Isocitric and 6-Phosphogluconic Dehydrogenases in Human Blood Serum. (23439)

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Although a number of dehydrogenases utilizing DPN† as a hydrogen acceptor have been demonstrated in the blood serum(1-4) and cerebrospinal fluid(5) of mammals, no information concerning TPN-linked enzymes in mammalian extracellular fluids is extant. Faulkner(6) found recently that silkworm blood contains a "malic" enzyme(7) and a polyhydric alcohol phosphate dehydrogenase both of which are specific for TPN.

We have found that both the serum and the erythrocytes of human and rat blood contain isocitric dehydrogenase (ICD):

Isocitrate + TPN ⇌ α-Ketoglutarate + CO_2 + TPNH + H^+

and 6-phosphogluconic dehydrogenase (PGD):

6-Phosphogluconate + TPN ⇌ Ribulose-5-phosphate + CO_2 + TPNH + H^+.

These enzymes are similar insofar as they both catalyze the reversible oxidative decarboxylation of their substrates and require TPN as a specific hydrogen acceptor. The properties of these enzymes in human blood serum were found to be similar to those reported for other animal tissues(8,9). Recent reports of large changes in activity of a number of glycolytic enzymes and transaminases of serum in certain diseases(2,3,10,11) prompted us to develop methods for determination of ICD and PGD in serum, which are here reported.

Methods. Human blood samples were drawn from the cubital vein without regard to the fasting state. The blood was transferred to polyethylene centrifuge tubes and allowed to clot at room temperature. The serum was separated by centrifugation, removed with a dropping pipette, and centrifuged again to remove all traces of erythrocytes. The millimolar extinction coefficient of reduced pyridine nucleotides at 340 mμ was assumed to be 6.22. PGD of rat liver was purified according to Glock and McLean(12); the preparations were devoid of glucose-6-phosphate dehydrogenase activity. ICD was purified from rat heart by the method of Ochoa(13). All spectrophotometric measurements were made with a Beckman model DU spectrophotometer with either quartz or corex cells of 1 cm light path. In most experiments the temperature of the cell compartment was maintained at 25° by circulating water at this temperature through thermospacers. Total serum protein was estimated by the quantitative biuret method(14).

Determination of ICD activity. For each determination a blank and an experimental cell was used. Both cells contained 0.5 ml Tris buffer pH 7.5 (0.1 M); 0.2 ml TPN (0.001 M) dissolved in 0.15 M NaCl and 0.3 ml $MnCl_2$ (0.01 M) dissolved in 0.15 M NaCl. The blank cell contained 0.05 ml 0.15 M NaCl and the experimental cell contained 0.05 ml of sodium DL-isocitrate (0.1 M). Usually these components were mixed in small tubes placed in a constant temperature bath at 25°. The reaction was initiated by the addition of 0.5 ml of serum to each tube. The components were mixed by inversion 3 times, the ends of the tubes being covered with a sheet of "parafilm". Immediately after mixing, the reaction mixtures were poured into the spectrophotometer cells. The absorbency of the experimental cell at 340 mμ versus that of the blank cell was measured at 3- to 5-minute intervals for 12 to 20 minutes. Sera with abnormally high ICD activities were diluted with 0.15 M NaCl just prior to their addition to the reaction mixture. ICD activities were calculated from zero order portions of plots of the change in absorbency of the experimental cell

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† The following abbreviations are employed: TPN = triphosphopyridine nucleotide; TPNH = dihydrotriphosphopyridine nucleotide; DPN = diphosphopyridine nucleotide; DPNH = dihydrophosphopyridine nucleotide; Tris = tris (hydroxymethyl) aminomethane.

versus time. The activity was expressed in terms of millimicromoles of TPNH formed per 1 ml of serum per hour at 25°. In those experiments where the temperature at which the reaction was run was other than 25°, the activities were corrected to the latter temperature, assuming a temperature coefficient (Q_{10}) of 1.7 over the range of 20°-30°. *Determination of PGD activity.* Both the blank and the experimental cells contained 0.1 ml Tris buffer pH 7.5 (0.5 M); 0.05 ml $MgSO_4$ (1 M); 0.1 ml TPN (0.004 M) dissolved in 0.15 M NaCl and 0.1 ml cysteine HCl (0.1 M, neutralized to pH 7.5 with $NaHCO_3$ just prior to use). The blank cell contained 0.7 ml 0.15 M NaCl while the experimental cell contained 0.6 ml 0.15 M NaCl and 0.1 ml of sodium 6-phosphogluconate (0.01 M). The reaction was initiated by the addition of 0.5 ml of serum to both tubes. The spectrophotometric method for the determination of substrate dependent formation of TPNH, and the method of expression of enzymatic activity was the same as that used for the determination of serum ICD.

Results. Properties of ICD and PGD in blood. Under the experimental conditions described, the rate of substrate dependent formation of TPNH obeyed zero order kinetics in both test systems until more than 50% of the added TPN was reduced. With both enzyme systems the initial rate of reaction was strictly proportional to the quantity of serum added. The rate of reduction of TPN in the absence of added substrate by the sera of apparently healthy adults was found to be negligible in comparison with the activity of ICD and PGD. No change in absorbency at 400 mμ was observed in either test system.

The concentrations of TPN, divalent cations and substrates employed appeared to be optimal insofar as increasing them 2-fold did not alter rate of reaction. Cysteine activated the serum PGD of normal humans from 3- to 5-fold at the concentrations used. This thiol was without influence upon serum ICD activity.

The ICD and PGD of normal human serum were found to be specific for TPN. Replacement of this nucleotide by DPN did not lead to a substrate-dependent formation of DPNH. A number of experiments were performed to detect pyridine nucleotide transhydrogenase (15) in serum. In these experiments, the TPN concentration in the ICD test system was reduced by a factor of 10, and the reaction allowed to proceed until all the TPN present (0.02 μmole) was reduced. The absorbency at 340 mμ remained constant after that time. DPN (1 μmole) was then added to both the blank and the experimental cells. No further change in absorbency at 340 mμ occurred, from which it was concluded that pyridine nucleotide transhydrogenase was absent from serum.

The ICD activity of serum was negligible in the absence of added Mn^{++} ions. When added in 10-fold molar excess, Mg^{++} ions permitted approximately 60% of the rate of reaction observed with Mn^{++} ions. With some sera, the formation of TPNH in the ICD test system showed a definite lag period for about the first 3 minutes. This lag period could be diminished by doubling the amount of Mn^{++} added, a procedure which did not affect the rate of reaction once the initial lag period was overcome. It must be emphasized that all reaction rates were calculated from the linear portion of plots of change in absorbency versus time. Mg^{++} ions induced definite but variable activations of serum PGD and hence were added to the test system for this enzyme.

When ICD purified from rat heart muscle was added together with serum to the ICD test system, the increase in rate of formation of TPNH was strictly additive. However, it must be mentioned that erratic results were obtained in attempts to measure the ICD activity of highly lipemic sera, and in these cases, the further addition of purified ICD did not result in the expected increase in activity. With normal sera, the addition of purified rat liver enzyme leads to the calculated increase in PGD activity.

No change in the ICD activity of normal serum was observed after storage at room temperature for 5 hours, or for many days at 4°. Serum PGD was more labile than ICD, approximately 50% of the activity was lost after storage at 4° for 4 days. Dialysis of serum against 0.15 M NaCl at 4° for 12 hours did not affect serum ICD.

TABLE I. Activity of ICD and PGD in Sera of Apparently Healthy Individuals.

Enzyme	Group	Age range (yr)	No. of subjects	Enzyme activity (mμmoles TPNH formed/1 ml of serum/hr at 25°C)
ICD	Males	21-49	22	110 ± 49* (55-224)
"	Females	21-49	22	109 ± 56 (47-264)
"	Term pregnancy	20-44	5	116 ± 36 (89-170)
"	Newborn infants		5	305 ± 91 (179-475)
PGD	Males	21-33	16	137 ± 36 (80-188)
"	Females	21-45	10	137 ± 60 (40-238)
"	Term pregnancy	16-25	3	178 (115,195,224)

* ± stand. dev.

Figures in parentheses represent range of values observed.

Stroma-free hemolyzates exhibited approximately 80 times the ICD and 450 times the PGD activity of the corresponding normal sera. Thus, reliable values for the activity of these enzymes can be obtained only with sera which show no visible trace of hemolysis. A number of experiments showed that contact of serum with erythrocytes for as long as 5 hours at room temperature did not change either ICD or PGD activity provided that no hemolysis had taken place.

ICD and PGD in the serum of apparently healthy individuals. The levels of ICD and PGD in the sera of apparently healthy individuals are summarized in Table I. It will be seen that the activity of both enzymes is of the same order of magnitude, *viz.* in the neighborhood of 50 to 250 mμmoles of TPNH formed per 1 ml of serum per hour at 25°. The statement in a preliminary communication(16) that the activity of these serum enzymes is one thousand times that depicted in Table I is due to a misprint. In adult subjects, no difference in the activity of either enzyme was found between the sera of males and females. Moreover, neither ICD nor PGD activity could be correlated with age, race, or total serum protein concentration. The sera of term pregnancies possessed activities for both enzymes which fell well within the normal range for adult females. However, sera from the cord blood of newborn infants showed somewhat elevated ICD levels. Repeated determinations of ICD and PGD in the serum of adults showed that the activity of these enzymes remained remarkably constant in a given individual.

Discussion. Although the presence of PGD in mammalian erythrocytes has been known for more than 20 years(17), the present findings make it clear that this enzyme and also ICD are true components of serum as well as of erythrocytes. The blood serum of normal individuals does not appear to contain all known TPN-linked enzymes. Unpublished experiments by us have shown that glucose-6-phosphate dehydrogenase activity of human serum is about the same as that of ICD and PGD. However, we have been unable to detect in the same samples any "malic" enzyme (7) or glutamic dehydrogenase which react with TPN.

When expressed in terms of moles of substrate transformed per 1 ml of serum per hour, the ICD and PGD levels found in normal individuals do not differ by a factor greater than 3 from the activities reported for the following serum enzymes: aldolase(10), glutamic-oxalacetic and glutamic-pyruvic transaminases(18), whereas the levels of lactic dehydrogenase(2,3) phosphohexoisomerase (11) and 5-phosphoriboseisomerase(19) are greater by orders of magnitude.

The vigorous ICD activity of human erythrocytes is in accordance with the observation of Rubinstein *et al.*(20) that certain enzymes of the tricarboxylic acid cycle including ICD are retained in the mature mammalian red cell, despite the fact that the respiratory metabolism of these cells is very feeble in the absence of unnatural electron carriers(21).

The factors which determine the extreme elevations in the activity of serum ICD in certain diseases of the liver(16) are under investigation.

Summary. Methods for determination of isocitric and 6-phosphogluconic dehydrogenases in human blood serum are described. The properties of these enzymes, and factors affecting their activity in the blood sera of

normal human adults are discussed.

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Received July 10, 1957. P.S.E.B.M., 1957, v96.

Mast Cell Changes in Animals Exposed to Cold. (23440)

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A marked increase in number of mast cells of the lungs and intestine of hedgehogs during hibernation was recently reported(1). Since mast cells are known to be an abundant source of heparin, the authors concluded that the increase of these cells was an important factor in preventing blood coagulation and thrombosis and in decreasing the work of the heart during hibernation. More recently a great deal of evidence has accumulated which strongly favors the opinion that mast cells are also an important source of histamine(2,3,4, 5).

We planned this study on mast cell changes in the cold for the following reasons. First, knowing that corticosteroids cause a degranulation of mast cells(6), it was suspected that during a long exposure to cold, when definite changes in the adrenal activity have been shown to occur(7), some changes in number or structure of these cells would take place. Secondly, measurements of histamine content in laboratory animals(8) and indirect evidence in humans(9) have shown that local application of a cold stress releases histamine from the stimulated tissues. Finally, the cold induced vasodilation normally observed after exposure to low temperatures, was associated with histamine release from the skin(9,10).

Methods. Experiments were conducted at 2 ± 1°C and at 6 ± 1°C; work to be reported elsewhere has shown that histological changes induced by cold are different at these 2 temperatures. White male rats, weighing approximately 300 g were used. In Exp. A, where animals were exposed to 2 ± 1°C, three groups of 12 rats were used. The first group, kept at 23°C, was the control group, whereas the second and third were respectively exposed to cold for 2 and 4 weeks. In Exp. B the same procedure was used with the exception of the temperature which was 6 ± 1°C instead of 2 ± 1°C.

The following procedure was used for all