Mechanism of Action of Thyroid Hormones on Erythrocyte 2, 3-Diphosphoglyceric Acid Synthesis

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ABSTRACT Normal erythrocytes, when incubated with thyroid hormone, were found to have increased levels of 2,3-diphosphoglyceric acid. In addition, a partially purified enzyme preparation, when incubated with either a 1,3-diphosphoglyceric acid generating system or 1,3-diphosphoglyceric acid directly, showed increased levels of 2,3-diphosphoglyceric acid when exposed to thyroid hormone. The hormonal effect was biphasic and was witnessed after 5 min of incubation. Substitution on the 3 and 5 positions of the basic thyronine molecule was essential for hormonal effect. It appears that thyroid hormone acts by directly stimulating the diphosphoglycerate mutase enzyme. The hormonal effect on 2,3-DPG synthesis may offer a biochemical explanation for the shift in the oxyhemoglobin dissociation curve observed in thyroid disorders.

INTRODUCTION

Benesch, Benesch, and Yu (1) and Chanutin and Curnish (2) described an increase in the dissociation of oxygen from hemoglobin upon the addition of 2,3-diphosphoglyceric acid to hemoglobin in a free solution. Gahlenbeck and Bartels (3) noted a decrease in the oxygen affinity of hemoglobin in blood of hyperthyroid patients as well as in euthyroid man and rats after treatment with triiodothyronine. The shift to the right in the oxygen dissociation curve facilitates oxygen release and can be explained if thyroid hormones increase 2,3-DPG. Recently, 2,3-diphosphoglyceric acid has been shown to be increased in normal red cells incubated with varying concentrations of thyroid hormone (4) and also in patients with hyperthyroidism (4, 5). In the present report, a direct effect of L-thyroxine and 3,5,3-triiodothyronine on a preparation of 2,3-diphosphoglycerate mutase from human erythrocytes has been demonstrated.

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METHODS

The following crystalline enzymes and substrates were obtained from Sigma Chemical Co., St. Louis, Mo.: monophosphoglycerate mutase, enolase, glyceraldehyde-3-phosphate dehydrogenase, lactic dehydrogenase (LDH) from rabbit muscle, alcohol dehydrogenase, phosphoglycerate kinase, diphosphopyridine nucleotide (NAD), *dl*-glyceraldehyde-3-phosphate (GAP), potassium pyruvate, disodium adenosine-5-triphosphate (ATP), adenosine diphosphate (ADP), 3-phosphoglyceric acid (3-PG), 2,3-diphosphoglycerate, pentacyclohexoammonium salt (2,3-DPG), triethanolamine-HCl (TEA), diethylaminoethyl-cellulose (DEAE-cellulose), 3,5,3-triiodothyronine (T3), L-thyroxine (T4), 3,5-diiodothyronine, thyronine, and 3,5-dinitrotyrosine. Phosphoric acid ^{32}P was obtained from Abbott Laboratories, N. Chicago, Ill. All other chemicals were of the reagent grade.

Preparation of 1,3-diphosphoglyceric acid (1,3-DPG) and labeled (l) ^{32}P 1,3-diphosphoglyceric acid. Synthesis was carried out by a modification of the method of Negelein (6) with glyceraldehyde-3-phosphate dehydrogenase coupled to an alcohol dehydrogenase. In an attempt to obtain the highest specific activity of 1,3-DPG (l) ^{32}P, the synthesis was carried out in two separate flasks. The nonradioactive flask contained a final concentration of 0.025 M neutralized GAP, 0.07 M phosphate buffer, pH 7.5, and 0.4 mM NAD diluted in a volume of 36 ml of 0.07 M TEA-HCl buffer, pH 7.5. The second flask contained labeled radioactive ^{32}P phosphoric acid, 5.4 mCi (specific activity 45 mCi/millimole), neutralized with TEA-HCl buffer and used as the only source of phosphorus, with GAP and NAD in the same concentrations as in the nonradioactive flask but diluted in 18 ml of TEA buffer, pH 7.5. Both flasks were maintained at 18°C. Glyceraldehyde-3-phosphate dehydrogenase, 5 U/4 cc of incubation solution, 0.02 M acetaldehyde, 5 U of alcohol dehydrogenase per 4 cc of incubation solution were added to each flask. Again after 2 and 4 min, 0.02 M acetaldehyde was added to each. The reaction was stopped in 25 min at which time 1 N H_2SO_4 was added. The flasks were combined and immediately poured into 660 ml of acetone (−20°C) and allowed to sit for one-half hour. The precipitate was centrifuged and washed once with ice

cold acetone and dried rapidly in a vacuum dessicator. The product was dissolved in 4 ml of cold distilled water and filtered. The clear solution was then neutralized with 1 N NaOH. The amount of 1,3-DPG was determined with glyceraldehyde-3-phosphate dehydrogenase according to the method of Negelein (6); in the same cuvette 3-PG was determined by adding neutralized 5 mM ATP, 5 mM $MgSO_4$, and 3-phosphoglycerate kinase (3.5 U), in that order, after observing the OD change due to 1,3-DPG. In a typical assay, 3 μmoles/ml of 1,3-DPG (1)^{32}P (an approximate 10% yield from the original 25 μmoles/ml of GAP) was obtained. The yield of 3-PG was 0.2 μmoles/ml or 1% yield of the original GAP. If stored at −80°C in small aliquots, there was essentially no loss of 1,3-DPG in a 2 wk period of time.

2,3-Diphosphoglyceric acid assay. 2,3-DPG was measured enzymatically by the modification by Beutler, Meul, and Wood (7) of Krimsky's (8) method and expressed as either nanomoles per milliliter of incubation solution or micromoles per gram of hemoglobin. The addition of 1 mM ADP, T3 (3.75×10^{-8}), and T4 (3.75×10^{-8}) directly to the assay mixture had no effect on the determination of 2,3-DPG.

Radioactive assay for 2,3-DPG (modification of Rose [9]). 2,3-DPG is both acid and heat stable and can be distinguished from 1,3-DPG and ATP on this basis. Incubations were carried out in a total of 1 cc volumes, at 25°C for 15 min. 2 mM glycyl glycine buffer, pH 7.8, 1 mM mercaptoethanol, 2 mM potassium phosphate buffer, pH 7.5, 120 μM 3-PG, diphosphoglycerate mutase enzyme (0.080 U/ml), 80 μM radioactive 1,3-DPG (1)^{32}P, and T3 and T4 were used in varying concentrations. The reaction was stopped by the addition of 0.2 ml of 10 N H_2SO_4 and heating at 100°C for 10 min. After cooling, water was added to a volume of 2.5 ml; 0.5 ml of a 5% ammonium molybdate and 10 ml of water-saturated isobutanol were added. This was mixed and centrifuged to obtain separation of the phases, the aqueous layers containing the heat- and acid-stable ^{32}P. 2,3-DPG was counted.

Preparation of intact erythrocytes. Fresh venous blood obtained from normal adults was collected in heparinized tubes (Vacutainer, Becton-Dickinson & Co., Rutherford, N. J.). The plasma was separated, the buffy coat was discarded, and the packed cells were washed three times in ice cold saline. Washed cells were readjusted in their own plasma to a hematocrit of 40, pH being maintained at 7.4 with supplemental Krebs-Ringer bicarbonate, and 4 mM glucose was added to the solution. The red cell suspension was gassed with 5% CO_2 and air, and the samples were divided into 4-ml aliquots. The tests were incubated with T3, 3.75×10^{-8} moles/liter. Samples were incubated (test and control) at 37°C in a water bath at 60 oscillations per min. 2,3-DPG was determined by the enzymatic method at zero, 1 and 2 hr.

Preparation of the erythrocyte enzyme fraction (modification of Hennessey, Waltersdorph, Huennekens, and Gabrio [10]). 200 ml of 6–7 day old banked whole blood collected in ACD was washed three times in ice cold saline, and the packed cells were adjusted to a hematocrit of 35–40 with Krebs-Ringer bicarbonate buffer, pH 7.4, to which has been added 10^{-4} M mercaptoethanol and 10^{-5} M EDTA. The adjusted cells were frozen and thawed three times and the stroma were removed by centrifugation at 30,000 *g* for 30 min. The hemoglobin-free enzyme fraction was obtained by repeated washings of an equal volume of hemolysate with a 10% DEAE-cellulose suspension, adjusted to pH 7.26 with a 0.003 M phosphate buffer. The final washing was clear and colorless. The enzymes were eluted from the resin by two 50-ml volumes of 0.5 M KCL. The hemoglobin-free, enzyme-rich supernatant was then dialyzed overnight against 0.15 M KCL, pH 7.26 and 10^{-4} M mercaptoethanol. Dialysis solution was changed every 2 hr for the first 6 hr. The following day, 2.5 mg of bovine serum albumin per cc of enzyme fraction was added, and diphosphoglycerate mutase enzyme was assayed according to the method of Rose (9). 2,3-DPG, 1,3-DPG, and 3-PG were measured by the enzymatic methods as described above and were found to be absent or below detectable levels

1,3-DPG. incubation system. The incubation mixture, containing 20 μmoles of GAP, 10 μmoles of NAD, 0.1 mM 3-PG, 1 mM $MgCl_2$, 2 μmoles of potassium phosphate, pH 7.5, 4 U of LDH, 40 μmoles of potassium pyruvate, 5 U of glyceraldehyde-3-phosphate dehydrogenase, 100 μmoles of TEA buffer, pH 7.8, and diphosphoglycerate mutase enzyme extract, 0.02 U/ml in a total volume of 2.2 cc, was incubated for 1 hr at 37°C.

Purification of the diphosphoglycerate mutase. The human erythrocyte diphosphoglycerate mutase was purified by DEAE-cellulose column chromatography and ammonium sulfate fractionation as described by Rose (9).

RESULTS

Effect of L-thyroxine on intact red cells. When normal, washed human erythrocytes were resuspended in their own plasma, buffered to pH 7.4 with the addition of 4 mM glucose, 2,3-DPG formation was increased by 35% in the first hr and 42% after the second hr of incubation with T4 (3.75×10^{-8} moles/liter) as compared with a paired control (Table I). 2,3-DPG remained constant during the 2 hr incubation in the control experiments. There essentially was no significant difference in results when washed, intact RBC were incubated in a Krebs-Ringer bicarbonate buffer, pH 7.4, with 4 mM glucose and 3 mM potassium phosphate added.

Effect of thyroid hormone on formation of 2,3-DPG in red cell crude enzyme preparation. A biphasic effect on 2,3-DPG synthesis (Table II) was obtained upon incubation with varying concentrations of thyroid hormones. The effect was first witnessed with T3 at concentrations of 3.75×10^{-14} mole/liter and is maximally stimulated at concentrations of 3.75×10^{-8} mole/liter, decreasing with increasing levels of thyroid hormone.

TABLE I

Effect of 3,5,3-Triiodothyronine (3.75×10^{-8}M) on 2,3-DPG Synthesis in Intact Normal Human Erythrocytes

System	2,3-DPG in micromoles ±SD per gram of hemoglobin		
	0	1 hr	2 hr
Control	15.0 ±1.6	14.8 ±1.3	15.0 ±1.30
3,5,3-Triiodothyronine	14.9 ±0.9	24.0 ±2.0	27.8 ±1.70

TABLE II

The Effect of Hormone Concentration on 2,3-DPG Synthesis Employing the 1,3-DPG Generating System as Compared with Control (n = 4)

Concentration of L-thyroxine	2,3-DPG	Concentration 3,5,3-triiodothyronine	2,3-DPG
M	*nmoles* ±SD/*ml per hr*	M	*nmoles* ±SD/*ml per hr*
3.75×10^{-5}	78 ±1.8	3.75×10^{-5}	115.7 ±15.8
3.75×10^{-6}	83 ±3.9	3.75×10^{-6}	105.6 ±19.8
3.75×10^{-8}	147 ±13.6	3.75×10^{-8}	189.6 ±2.1
3.75×10^{-10}	162.5 ±8.8	3.75×10^{-10}	166.5 ±5.1
3.75×10^{-12}	75 ±2.7	3.75×10^{-12}	102.5 ±1.6
3.75×10^{-14}	41.3 ±2.2	3.75×10^{-14}	93.2 ±2.2
0 (control)	44.2 ±1.3	3.75×10^{-16}	46.2 ±6.0
		0 (control)	45.3 ±0.2

T4 effect was noted initially at 3.75×10^{-12} mole/liter and peaked at 3.75×10^{-10} mole/liter.

Effect of substrates on 2,3-DPG formation. The dependence of 2,3-DPG synthesis on either elimination or addition of the substrates in the 1,3-DPG generating system was examined. Table III depicts that when GAP was omitted, essentially no 2,3-DPG was synthesized. 3-PG (a cofactor in the diphosphoglycerate mutase step), when omitted, blunted the production of 2,3-DPG by approximately 50% in both the test and the control. When the NAD and NADH oxidizing system was omitted, a marked decrease in the 2,3-DPG was noted. Thus the full 1,3-DPG generating system must be present in order to witness complete thyroid hormonal effect on 2,3-DPG formation. Upon the addition of 0.75 mM ATP to the complete incubation system with T4, no increased synthesis of 2,3-DPG was noted. This demonstrated that the back reaction involving phosphoglycerate kinase is not an important means of producing 2,3-DPG.

TABLE III

The Effect of the Elimination of Different Components of the 1,3-DPG Generating System upon 2,3-DPG Synthesis (n = 4)*

System	2,3-DPG
	nmoles ±SD/*ml per hr*
Control	40.5 ±0.1
Control plus T4	132.8 ±1.2
Control plus T4 plus 0.75 mM ATP	129.3 ±4.6
Omit 1,3-DPG generating system	6.4 ±0.3
Omit 1,3-DPG generating system and T4	5.9 ±0.7
Omit GAP	7.0 ±0.1
Omit GAP and T4	7.4 ±0.6
Omit 3-PG	71.1 ±2.3
Omit 3-PG and T4	29.5 ±0.2
Omit DPN, LDH, K-pyruvate	9.8 ±0.1
Omit DPN, LDH, K-pyruvate, and T4	10.6 ±1.2

* 3.75×10^{-8} M T4 was added as noted. For other details, see Methods.

Hormonal effect employing 1,3-DPG as a substrate employing the partially purified enzyme. To evaluate the effect of thyroid hormone on 2,3-DPG synthesis employing 1,3-DPG as a substrate rather than a 1,3-DPG generating system, the following experiment was carried out (Table IV). When substrate 1,3-DPG was omitted, essentially no synthesis of 2,3-DPG was noted; however, when a standard concentration of 2,3-DPG was added to the same cuvette, the optical density changes were noted. When 3-PG was omitted, approximately 50% of the production of 2,3-DPG was assayed.

Thyroid hormonal effect employing radioactive 1,3-DPG (l)^{32}P as a substrate (Table V). The enzymatic assay results were corroborated by employing a radioactive assay for 2,3-DPG. The results correlate well with the enzymatic findings demonstrating a T3–T4 effect with levels of 10^{-6} and 3.75×10^{-8}, with higher values noted at lower concentrations.

Effect of thyroid hormone as a function of time. A hormonal effect was detected as early as 5 min after incubation with a maximal effect witnessed after 40 min (Fig. 1). In the control experiments, the rise in

TABLE IV

Thyroid Hormonal Effect Using 1,3-DPG as a Substrate (n = 4)

1,3-DPG	3-PG	System tested	2,3-DPG ±SD
μM	μM	M	*nmoles/ml per 20 min*
40	100	T4 3.75×10^{-8}	42.3 ±0.1
40	100	0	28.1 ±0.3
0	100	T4 3.75×10^{-8}	no change
40	0	T4 3.75×10^{-8}	25.1 ±4.8

TABLE V

Radioactive Assay for 2,3-DPG with 1,3-DPG (l) ^{32}P as a Substrate Incubated at 25°C for 20 Min (n = 3)

Compound tested	CPM ±SD Aqueous layer 2,3-DPG ^{32}P
M	
T4 3.75×10^{-6}	65,997 ±668
T4 3.75×10^{-8}	70,646 ±523
T3 3.75×10^{-6}	65,348 ±986
T4 3.75×10^{-8}	69,855 ±931
Control	49,053 ±740

2,3-DPG occurred early, stopped after 15 min, and remained unchanged throughout the remainder of the incubation period.

The effect of analogues on thyroid hormone stimulation of 2,3-DPG. In order to determine whether stimulation of 2,3-DPG was a specific thyroid hormonal effect, further studies were carried out with thyronine, potassium iodide, 3,5-diiodothyronine, and dinitrotyrosine in comparable concentrations (Table VI). As shown, none of these compounds except 3,5-diiodothyronine produced stimulation of 2,3-DPG production. It appears that substitutions at the 3 and 5 positions of the thyronine molecule are essential for hormonal effect on 2,3-DPG synthesis.

Effect of thyroid T4 on 2,3-DPG synthesis employed purified diphosphoglycerate mutase and 1,3-DPG as a substrate. The diphosphoglycerate mutase showed 2.5

TABLE VI

The Effects of Analogues on 2,3-DPG Synthesis Employing the 1,3-DPG Generating System, Incubated for 1 Hr at 37°C (n = 4)

Compound tested	Concentration	2,3-DPG
	M	*nmole* ±SD/ *ml per hr*
L-Thyroxine	3.75×10^{-6}	95.5 ±1.2
	3.75×10^{-8}	119.3 ±0.7
Thyronine	3.75×10^{-6}	45.0 ±0.3
Potassium iodide	3.75×10^{-6}	42.3 ±0.1
3,5-diiodothyronine	3.75×10^{-6}	81.0 ±0.3
	3.75×10^{-8}	88.3 ±1.1
Dinitrotyrosine	3.75×10^{-8}	40.0 ±1.3
Control	None	44.0 ±1.2

U/mg of protein. When the mutase enzyme was incubated with 40 μM 1,3-DPG with 2 mM glycyl glycine buffer, pH 7.8, 1 mM mercaptoethanol, 2 mM potassium phosphate, pH 7.5, and 0.1 mM 3-PG when T3 (3.75×10^{-8}) was added, 4.7 nmoles/ml per 20 min was synthesized as compared to a control of 3.2 nmoles/ml per 20 min. Furthermore, when the purified dimutase enzyme was incubated with 6μM 2,3-DPG for 20 min there was no formation of 3-PG or a decrease in 2,3-DPG.

DISCUSSION

There have been no consistent reports of the effect of thyroid hormone on glycolysis in the human erythro-

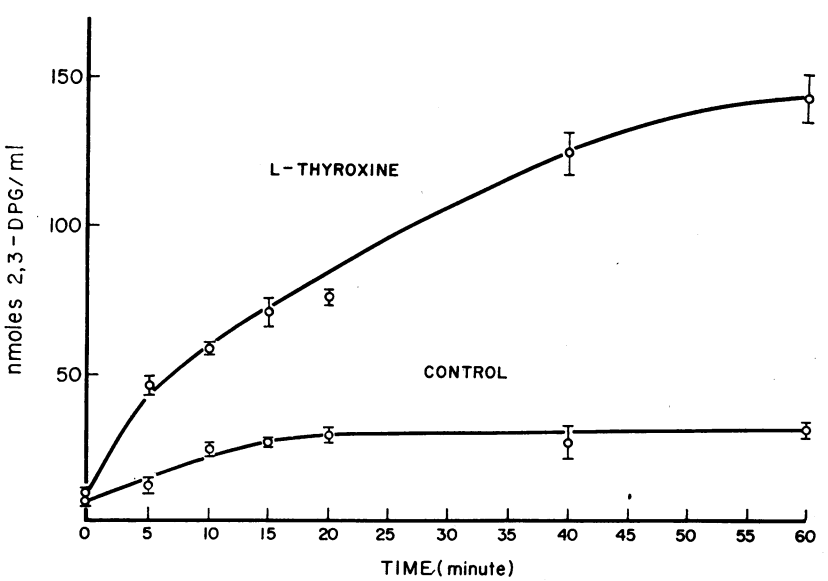

FIGURE 1 The effect of 3.75×10^{-8} M T4 on 2,3-DPG formation employing a crude erythrocyte enzyme preparation incubated with a 1,3-DPG generating system as a function of time. Φ = mean ±SD; n = 4.

cyte. A biphasic action of triiodothyroacetic acid was observed in human erythrocytes (11). 10^{-16}–10^{-14} M hormone increases the rate of ^{32}P utake, whereas 10^{-6} mole/liter showed a decrease. Dinitrophenol in concentrations of 10^{-6} mole/liter had no effect on ^{32}P uptake, as expected, since oxidative phosphorylation does not occur in the red cells (11). Further studies have shown that red cells of human thyrotoxic subjects demonstrate a more rapid uptake of ^{32}P in vitro than those from euthyroid persons; whereas cells from myxedematous patients take up ^{32}P more slowly (12). Erythrocytes of rabbits, receiving intravenous daily injections of L-triiodothyronine, aerobically glycolyzed more rapidly than untreated controls in the first 24 hr, less rapidly in the second 24 hr, and less than normal after 7 days (13).

Necheles and Beutler (14) reported an increase in oxygen consumption and a slight increase in CO_2 production following incubation of human erythrocytes with 3.75×10^{-8} M triiodothyronine, and they concluded that the increase in glycolysis was due to stimulation of the hexose monophosphate shunt. However, Crevasse, Hewson, Hazouri, and Shipp (15) demonstrated no significant change in either CO_2 production or lactate formation upon incubation of human erythrocytes with T3 in varying concentrations of 10^{-6}–10^{-8} mole/liter, concluding that the glycolytic and oxidative enzymes of the hexose monophosphate, Embden-Myerhoff pathways were not directly affected by thyroid hormone. Differences in incubation media, oxygen tensions, and pH employed may account for variations in results of previous studies.

In the present study, thyroxine treatment of intact red cells incubated either in a fortified Krebs-Ringer bicarbonate buffer or autologous plasma resulted in increased levels of 2,3-DPG after 1 and 2 hr of incubation, whereas the levels in control samples did not change (Table I). A similar effect was obtained with a crude enzyme preparation which was hemoglobin free. With this preparation a maximum stimulation was found at 3.75×10^{-8} and 3.75×10^{-10} M T4 (Table II). Higher concentrations of these hormones resulted in a progressive decrease. These findings are consistent with the biphasic action of thyroid hormones noted in physiological experiments and may represent competition of hormone molecules for the same binding sites analogous to substrate inhibition (16).

The complete 1,3-DPG generating system of 1,3-DPG itself was required for the effect to be evident in the crude enzyme preparation (Tables III and IV). This indicates that the hormones either (*a*) stimulated diphosphoglyceromutase, (b) increased the K_i for 2,3-DPG, or (*c*) inhibited the diphosphoglycerate phosphatase. The greater initial reaction velocity and the continued increase with time (Fig. 1) in the presence of T4 strongly indicate a direct effect on the diphosphoglyceromutase and an increase in the K_i of 2,3-DPG. Rose (9) has shown that the K_i of diphosphoglycerate mutase for 2,3-DPG is 0.85 μmole/liter. If the effect of thyroid hormones were only to inhibit the phosphatase, then a greater initial reaction velocity and a plateauing at the same level as observed in the control would have been expected (Fig. 1). The increase in the initial reaction velocity would be expected, but the absolute levels obtained would be the same since the K_i would not be modified. If the only action of the hormones was to release 2,3-DPG inhibition of diphosphoglyceromutase, then no change in the initial reaction velocity could be expected under conditions where little or no 2,3-DPG is present initially, as was shown to be the situation in Fig. 1.

In subsequent experiments, a purified preparation of diphosphoglycerate mutase gave increased levels of 2,3-DPG when incubated with thyroxine. This preparation did not possess phosphatase activity since incubation with 2,3-DPG did not result in any decrease in 2,3-DPG or the formation of any 3-PG. The effect on diphosphoglycerate mutase activity was obtained with T4, T3, and 3,5-diiodothyronine. These studies, although limited, indicate a relatively specific effect which requires iodination at the 3 and 5 positions of thyronine. Presumably, the mature human erythrocyte does not synthesize protein and *de novo* protein synthesis cannot be implicated; therefore, the effect must represent enzyme activation.

The biochemical mechanism of thyroid hormones has been extensively investigated (17–19), and in general the conclusion is that the transformation of energy derived from oxidative reactions into metabolically utilizable phosphate bonds (e.g., ATP) is altered. The present authors are proposing a mechanism of action of thyroid hormones in the human erythrocytes which involves the indirect hydrolysis of 1,3-DPG via 2,3-DPG to 3-PG. This mechanism acts as an ATP sink. effectively uncoupling ATP from glycolysis.

Harrary (20) has postulated a molecular mechanism for the action of thyroid hormones in the rat liver and muscle whereby they activate an acetylphosphatase which hydrolyses 1,3-DPG to 3-PG and P_i. His mechanism is similar but not identical with the mechanism proposed by the present authors for the human erythrocyte. Many of the effects of thyroid hormones in other tissues can be related to the action demonstrated herein, and this observation may be suggested as a general hypothesis for the mechanism of action. Further studies will be necessary to test this hypothesis and also to define further the nature of the interaction of the thyroid hormones with diphosphoglyceromutase.

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