ciency,[13] is still obscure. For example, Bellows and Chinn[14] indicate that intraocular hemorrhages were obtained in young rats raised on a diet low in choline and methionine while Zunz and Vessoelovsky[15] did not observe any change in the clotting time of rabbits injected intravenously with choline or its homologues. In unpublished studies, it was found that choline and methionine had no effect on the hypoprothrombinemia induced in rats by 3,3′-methylenebis (4-hydroxycoumarin) and gave inconclusive results in correcting the hypocoagulability (prothrombin and fibrinogen deficiency) induced by chloroform liver intoxication in dogs maintained on an adequate dietary regime.‡

Summary. 1. The hypoprothrombinemia induced in chicks by feeding a vitamin K-free diet was not significantly influenced by the addition of choline to the diet. 2. Chicks raised on a vitamin K-free diet exhibited the typical hypoprothrombinemia but their fibrinogen levels and total liver lipids resembled those of control chicks. 3. The total lipids of the livers of chicks receiving synthetic diets were somewhat increased over the lipids from chicks given a natural ration but this condition was not corrected by vitamin K.

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Inhibition of Cytochrome Oxidase (Paraphenylendiamine Oxidase) of the Thyroid Gland by Thiouracil and Other Compounds.

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It is believed that an oxidizing enzyme plays an important role in the formation of thyroid hormone. Inasmuch as thiourea, thiouracil, sulfonamides, and other compounds have been found to suppress formation of thyroid hormone[1,2,3] it appeared of interest to study the influence of these agents on oxidizing enzymes of the thyroid gland. The experiments reported here deal with the influence of thiouracil, sulfonamides, para-aminobenzoic acid, glutathione, cysteine, ascorbic acid, and certain xanthines on the cytochrome oxidase (paraphenylendiamine oxidase) in the thyroid gland.

Methods. The cytochrome oxidase activity of thyroid tissue was determined by a modification of the method of Galli-Mainini.[4] Rats were stunned by a blow on the neck and exsanguinated by opening the chest and cutting the heart. The thyroid glands were dissected free from connective tissue, rapidly weighed on a torsion balance, and immediately dropped into a test tube containing 5 cc phosphate buffer solution pH 7 and a small amount of sand. The tissue was triturated with a glass rod. Five cubic centimeters of a freshly prepared 0.2% aqueous paraphenylendiamine solution was added. The test tubes were then shaken in a mechanical shaker for 45 minutes at 37°C. The samples were immediately filtered into a colorimeter tube and read in the Evelyn photoelectric colorimeter against a blank treated in identical manner (blank =

* J. Ewing Mears Fellow in Physiology and Medicine.

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[2] Astwood, E. B., Sullivan, J., Bissel, A., and Tyslowitz, R., *Endocrinol.,* 1943, **32**, 210.

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TABLE I.
Inhibition of Cytochrome Oxidase Activity (Paraphenylendiamine Oxidase) of Rat Thyroid Gland *in Vitro*. One thyroid gland of each rat served as control.

Exp. No.	No. of rats	Inhibitor	Oxidase index (per mg thyroid) Controls M	Experimental M	Difference	P of Difference*
1	11	Thiouracil 0.002 M	35.9	16.9	19.0	<0.01 S
2	8	Glutathione 0.001 M	29.7	2.7	27.0	<0.01 S
3	8	Cysteine HCl 0.001 M	42.2	38.1	4.1	>0.9
4	4	Cysteine HCl 0.002 M	28.5	15.8	12.7	<0.01 S
5	8	Na Sulfadiazine 0.001 M Na Sulfathiazol 0.001 M	35.8	26.8	9.0	<0.01 S
6	8	Na Sulfadiazine 0.0005 M Na Sulfathiazol 0. 0005 M	33.3	21.5	11.8	<0.05 S
7	18	Na Sulfathiazol 0.001 M	23.1	17.7	5.4	<0.01 S
8	14	Na Sulfadiazine 0.001 M	21.0	17.9	3.1	<0.4
9	14	Paraaminobenzoic Acid 0.001-0.003 M	28.6	30.5	insignificant increase	<0.5
10	4	Ascorbic Acid 0.001 M	25.5	0	complete inhibition	
11	2	Paraxanthin 25 mg %	32.4	36.7	insignificant increase	<0.1

* P values calculated according to "Student's t-method," R. A. Fisher: *Statistical Methods for Research Workers*, 1932.
Significant values are marked in the table by "S."

TABLE II.
Oxidase Index of Thyroids of Rats Treated with Thiouracil for 11 to 21 Days, as Compared with Normal Controls.

Normal controls No. of animals	Normal controls Oxidase index* mean	Thiouracil treated No. of animals	Thiouracil treated Oxidase index* mean	Diff. of indices	P of Diff.†
55	28.9	25	21.6	7.3	<0.01 S
	Corr.‡ 49.8		Corr.‡ 22.9	26.9	

* Oxidase index calculated per mg thyroid weight.
† See footnote *, Table I.
‡ Index corrected to give oxidase activity per mg epithelial weight. See text.

100) using filter 635. An index of enzyme activity per mg thyroid was calculated as follows:

$$I = \frac{2 - \log G}{(\text{weight in mg})}$$

In one series of experiments the influence of thiouracil,† sodium sulfathiazole, sodium sulfadiazine, glutathione, cysteine hydrochloride, paraaminobenzoic acid, ascorbic acid, and xanthin‡ compounds was studied *in vitro*. These were dissolved in the buffer solution, prior to the addition of the thyroid tissue, in amounts to give final concentrations as indicated in Table I. Blanks were treated in the same manner. One thyroid gland of each animal was used with the test material, the other serving as a control. A blank containing no inhibitor was used for the control glands.

In a second group of experiments the oxidase activity of the thyroid glands of rats treated with thiouracil was compared with that of normal control rats. Adult male and female rats were used, maintained on a stock diet of Purina dog chow, with lettuce added twice weekly. The experimental animals received thiouracil in 0.05% solution as drinking solution for a period of 11-21 days.

† Thiouracil was supplied by the Lederle Laboratories through the courtesy of Dr. Stanton M. Hardy.

‡ Paraxanthin was kindly prepared for us by Dr. D. Turner, Jefferson Medical College, Philadelphia.

Results. Results of *in vitro* experiments are summarized in Table I. Thiouracil, sodium sulfathiazole and a mixture of sodium sulfathiazole and sodium sulfadiazine inhibited oxidase activity significantly. Ascorbic acid produced complete, and glutathione almost complete inhibition; in 0.001 M solution both were much more potent inhibitors *in vitro* than were the antithyroid drugs. It is interesting that cysteine hydrochloride, the reducing power of which is similar to that of glutathione, did not inhibit in equimolecular solution (0.001 M). However, it did inhibit in 0.002 M solution, its inhibiting action in this concentration being of the same magnitude as that of 0.002 M thiouracil solution.

Paraaminobenzoic acid and paraxanthin failed to inhibit oxidase activity in the concentrations employed. Not included in the table are several experiments with other xanthin derivatives, theophyllin and theobromine as well as a mixture of both, which also failed to inhibit the oxidase.

The *in vivo* experiments are summarized in Table II. Adult animals were used for controls and treated. Inasmuch as there was no apparent difference between males and females and the results obtained with treatment periods of 11-21 days were uniform, all values for this period were pooled. The cytochrome oxidase activity of the thyroids of the treated animals were significantly lower than that of the controls. Data on rats treated for shorter or considerably longer periods are not given in the table since these data are incomplete. It does appear, however, that after treatment for many months the oxidase activity of the thyroid glands increases, approaching normal. In these as in all experiments, the oxidase index of the thyroid was calculated per unit of weight. It may be assumed that the enzyme is present largely in the cells rather than the colloid. Therefore, comparison of the enzyme activity per weight unit of normal control glands rich in colloid, and with low epithelium with that of glands of thiouracil-treated animals, devoid of colloid and with hyperplastic epithelium may be misleading. Since in most experimental and control animals both glands had been used for the enzyme studies correction could not be made for each thyroid gland. However, representative sections of thyroids of normal and of thiouracil-treated rats were drawn on paper by means of a projector; colloid and interstitial tissue areas were cut out and the epithelial area weighed. The ratio of total area weight to epithelial area weight was used to calculate the oxidase index per mg epithelial weight instead of per mg thyroid weight. These figures, admittedly representing only a gross approximation, are presented in Table II as "corrected indices." This correction emphasizes the diminution in cytochrome oxidase activity in the treated glands, which was apparent even when calculated on the basis of weight of the entire gland.

Discussion. Of the compounds studied by us, thiouracil and sulfonamides are known to suppress formation of thyroid hormone[1,2] and to decrease the uptake of iodine by the thyroid gland of treated subjects.[5-9] Thiouracil[10] and sulfonamides[11] inhibit the formation of diiodotyrosin and thyroxin but not the uptake of inorganic iodine, by thyroid slices *in vitro*. The mechanism of formation of diiodotyrosin and thyroxin is not fully understood but there is evidence that an oxidative enzymatic mechanism is involved, possibly in the freeing of I from inorganic iodide.[12] Dempsey[13] showed that the histological peroxidase reaction of thyroid tissue can be inhibited by immersing sections in thiourea or thiouracil solutions. However, he found no difference between the histological peroxidase reaction in thyroids

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[13] Dempsey, E. W., *Endocrinol.*, 1944, **34**, 27.

from thiouracil-treated rats and normal controls. It has been suggested on theoretical grounds that the formation of thyroxin from diiodotyrosin may be dependent on peroxidase action.[14] However, Glock[15] has shown that the thyroid gland (of the horse) contains no peroxidase.

Polyphenol oxidase (tyrosinase) is inhibited *in vitro* by phenylthiourea,[16] sulfonamides,[17] and by thiouracil, and a number of other compounds[18] but since this enzyme is not present in thyroid tissue these observations merely indicate that thiourea and thiouracil inhibit various oxidizing enzymes but throw no light on the mechanism of inhibition of thyroid hormone formation. Schachner *et al.*[12] have found that *in vitro* formation of thyroxin and diiodotyrosin in thyroid tissue slices is inhibited by KCN, H_2S, CO, and NaN_3. Only two oxidizing enzymes are known to be inhibited by all of these inhibitors, *i.e.*, cytochrome oxidase and polyphenol oxidase. Because of the absence of polyphenol oxidase in thyroid tissue and because of the type of inhibition produced by CO in their experiments, these authors concluded that cytochrome oxidase is the oxidizing enzyme involved in the formation of thyroid hormone.

Our data, a preliminary report of which has appeared previously,[19] indicate that two groups of antithyroid drugs, thiouracil and sulfonamides, which are known to inhibit formation of thyroid hormone, actually inhibit the cytochrome oxidase (paraphenylendiamine oxidase) activity of the thyroid gland *in vitro*. The oxidase activity of thyroid glands of animals treated with thiouracil is likewise decreased. Since there is suggestive evidence that the cytochrome oxidase system is involved in the formation of thyroid hormone[12] and since we have found that this enzyme system is inhibited by thiouracil and sulfonamides, it appears possible that inhibition of this enzyme may constitute the basis for the antithyroid effect of these agents.

It should be noted that the experimental procedure employed by us reflects upon the entire cytochrome oxidase system, no attempt having been made to isolate either cytochrome or cytochrome oxidase, both of which are involved in the oxidation of paraphenylendiamine.

A number of compounds were studied in addition to thiouracil and sulfonamides. Of these, paraminobenzoic acid is of interest because Astwood[3] found that it induced moderate thyroid hyperplasis and because of recent claims[20] that it is useful therapeutically in thyrotoxicosis. The agent had no inhibitory effect on the cytochrome oxidase activity of the thyroid in concentrations employed by us, in striking contradistinction to its inhibitory effect on potato tyrosinase.[18] The effect of paraxanthin was investigated, with negative results, because of the report of Carter *et al.*[21] Glutathione, and ascorbic acid, and to a lesser extent cysteine hydrochloride, proved to be potent inhibitors *in vitro*. Whether this fact has any physiological significance is questionable. Astwood obtained no antithyroid effect in rats with ascorbic acid[3] whereas Heyl[22] reported morphological effects in guinea pigs similar to those subsequently obtained with antithyroid drugs. Our own observations[23] in guinea pigs have failed to show significant hyperplasia after administration of ascorbic acid.

Summary and Conclusions. 1. Thiouracil and sulfonamides inhibit the cytochrome oxidase activity (paraphenylendiamine oxidase) of thyroid tissue (rat) *in vitro*. 2. The cytochrome oxidase activity of thyroid glands of rats treated with thiouracil is subnormal. 3. Inhibition of cytochrome oxidase activity

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22 Heyl, J. G., *Acta brev. Neerland*, 1934, **4**, 12.

23 Paschkis, K. E., unpublished observations.

may possibly be the factor responsible for the antithyroid activity of these drugs. 4. Glutathione, cysteine hydrochloride, and ascorbic acid inhibit cytochrome oxidase activity (paraphenylendiamine oxidase) of thyroid tissue *in vitro*. 5. Paraminobenzoic acid and paraxanthin do not influence the cytochrome oxidase activity of the thyroid gland.

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Antibody Response of Swine to Repeated Vaccination with Formalin-Inactivated, Purified Swine Influenza Virus.*

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The results of studies on the relation of antibody response of swine to dose of purified swine influenza virus treated with formalin have been described in a recent report.[1] Within the range of dosage employed, 0.1 to 5.0 mg of formolized virus per 100 lb of animal weight, the relation of log antibody titer to log dose was essentially linear. In a single vaccination, a 10-fold difference in dose was associated with a 2.5-fold difference in titer. The maximum titer of serum taken at weekly intervals occurred in the serum obtained 1 week after vaccination; within 3 weeks after vaccination the average titers associated with all doses had declined to similar levels, which were about three 2-fold dilutions below the maximum average titer of the animals receiving the largest doses.

Because of the relatively small influence of the magnitude of the dose and the surprisingly rapid decline in titer, a second vaccination was made 6 weeks after the first. The relation of log antibody response to log dose was again apparently linear and a 10-fold difference in dose was associated with a 1.6-fold difference in titer. In contrast with the results of the first vaccination, however, the maximum titer, which occurred 7 to 9 days after the second vaccination, greatly exceeded that following the single vaccination. For comparable doses per unit weight, the antibody titer after the second vaccination rose to a level approximately three 2-fold dilutions above that seen after the first vaccination. Furthermore, the titers observed 6 weeks after the second vaccination, except those resulting from the smallest doses of vaccine, had not sunk to the level reached 3 weeks after the initial vaccination.

These and other results demonstrated that the degree of antibody response was far more dependent on the status of the host with respect to previous experience with the influenza virus or its derivatives than on the mass of formolized virus introduced at vaccination. In this respect it was observed that the maximum response to the dose of 0.125 mg per 100 lb at the second vaccination was approximately twice that to the dose of 2.0 mg given in the first vaccination. Inasmuch as the cost

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