and so leads to an elevated $H^3 : C^{14}$ ratio. The change in this ratio bears a theoretical and experimentally demonstrated linear relation to the amount of tissue SAMe added to the incubation mixture, and

Table 1. Hematologic findings and *S*-adenosylmethionine in white blood cells of eight patients with chronic myelogenous leukemia.

Total white cells (cells/mm³)	Cells younger than myelocytes (%)	SAMe (μg/g)
	Controls	
2500–8500	0	1.0–3.0
	"Blast"-cell preparation	
	> 60	7.0
	Patients	
137,000	31	6.1
136,000	27	17.3
144,000	24	8.2
195,000	22	3.9
194,000	19	7.1
156,000	8	4.5
67,100	6	13.9

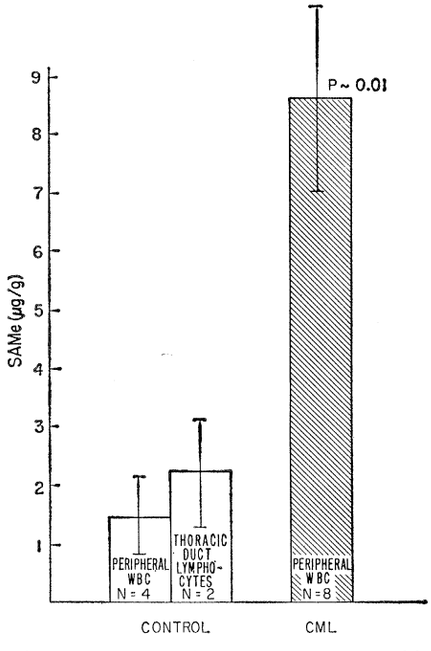

Fig. 1. Concentration of *S*-adenosylmethionine in the white blood cells of normal individuals and those of patients with chronic myelocytic leukemia (*CML*). *N*, number of patients.