THE EFFECT OF TRIIODOTHYRONINE ON THE OXIDATIVE METABOLISM OF ERYTHROCYTES. I. CELLULAR STUDIES * †

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The mature, non-nucleated mammalian erythrocyte is a useful system in which to study the control of both anaerobic glycolysis and the hexose monophosphate shunt in an intact-cell preparation. The physiologic and metabolic properties of this cell have been reported extensively (1, 2). The final suspension can be obtained conveniently in relatively pure culture from man or experimental animals, and the quantity of cells studied can be measured easily. Inasmuch as the erythrocyte has only slight remnants of the citric acid cycle, cytochrome system, and pathway for protein synthesis, these cannot be evaluated. However, the cell can carry out the reactions of the glycolytic pathway and of the hexose monophosphate shunt. When the erythrocyte is incubated with methylene blue, the hexose monophosphate shunt is activated by increasing the rate of oxidation of reduced triphosphopyridine nucleotide (TPNH). The rate of metabolism via this path can be estimated by determining the rate of oxygen consumption of the cells (3, 4).

This report describes the use of this system in an attempt to define the locus of action of the thyroid hormone.

MATERIALS AND METHODS

Reagents. Glucose-6-phosphate (G-6-P), triphosphopyridine nucleotide (TPN), 3,5,3'-triiodo-L-thyronine (TRITh), L-thyroxine, 3,5-diiodo-L-thyronine (DITh), 3,5-diiodo-L-tyrosine (DIT), and 3,5-dinitro-L-tyrosine (DNT) were obtained from Sigma Chemical Co. The disodium salt of ethylenediaminetetraacetic acid (Disodium Versene®) was obtained from Versenes, Inc. All of these reagents were dissolved in 0.9 per cent sodium chloride solution prior to use. A few drops of 1 N NaOH were used and the solution was heated to 55° C. to aid solution of TRITh when used in the highest concentration, 3.75×10^{-6} M. The buffered saline used in these experiments was prepared by the method outlined by Umbreit, Burris and Stauffer (5). The buffer was prepared by dissolving 1.78 Gm. of $Na_2HPO_4 \cdot 2H_2O$ in about 90 ml. of distilled water. The pH was adjusted to 7.4 with approximately 2.0 ml. of 1 N HCl, and sufficient water was added to bring the total volume to 100 ml. Twenty ml. of this buffer was added to 109 ml. of 0.9 per cent NaCl. The final pH in all reaction flasks before incubation was 7.4; the fall during incubation was not more than 0.2 pH units.

Glucose-1-C^{14} and glucose-6-C^{14} were obtained from Volk Radiochemical Co. The labeled components were added to the reaction vessels together with carrier glucose to a final concentration of 5.55×10^{-4} M. The $C^{14}O_2$ evolved on incubation was absorbed in 10 per cent sodium hydroxide. The carbonate was precipitated and plated as barium carbonate, following the method described by Krahl (6). All counts were corrected for self-absorption and coincidence (7) and are expressed as total cpm for the $C^{14}O_2$ formed per flask in 90 minutes.

Glucose was determined as total reducing substance by the method of Folin and Malmros as described in Umbreit (5). Aliquots were removed at intervals and the results were calculated as μg. glucose consumed per ml. erythrocytes. Appropriate corrections were made (5) when glucose-6-phosphate was used as substrate.

Blood from normal human volunteers was drawn into a flask containing 0.5 ml. (500 units) heparin solution and was immediately centrifuged at $3,000 \times G$ for 10 minutes. The plasma and buffy coat were discarded, and the red cells were resuspended in an equal volume of cold buffered saline. The cells were put into the Warburg flasks within 30 minutes after they had been drawn. For studies in which hemolysates were used, the packed red cells were resuspended in an equal volume of buffered saline containing 478 mg. per cent nicotinamide to reduce hydrolysis of the nucleotides (8). The suspension was then hemolyzed by freezing and thawing three times. Aliquots equal to 1 ml. packed cells were used in all experiments.

Oxygen consumption was determined in a Warburg respirometer. Each flask contained approximately 1 ml. of erythrocytes and buffered saline, pH 7.4, in a total volume of 3.6 ml. Unless otherwise noted, the final

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TABLE I

Oxygen and glucose consumption of human erythrocytes in the presence of triiodothyronine (TRITh)

Experiment	Flask pairs	Oxygen consumption		p*	Glucose consumption	
		Control	TRITh		Control	TRITh
		μl./min./ml. erythrocytes			*μg./min./ml. erythrocytes*	
		Whole erythrocytes without methylene blue				
1	3	0.18	0.19			
2	3	0.43	0.46			
9	2	0.24	0.32			
16	2	0.44	0.61			
21	3	0.20	0.27	0.002		
		Whole erythrocytes plus methylene blue				
1	3	1.65	1.79			
2	2	1.83	2.05			
3	3	3.07	3.56			
7	3	2.18	2.71			
8	3	2.64	2.82			
9	4	1.80	2.02			
10	3	2.45	2.61			
11	3	2.57	3.04			
12	3	2.63	2.81			
13	3	2.17	2.49			
14	3	3.14	3.45			
15	3	1.93	2.85		17.8	24.1
16	4	2.00	2.21		18.5	23.4
18	3	3.04	3.55		22.1	26.3
21	3	1.25	1.41		12.2	12.2
22	3	4.10	4.83	0.001	30.4	32.9
		Hemolysates plus methylene blue				
4	3	1.38	2.40			
5	3	2.55	3.43			
6	3	1.37	2.49			
7	3	2.04	2.84			
13	3	2.98	3.21			
23	3	1.82	2.48		15.7	21.4
24	3	1.47	1.82			
25	3	1.93	2.58	0.002		

* Calculated by student's "t" test on the basis of per cent increase in paired individual flasks within each experiment.

concentrations were: 3.75×10^{-6} M TRITh, 10^{-6} M methylene blue (MeB), and 5.55×10^{-4} M glucose. The flasks and contents were equilibrated under air for 10 minutes at 37° C. in the water bath, and the oxygen consumption was then measured at 10 to 15 minute intervals for 90 minutes. When hemolysates were used, 5.55×10^{-4} M glucose-6-phosphate was substituted for the glucose. Microhematocrit or hemoglobin determinations were carried out on all flasks. Oxygen uptake was expressed as μl. per minute per ml. cells.

RESULTS

Table I summarizes the data obtained when erythrocytes were incubated with TRITh. As has been reported repeatedly (9), the oxygen consumption of mature mammalian erythrocytes in the absence of methylene blue was extremely small. Upon incubation with TRITh, the rate of oxygen utilization increased slightly.

In the presence of methylene blue, human erythrocytes incubated with physiologic amounts of TRITh exhibited consistently a small but statistically highly significant increase in the rate of oxygen consumption (Table I). Hemolysates of cells from the same donors incubated with glucose-6-phosphate revealed similar or greater stimulation when TRITh was added. The data obtained from a representative experiment are presented in Figures 1 and 2. When the concentration of TRITh was increased 100-fold, to 3.75×10^{-6} M, more than 100 per cent stimulation of O_2 consumption was demonstrated. The relationship between the magnitude of stimulation observed and the concentration of TRITh in the reaction mixture is illustrated in Figure 3.

In five out of six experiments, glucose consump-

tion was found to increase comparably to the increases in oxygen consumption (Table I).

Previous workers have demonstrated that the oxidation of glucose, which occurs in erythrocytes when incubated with methylene blue, involves carbons 1–3 and occurs in the hexose monophosphate shunt (4). In order to determine whether increased oxygen consumption represents stimulation of metabolism in this pathway, rather than alternate pathways of oxidative metabolism, specifically labeled glucose was added to the red cell suspension. Incubation was carried out with and without methylene blue and with and without TRITh. The radioactivity of the carbonate obtained from cells incubated with glucose-1-C^{14} and glucose-6-C^{14} was compared. The results (Table II) indicated that carbon-1 was preferentially oxidized, supporting the interpretation that the hexose monophosphate shunt was stimulated by TRITh.

To determine whether the stimulation of metabolism in this pathway paralleled the known metabolic activities of these compounds, further studies were carried out with substances related to triiodothyronine: thyroxine diiodotyrosine, dinitrotyrosine, and inorganic iodide were tested in comparable concentrations. The results are shown in Tables II and III, and in Figure 4. In concentrations up to 20 times that of TRITh used in these experiments (7.5×10^{-7} M *vs.* 3.75×10^{-8} M), none of the compounds except L-thyroxine produced significant stimulation. Although stimulation was demonstrated when thyroxine was added, it was much less than that observed with comparable concentrations of TRITh. This finding agrees with the comparative *in vivo* activities of these compounds. Evaluation of stimulation using iodinated thyronine analogues of TRITh (*i.e.*, diiodothyronine and monoiodothyronine) could not be made because the commercially obtainable preparations of these compounds are not altogether free of the tri- and tetra-iodo-compounds and thus show biologic activity. The chelating agents, citrate and Versene®, were devoid of stimulating activity (Figure 4).

It has been suggested that the thyroxine analogues may act as accessory electron transporting

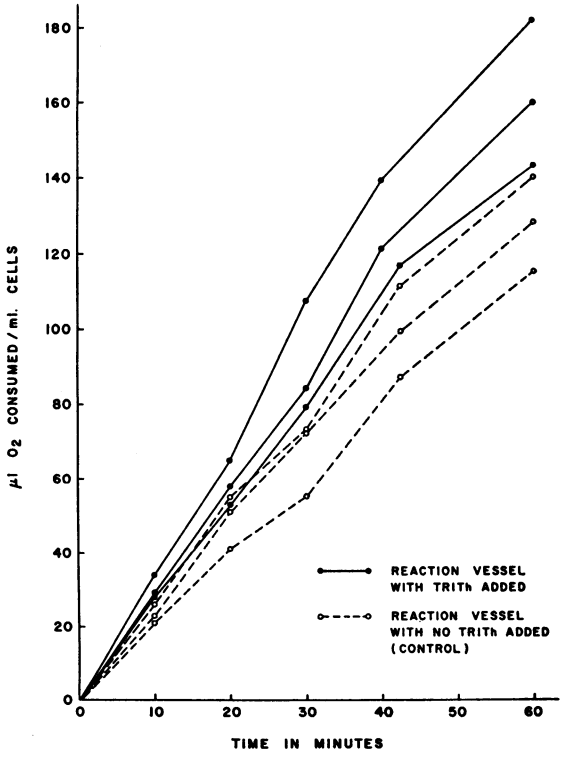

FIG. 1. THE EFFECT OF TRIIODOTHYRONINE (TRITH) ON THE OXYGEN CONSUMPTION OF ERYTHROCYTES

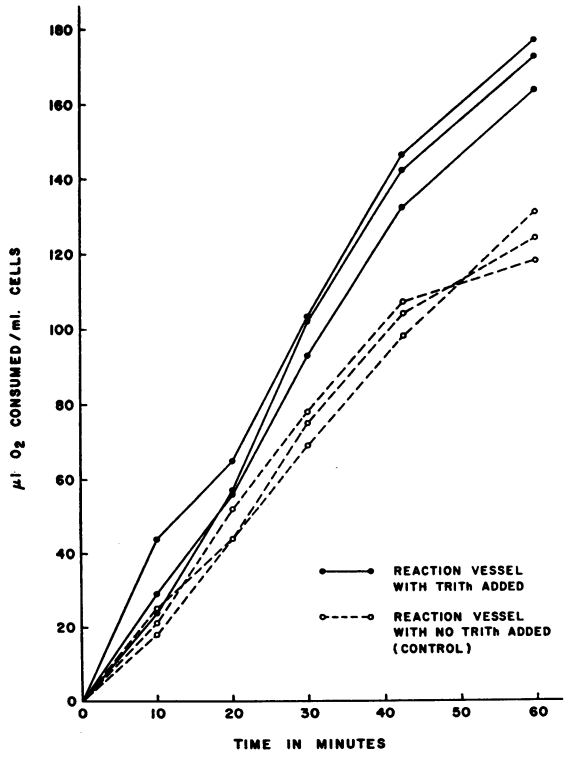

FIG. 2. THE EFFECT OF TRIIODOTHYRONINE (TRITH) ON THE OXYGEN CONSUMPTION OF HEMOLYSATES PREPARED FROM THE SAME BLOOD SAMPLE

TABLE II

The production of $C^{14}O_2$ by erythrocytes incubated with methylene blue

Experiment	Sample*	Oxygen consumption	Cpm from: Glucose-1-C^{14}	Cpm from: Glucose-6-C^{14}	Cpm added to flask
		µl./min./ml. erythrocytes			
9	Cells	0.24	3,573	135	1.9×10^5
	Cells, TRITh	0.32	3,240	117	
	Cells, MeB	1.80	95,044	285	
	Cells, MeB, TRITh	2.02	120,385	145	
14	Cells, MeB	3.14	1,129		1.3×10^4
	Cells, MeB, TRITh	3.44	1,315		
	Cells, MeB, DIT	3.15	1,125		
	Cells, MeB, DNT	3.06	1,120		
	Cells, MeB, KI	3.25	1,140		
15	Cells, MeB	1.93	1,880		4.7×10^4
	Cells, MeB, TRITh	2.58	2,493		
	Cells, MeB, DIT	2.25	1,908		
	Cells, MeB, DNT	2.02	1,661		
	Cells, MeB, KI	2.19	1,860		
16	Cells, MeB	2.00	28,908	135	4.7×10^5
	Cells, MeB, TRITh	2.21	32,320	199	
18	Cells, MeB	3.04	50,130		4.7×10^5
	Cells, MeB, TRITh	3.55	54,591		
	Cells, MeB, DIT	2.91	50,100		
	Cells, MeB, KI	3.28	50,073		

* Abbreviations are as follows: TRITh, 3,5,3′-triiodo-L-thyronine; MeB, methylene blue; DIT, 3,5-diiodo-L-tyrosine; DNT, 3,5-dinitro-L-tyrosine; KI, potassium iodide.

p value calculated by student's "t" test on the basis of per cent increase in counts per minute in paired individual flasks within each experiment.

	TRITh	DIT	KI
p =	0.003	>0.50	>0.50

mechanisms, perhaps replacing TPN (10, 11). To evaluate the importance of an intact electron transferring system in the mechanism of TRITh stimulation, the following experiment was carried

TABLE III

The effect of various analogues of TRITh upon oxygen consumption of erythrocytes incubated in methylene blue *

Experiment	No. of flask pairs	Oxygen consumption: Control	TRITh	DIT	KI
		µl./min./ml. cells			
10†	3	2.45	2.61	2.43	2.50
11	3	2.57	3.04	2.70	2.63
14	3	3.14	3.45	3.15	3.25
15	3	1.93	2.58	2.25	2.19
18	3	3.04	3.55	2.91	3.28
p			<0.001	>0.30	0.10

* Abbreviations and concentrations are as follows: TRITh, 3,5,3′-triiodo-L-thyronine, 3.75×10^{-8} M; DIT, 3,5-diiodo-L-tyrosine, 7.5×10^{-7} M; DNT, 3,5-dinitro-L-tyrosine, 7.5×10^{-7} M; KI, potassium iodide, 7.5×10^{-7} M.

† TRITh and other compounds were all tested at the same concentration, 3.75×10^{-8} M, in this experiment.

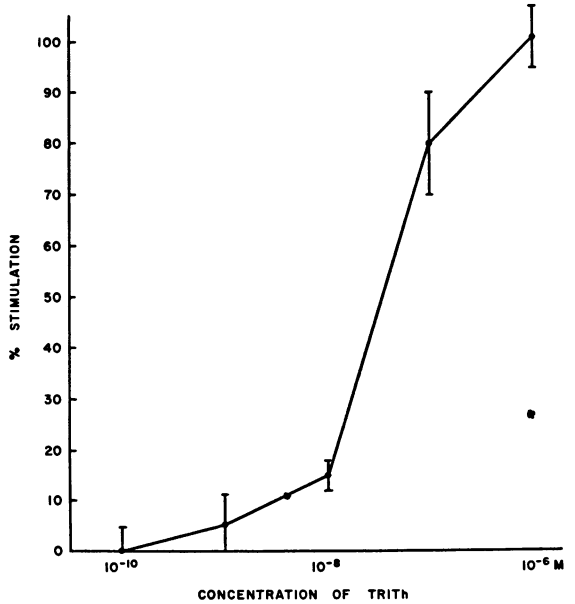

FIG. 3. STIMULATION OF OXYGEN CONSUMPTION *VS.* CONCENTRATION OF TRIIODOTHYRONINE (TRITH) IN REACTION MIXTURE

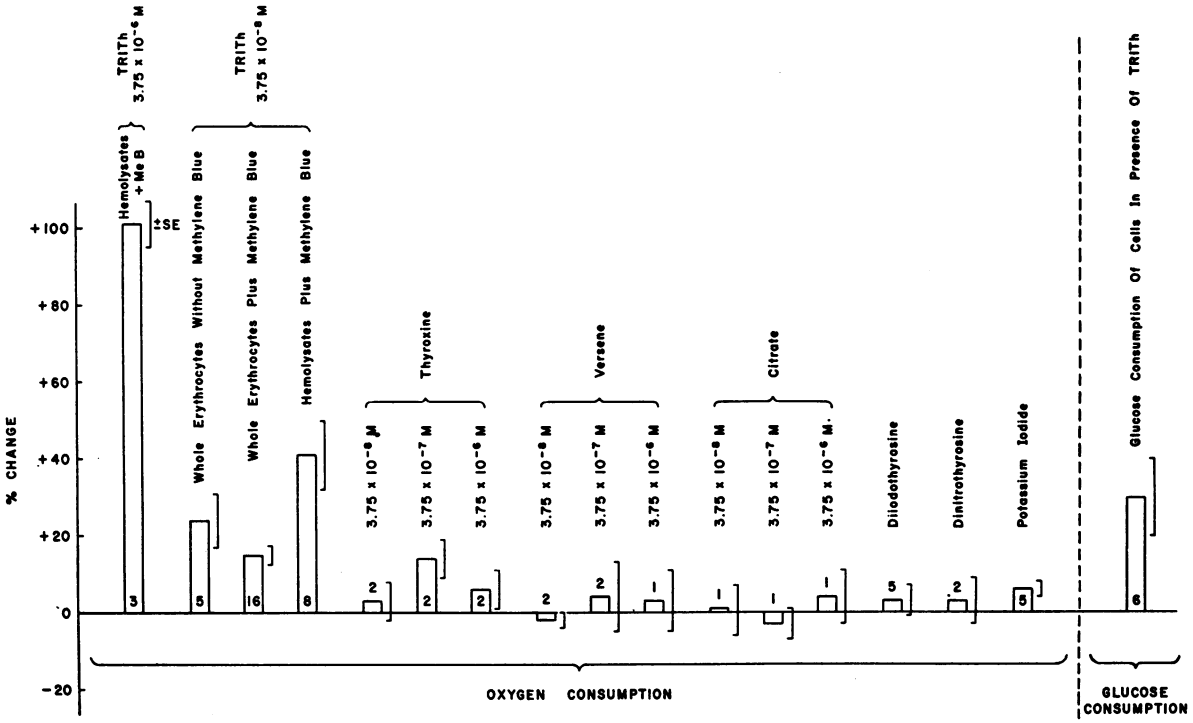

FIG. 4. PER CENT CHANGE IN OXYGEN AND GLUCOSE CONSUMPTION IN THE PRESENCE OF TRIIODOTHYRONINE (TRITH) AND ANALOGUES

Average of number of experiments indicated.

out. Erythrocytes were hemolyzed in the absence of nicotinamide. This promotes hydrolysis of TPN (8). Aliquots of the hemolysate were incubated with glucose-6-phosphate and methylene blue in Warburg reaction vessels. TRITh was added to one-half of the samples, and oxygen consumption was measured for 60 minutes. At the end of this period, TPN was tipped into the reaction mixture to give a final concentration of 2.5×10^{-7} M, and oxygen consumption was measured for an additional 30 minutes. Before the addition of TPN, no difference in oxygen consumption was observed (0.04 *vs.* 0.04 μl. oxygen per minute per ml. cells) in the presence or absence of TRITh. However, when TPN was introduced into the system, the rate of oxygen utilization returned to normal and stimulation of O_2 utilization was observed in the presence of TRITh (4.81 *vs.* 3.53 μl. oxygen per minute per ml. cells). The results are shown in Figure 5.

DISCUSSION

The mechanisms involved in the actions of the hormones upon specific target organs has long been a subject of speculation. Within the past several years, experimental evidence has been obtained regarding the possible mechanisms of action of several hormones. Among these are epinephrine (12), adrenocorticotropic hormone (ACTH) (13), and estradiol (14). The site of action of the thyroid hormone has not been elucidated, however. The studies reported here represent the first part of an investigation of the locus of action of this hormone.

Because of the relative simplicity of the mammalian erythrocyte, this cell was chosen in an attempt to localize some of the immediate metabolic effects of TRITh. This form of thyroid hormone was chosen because it acts more rapidly than thyroxine *in vivo*. The effects of thyroid hormone or its analogues has not been studied previously *in vitro* in the red blood cell. Erythrocytes have been found to have an increased rate of glycolysis when thyroxine was administered to experimental animals, but no attempt was made to investigate the mechanism of this action (15). In the present investigation, it has been shown that

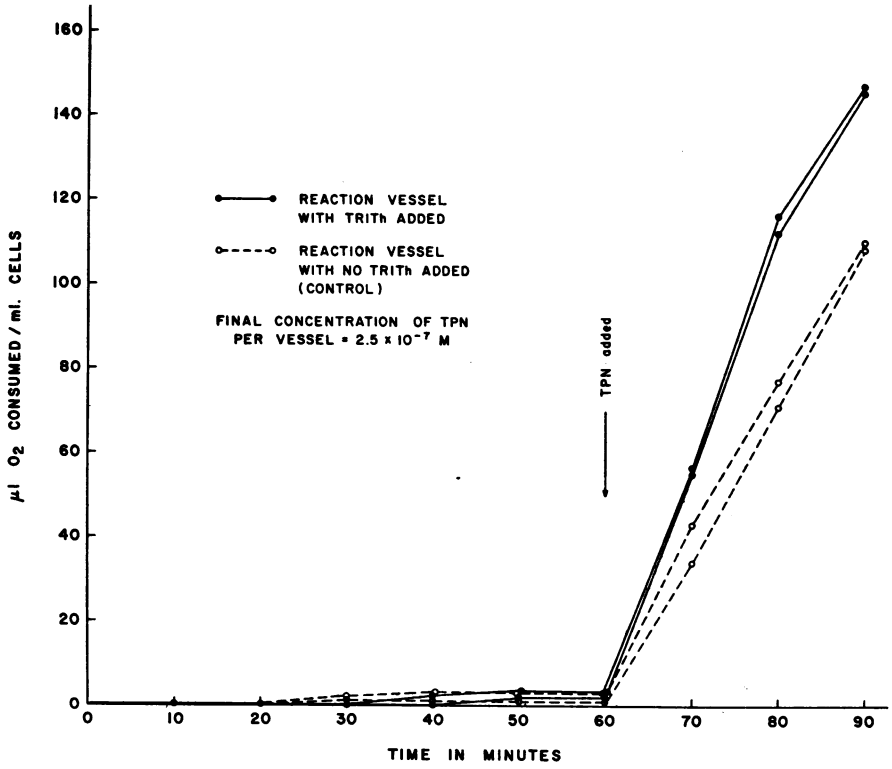

FIG. 5. STIMULATION OF OXYGEN CONSUMPTION OF HEMOLYZED ERYTHROCYTES BY TRIIODOTHYRONINE TRITH IN THE PRESENCE AND ABSENCE OF TRIPHOSPHOPYRIDINE NUCLEOTIDE (TPN)

TRITh stimulates the oxygen consumption of human erythrocytes *in vitro*.

Because so many of the mechanisms proposed to explain the action of thyroxine on the basis of *in vitro* experimental evidence have been shown to be the result of rather nonspecific action on various systems (16), several metabolically inert structural analogues of TRITh were tested. DIT and DNT were found to have little or no effect, both in terms of oxygen consumption and $C^{14}O_2$ production, even though they were present in concentrations 20 times that of TRITh. The same was found to be true for inorganic iodide and the chelating agents, Versene® and citrate. In addition, it was found that although thyroxine produced stimulation of oxygen consumption, concentrations required were much higher than those of TRITh, an observation which agrees well with the comparative *in vivo* action of these compounds.

Several alternative mechanisms have been suggested to explain how thyroxine increases oxygen consumption. These proposed mechanisms can be classified into two broad categories: *1*) those that postulate a direct or primary action on the permeability characteristics of one or more of the cellular and intracellular barriers, and *2*) those that postulate an action on one or more enzyme systems.

Several workers (17) have proposed that the specific action of thyroxine is to change the permeability characteristics of the mitochondria, producing swelling and thus changing the surface organization of these subcellular particles. However, it is immediately apparent that such a mechanism is not the operative one in the system that we have studied. Mitochondria have not been demonstrated to exist in mature erythrocytes, and TRITh produces the stimulation even after the removal of the physiologically intact membrane (by hemolysis). It seems more probable that, as suggested by the work of Lehninger and Ray (18), the swelling observed in mitochondria exposed to thyroxine *in vivo* or *in vitro* is directly related to the oxidation-reduction state of the mitochondrial electron carrier system. Thus, while the thyroid

hormone may well influence the permeability of membranes, this is apparently an indirect effect.

Direct action of the hormone upon individual enzymes or enzyme systems has often been suggested as an alternative possibility for the mode of action of the thyroid hormone at the subcellular level. Various enzymes have been found to be affected by the *in vivo* administration of thyroxine. Among these are intestinal glucokinase (19), acyl phosphatase (20), succinoxidase, glutamic dehydrogenase, cytochrome oxidase (21), DPNH-oxidase (22), steroid reductase (23, 24), glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase (25), TPNH-cytochrome C reductase (26), and hexokinase (17). Data obtained by such means are always difficult to interpret, since many indirect mechanisms may affect enzyme activity when thyroxine is administered to the animal. Although succinoxidase activity was found to be stimulated in mitochondrial preparations obtained from thyroxine-injected animals (27), no direct action of the hormone was found in submitochondrial preparations. Wolff, Wolff and Ball, on the other hand (28, 29), report inhibition of various dehydrogenases by thyroxine and various analogues *in vitro*. Although they point out that this mechanism could increase the oxygen consumption of tissues *in vivo* by diverting metabolites into primarily oxidative pathways, they question the actual operation of this mechanism under physiologic conditions.

In the intact organism and in most tissues, several routes for oxygen utilization are available. In the human erythrocyte, on the other hand, oxygen utilization occurs almost entirely through one pathway, the hexose monophosphate shunt. Metabolism through this pathway is enhanced in the presence of methylene blue. Since this is the only known pathway of oxygen consumption, we may presume that stimulation of oxygen consumption by TRITh is due to stimulation of metabolism in this pathway. The glucose consumption in the stimulated cells indicates that the increased metabolism via the hexose monophosphate pathway is not merely a secondary manifestation of inhibition of the glycolytic pathway. The finding that the increased CO_2 produced originated from the 1-carbon but not from the 6-carbon strongly supports the conclusion that stimulation of oxygen consumption occurred in the hexose monophosphate shunt. Other known mammalian oxidative pathways produce CO_2 from the 6- as well as the 1-carbon. Furthermore, the dissociation of phosphorylation and oxygen consumption observed in the intact animal is well explained by stimulation of the hexose monophosphate shunt since the oxidation of TPNH is not coupled to phosphorylation (30).

The steps through which substrate is thought to be oxidized in the hexose monophosphate pathway in the mature mammalian erythrocyte are illustrated in Figure 6. The studies reported here do not show what the exact mechanism of the stimulation of this pathway is. Our studies indicate that stimulation of oxygen consumption can be demonstrated only when TPN is present, but that it may be observed both in the presence and absence of methylene blue. These findings must be taken into account when further efforts are made to delineate clearly the site of action of the thyroid hormones in the human erythrocytes. It is hoped that further studies now in progress will provide an explanation of the action of thyroxine in this system on an enzymatic level.

It should be emphasized that the system used for these investigations is not typical of the majority of the cells affected by the thyroid hormone(s) and thus the mechanisms observed may not be general. More than one mechanism of action may exist in complex tissues. Using liver slices from thyrotoxic rats, Glock, McLean and Whitehead (31) have concluded, on the basis of rates of carbon dioxide production from specifically labeled glucose, that thyroxine enhances metabolism via the glycolytic pathway. However, samples were removed for counting only after a 90 minute interval. More recently Spiro and Ball (32), studying the rate of labeled carbon dioxide production in intact animals to which specifically labeled glucose had been administered, found an initial increase in the proportion of glucose carbon-1 oxidized to carbon dioxide. Within 30 minutes, however, the ratio of carbon-1 to carbon-6 in the expired carbon dioxide approached that found in the normal animal. From their data, these authors concluded that both the glycolytic and hexose monophosphate pathways were stimulated by thyroxine *in vivo*. As has been empha-

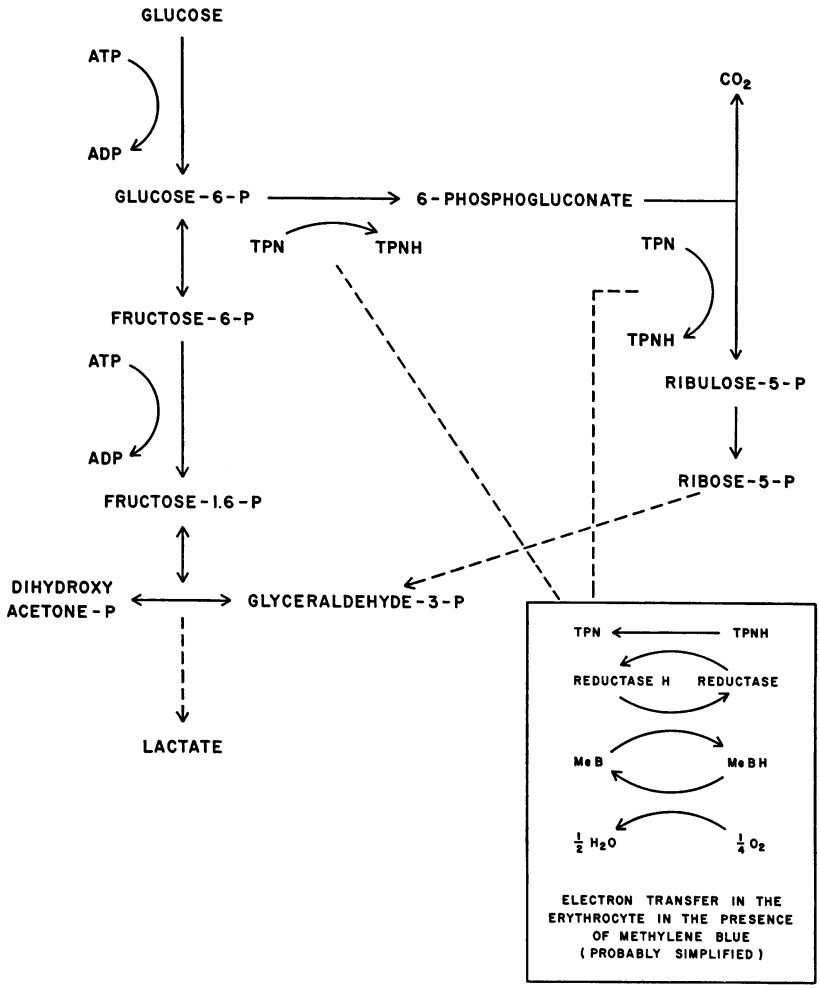

FIG. 6. SIMPLIFIED SCHEME OF CARBOHYDRATE METABOLISM IN THE HUMAN ERYTHROCYTE (1)

sized recently by Wood and Katz (33), however, after a short initial period randomization of the label occurs within the carbohydrate pools. In other words, once hexose has been decarboxylated to pentose, further metabolism of the pentose moiety takes place rapidly. Recycling is known to occur in the hexose monophosphate pathway and, in addition, further oxidation of triose fragments formed may occur by the Krebs' cycle reactions. Thus, over extended periods the appearance of carbon dioxide from specifically labeled glucose must be interpreted with caution. It is our opinion, therefore, that the data obtained by Spiro and Ball (32) would be entirely compatible with the interpretation that the primary locus of action of thyroid hormone in tissues other than the erythrocyte is in the hexose monophosphate pathway.

While it is certainly not permissible to extrapolate our findings in human erythrocytes to other cell types, these investigations may help to focus attention on the hexose monophosphate pathway as a possible locus of thyroid hormone action in other tissues.

SUMMARY

1. Significant stimulation of oxygen consumption of intact human erythrocytes incubated in the presence of glucose and methylene blue was observed upon the addition of physiologic amounts of triiodothyronine (TRITh), a more physiologically active analogue of thyroxine. The de-

gree of stimulation varied directly with the concentration of TRITh used.

2. Equal or greater stimulation of oxygen uptake was observed when hemolysates were incubated in the presence of methylene blue, glucose-6-phosphate, and TRITh, indicating that the observed effect was not due primarily to alterations in the transmembrane transfer of glucose or to changes in the rate of phosphorylation.

3. Glucose consumption was also increased when erythrocytes were incubated with TRITh.

4. This stimulation was reflected in an increased $C^{14}O_2$ production from glucose-1-C^{14} but not from glucose-6-C^{14}, suggesting that the increase in oxygen utilization was the result of stimulation of the hexose monophosphate shunt.

5. Stimulation observed with TRITh was not elicited by physiologically inactive analogues of the hormone including dinitrotyrosine, diiodotyrosine, and potassium iodide. Chelating agents, citrate and Versene®, also had no effect on oxygen consumption. Thyroxine showed some stimulatory effect when present in relatively high concentrations.

6. Triiodothyronine was not capable of replacing triphosphopyridine nucleotide (TPN) in the electron transferring system of the erythrocyte.

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