V) on metal toxicities is important for another reason also. From the present data it is difficult to say whether the effect of Mg in Co and Ni toxicities is primarily indicative of a Mg deficiency since both Fe and Mg can reverse these toxicities. However, it is possible that the effect of each of these 2 metals, *viz.*, Fe and Mg, is itself dependent on the physiological status of the organism with respect to the other metal. For instance, it has been found recently that in the mold *N. crassa*(5), wherein the level of Mg determines the concentration at which Co, Ni or Zn becomes toxic, reversal of these toxicities by Fe requires a very much higher amount of Fe on a low-Mg medium as compared to one wherein the Mg content is 10-fold higher.

It may be conjectured that excess Fe and Mg influences the intracellular accumulation of toxic metals by tissues. In this context, the demonstration by Abelson and Aldous(3) of such a control of metal ion uptake in certain organisms is of interest.

In any event, the present data indicate that the toxicity of Co, Ni and Zn is associated with their interference with the functioning or utilization of physiological active elements like Fe and Mg. It is likely that such derangements, in turn, bring about far reaching metabolic defects by affecting the rate of synthesis or degradation of vital metabolites. The complete reversal of Zn toxicity in *Corcyra* larvae by nucleic acids without any concomitant effect on accumulation of $Zn^{65}$(12) is indicative of such a possibility.

*Summary.* The range of toxicity of Co and Ni to *Corcyra* larvae has been determined. It has been shown that under conditions wherein excess Co, Ni and Zn produce severe inhibition of growth in these larvae, supplementation with Fe uniformly restores growth to normal in all 3 metal toxicities and that Mg is effective only in Co and Ni toxicities. The significance of these results in relation to metal interrelationships in *Corcyra* larvae has been discussed.

1. Hewitt, E. J., *Nature,* 1948, v161, 489.
2. Löhnis, M. P., *Plant and Soil,* 1960, v12, 339.
3. Abelson, P. H., Aldous, E., *J. Bacteriol.,* 1950, v60, 401.
4. Healy, W. B., Cheng, S., McElroy, W. D., *Arch. Biochem. and Biophys.,* 1955, v54, 206.
5. Sivarama Sastry, K., Adiga, P. R., Venkatasubramanyam, V., Sarma, P. S., 1960, Abst. 182, V$^{th}$ Intern. Congr. Nutr., Washington, D.C., p40.
6. Adiga, P. R., Sivarama Sastry, K., Venkatasubramanyam, V., Sarma, P. S., *Biochem. J.,* 1961, v81, 545.
7. Smith, S. E., Larson, E. J., *J. Biol. Chem.,* 1946, v163, 29.
8. van Reen, R., Pearson, P. B., *Arch. Biochem. and Biophys.,* 1953, v46, 337.
9. Cox, D. H., Harris, D. L., *J. Nutr.,* 1960, v70, 514.
10. Sarma, P. S., *Souvenir Soc. Biol. Chem. (India),* 1955, p49.
11. Adiga, P. R., Sarma, P. S., *J. Sci. and Ind. Res.,* 1961, v20A, 319.
12. Sivarama Sastry, K., Radhakrishnamurthy, R., Sarma, P. S., *Biochem. J.,* 1958, v69, 425.
13. Sivarama Sastry, K., Sarma, P. S., *Nature,* 1958, v182, 532.

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## Thyroid State and Glucose Oxidation by Blood.* (27134)

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The *in vitro* glucose oxidation rate of erythrocytes is changed by prior treatment of the animal with triiodothyronine and thyroxine(1,2). Oxidation is increased during the first 4 days of treatment with high doses of triiodothyronine, but continued treatment causes the oxidation rate to decrease until it reaches 62% of normal by the 21st day when thyrotoxic symptoms first appear(2). *In vitro* addition of physiological doses of triiodothyronine to solutions containing eryth-

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TABLE I. Percentage of Glucose-1-$C^{14}$ Released as $C^{14}O_2$ over a 3-Hour Period from 5 ml of Blood.

| | | 1 hr | 2 hr | 3 hr | Total |
|---|---|---|---|---|---|
| Normal subject | 1 | 8.77 | 18.85 | 12.76 | 40.38 |
| | 2 | 8.08 | 14.15 | 11.67 | 33.90 |
| | 3 | 13.62 | 18.73 | 10.48 | 42.83 |
| | 4 | 10.43 | 15.31 | 15.27 | 41.01 |
| Thyrotoxic | 1 | 4.81 | 4.94 | 16.07 | 25.82 |
| | 2 | 4.80 | 13.46 | 17.86 | 36.12 |
| | 3 | 4.56 | 10.78 | 11.33 | 26.67 |

rocytes and methylene blue will also cause a significant increase in oxygen consumption, glucose utilization and carbon dioxide production from glucose-1-$C^{14}$ but not from glucose-6-$C^{14}$(3). We have added glucose-1-$C^{14}$ and methylene blue to blood from normal and thyrotoxic patients to determine the effect thyroid hormone has on production of radioactive carbon dioxide(4). The radioactive carbon dioxide produced can be used to estimate hexosemonophosphate shunt (HMP) activity(4).

*Methods.* Five ml of heparinized blood was obtained from normal subjects and patients with the clinical syndrome of thyrotoxicosis. Diagnosis of thyrotoxicosis was by clinical examination, $I^{131}$ uptake, radioactive triiodothyronine serum binding test and protein bound iodine. Blood was obtained also from 3 normal individuals who received 100-200 $\mu$g of L-triiodothyronine daily for 4 to 7 days. This resulted in no demonstrable change in white blood cell count or differential.

After a 15-minute pre-incubation period in a Warburg flask 0.5 ml of a 1% solution methylene blue and 5 $\mu$c of 1-$C^{14}$ glucose (0.08 mg/$\mu$c) was added. Incubation was continued for 1-3 hours at 37°C in a continuous stream of 100% oxygen. The evolved carbon dioxide was collected by bubbling it into 10 ml of 0.5 M hyamine hydroxide. The radioactivity present in 1 ml of hyamine solution was determined by using a liquid scintillation detector. Results are expressed as percentage of injected $C^{14}$ appearing as radioactive carbon dioxide.

*Results.* During the first hour of incubation 10.1 $\pm$ 0.4% (mean $\pm$ standard error) of the $C^{14}$ from glucose-1-$C^{14}$ was collected as carbon dioxide from blood of 12 normal subjects and 2.9 $\pm$ 0.5 from 11 hyperthyroid individuals. There is a statistically significant difference in these 2 means, $P<.001$. No overlap was found between the 2 groups.

In Table I percentage of $C^{14}$ $O_2$ produced during 3 hours of incubation is shown. The marked inhibition of carbon dioxide production by thyrotoxic blood during the first hour of incubation is present to a lesser extent during the second hour of incubation but not seen during the third hour of incubation.

Table II shows percentage of carbon dioxide produced by blood from normal subjects who had received 100 $\mu$g of triiodothyronine daily. The depression of $C^{14}$ $O_2$ production which followed triiodothyronine treatment has disappeared 10 days after therapy. Subject 2 showed a continued decrease for at least 48 hours after stopping triiodothyronine.

*Discussion.* Using radioactive C-1-glucose Necheles and Beutler(3) found that production of radioactive $CO_2$ by washed human red cells could be increased by the acute addition of triiodothyronine ($3.75 \times 10^{-8}$M con-

TABLE II. Effect of 100 $\mu$g of Triiodothyronine Daily on $C^{14}$ $O_2$ Production from Blood Treated with 1-$C^{14}$ Glucose.

| | Normal subject | Days of therapy 0 | 1 | 2 | 3 | 4 | 5 | 10 | 10 days post therapy |
|---|---|---|---|---|---|---|---|---|---|
| 1st hr of incubation | 1 | 10.49 | 10.43 | 8.11 | 6.12 | 5.70 | 5.79 | — | 10.36 |
| | 2* | 10.35 | 13.62 | 8.30 | 5.84 | 6.53 | 8.45 | — | 11.35 |
| | 3 | 10.17 | 9.90 | 2.91 | 9.40 | 2.52 | 5.42 | 5.98 | — |
| 2nd hr of incubation | 1 | — | 15.31 | 10.49 | 15.32 | 11.83 | 11.53 | — | 11.09 |
| | 2* | — | 18.73 | 9.96 | 12.34 | 14.49 | 12.90 | — | — |
| | 3 | 13.10 | 15.56 | 11.25 | 13.25 | — | 13.78 | 12.60 | — |

* Subject 2 received triiodothyronine for only 3 days.

Results are percentage of added $C^{14}$ collected as carbon dioxide.

centration) suggesting stimulation of the HMP shunt. Others have shown that washed red cells taken from rabbits treated with 125 $\mu$g/kg of thyroxine per day show, during the first few days of treatment, increase in glucose utilization followed by a depression as treatment continues(2). The HMP shunt pathway of the intact rats as measured by the ratio of radioactive C-1 to C-6 glucose oxidation is depressed after chronic thyroxine administration(5). Our results with whole blood show a suppression of the HMP pathway in thyrotoxic humans and after acute administration of triiodothyronine. Combining these data suggests that administration of thyroid hormone may produce an early increase in the HMP shunt followed by marked depression. The disease of thyrotoxicosis is associated with a decrease in HMP activity.

Glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase, 2 of the earliest enzymes used in the HMP shunt, have been shown to be increased in thyrotoxic individuals and in experimentally produced hyperthyroidism(5,6). In erythrocytes, these enzymes increase within 48 hours of administration of thyroxine or triiodothyronine(6). An increase in these enzymes should be reflected by a comparable increase in oxidation of 1-$C^{14}$-glucose. Our data demonstrate a reduction in $CO_2$ production during the first hour of incubation of blood from thyrotoxic patients and from normal subjects treated with triiodothyronine. Glucose oxidation during the second hour of incubation is inconsistently depressed and no depression is noted during the third hour of incubation. This indicates that erythrocytes from thyrotoxic individuals are capable of oxidizing glucose normally.

The activity of glucose-6-phosphate dehydrogenase is dependent on the presence of triphosphopyridine nucleotide (TPN) and perhaps an activator substance(7,8,9). Glucose-6-phosphate dehydrogenase reduces TPN following which TPN is oxidized by the cytochrome oxidase system. This system is not present in mature erythrocytes. Addition of methylene blue to the incubation mixture is designed to perform this reduction and in this way stimulate HMP activity(4).

There are several possible explanations for decreased HMP activity of blood from thyrotoxic individuals even though it has been reported that the activity of glucose-6-phosphate dehydrogenase is increased in thyrotoxic erythrocytes. There may be a net decrease in TPN and TPNH; there may be a decrease in other HMP enzymes; or the added methylene blue may not be adequate for thyrotoxic blood. These data suggest that the increase in glucose-6-phosphate dehydrogenase activity found in thyrotoxic blood might be compensatory to a decrease in TPN or activator substance. TPN levels are known to be decreased in thyrotoxic liver(10) but similar studies have not been made in blood. It is unlikely that the changes noted here are due to inability of glucose to enter the cell, changes in the white blood cell count or the slight differences in blood sugar(11,12, 13).

*Summary.* 1. Oxidation of glucose by the hexose monophosphate shunt pathway has been estimated by measuring the radioactive carbon dioxide produced from 1-$C^{14}$-glucose added to blood treated with methylene blue. 2. A significant decrease in early $C^{14}$ $O_2$ production has been found in blood obtained from thyrotoxic patients and normal subjects treated with triiodothyronine.

1. Macho, L., *Clin. Chem. Acta,* 1957, v2, 345.

2. Calesnick, B., Altarelli, V. R., Spirtes, M. A., *Endocrinology,* 1960, v66, 517.

3. Necheles, T., Beutler, E., *J. Clin. Invest.,* 1959, v38, 788.

4. Brin, M., Yonemoto, R. H., *J. Biol. Chem.,* 1958, v230, 307.

5. Dow, D. S., Allen, C. E., *Canad. J., Biochem.* and Physiol., 1961, v39, 981.

6. Glock, G. E., McLean, P., *Biochem. J.,* 1955, v61, 390.

7. Pearson, H. A., Druyan, R., *J. Lab. and Clin. Med.,* 1961, v57, 343.

8. Rimon, A., Askenazi, I., Ramot, B., Sheba, C., *Biochem. Biophys. Res. Comm.,* 1960, v2, 138.

9. Marks, P. A., Szeinberg, A., Banks, J., *J. Biol. Chem.,* 1961, v236, 10.

10. Cahill, G. F., Hastings, A. B., Ashmore, J., Zattu, S., *Am. J. Physiol.,* 1958, v192, 491.

11. Glock, G. E., McLean, P., *Biochem. J.,* 1955, v61, 397.

12. Murphy, J. R., *J. Lab. and Clin. Med.*, 1960, v55, 781.

13. Ultmann, J. E., Hyman, G. A., Harvey, J. L., Dente, A. R., *J. Hemat.*, 1957, v12, 1114.

14. Buffa, F., Maraini, G., *Experientia*, 1958, v14, 283.

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# The Use of Fixed, Sensitized, Lyophilized Erythrocytes for the Middlebrook-Dubos Tuberculin Test.* (27135)

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The purpose of this paper is to describe the use of fixed sensitized sheep erythrocytes in the Middlebrook-Dubos hemagglutination test for tuberculosis. The classical test described by Middlebrook and Dubos(1), and the modified test by Scott and Smith(2), and others(3,4) involves the use of fresh unfixed erythrocytes. As such, the preparation for the individual tests is rather time-consuming. A diagnostic reagent which could be directly used would be of great advantage.

With a view toward development of a rapid, convenient screening test for toxoplasmosis, the author has previously described a stable preparation of alcohol-formalin fixed, Toxoplasma-sensitized lyophilized sheep erythrocytes(6). These antigen coated cells could be stored succesfully for a period of at least 2 months. Encouraged by these results the author has attempted to adapt this fixation technic to the modified Middlebrook and Dubos test.

*Material and methods.* 1. *Sheep erythrocytes.* Sheep blood collected aseptically in 1.2 volumes of modified Alsever's solution(1) was kept for at least 3 days before use, and was used thereafter for a period up to 2 weeks. Before fixation, the cells were washed 3 times with normal saline by serial centrifugation. When the fresh washed cells were used in the test, the final suspensions were centrifuged at 2,000 rpm for 10 minutes.

2. *Fixation of washed cells.* One volume of washed packed cells was suspended in 9 volumes of isotonic phosphate-buffered saline (pH 7.2). Ten volumes of the fixative described below were added to the sheep cell suspensions, and the mixture was left at room temperature overnight.

Alcohol-Formalin in Fixative Solution (pH 7.2)

| | | |
|---|---|---|
| 40% formaldehyde | 20 | cc |
| 95% ethyl alcohol | 5 | cc |
| Acid sodium phosphate, monohydrate | .4 | g |
| Anhydrous disodium phosphate | .64 | g |
| Distilled water | 75 | cc |

Details of the fixation and washing procedure have been described(6). It was imperative that all traces of formaldehyde be removed. The cells were not tanned.

3. *Preparation of antigen.* Old tuberculin (Lederle),‡ 4 times standard strength, was diluted 1:10 in buffered saline solution (pH 7.2); 0.5 ml of tuberculin being diluted in 4.5 ml of buffered saline. To this, 1.0 ml of a 10% fixed cell suspension in buffered saline was added and incubated for 2 hours in a water bath at 37°C. The mixture was thoroughly shaken at approximately 15-minute intervals. This cell suspension was removed from the water bath and centrifuged. The cells were washed 3 times with 6 ml volumes of buffered saline and finally resuspended in 50 ml of buffered saline (0.2% cell suspension).

For comparison, fresh, unfixed erythrocytes were sensitized by the technic of Mid-

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