quate to supply the necessary amount of essential fatty acid. The fatty acid patterns of the bile lipid fractions have recently been reported to reflect those of the dietary lipids (14). Our study, however, indicated that they more closely resembled the fatty acid patterns of plasma lipoproteins.

Summary. The effect of bile duct cannulation on the plasma lipoproteins was markedly influenced by the type and level of dietary fat and by the age of the birds. The low density lipoprotein level of cannulated chicks fed 1% fat decreased, while the level in cannulated chicks fed 10% fat increased; the greater increase occurred in the group fed corn oil. High density lipoprotein levels were unaffected. The increases in level of low density lipoproteins in the chicks fed 10% fat were followed by a stationary period and by a gradual decline as observed in cannulated adult birds.

The fatty acid composition of the lipoproteins also changed after cannulation. In all cannulated birds except those fed 10% corn oil the linoleic acid content of the low density lipoproteins decreased. Fatty acid composition of the high density lipoproteins showed closely parallel changes. The mixed fatty acid composition of the bile from cannulated chicks and that from corresponding serum lipoproteins of non-cannulated chicks were very similar.

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Growth Stimulating Effect of 4-Methyl-5(2-Hydroxyethyl) Thiazole on Thiamine-Deficient Rats.* (29580)

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It is well known that 4-methyl-5(2-hydroxyethyl) thiazole (MHET) can replace thiamine as a nutrient for some bacteria(1). Abderhalden(2,3) without success attempted to demonstrate that MHET could replace thiamine in the diet of the rat. Since then, MHET and the pyrimidine moiety of thiamine (2-methyl-4-amino-5-hydroxymethyl pyrimidine) have been isolated from urine of rats after giving large doses of thiamine(4,5), suggesting that the rat is capable of cleaving thiamine to give these two products. Also Van Eys(6) has identified MHET as a component of glycerol phosphate dehydrogenase, which indicates a strong possibility that MHET is biologically active. It, therefore, seemed desirable to investigate once again the possibility that MHET is biologically active. As workers in the past have apparently achieved essentially negative results(2,3,6,8)

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our experimental approach involved 3 special considerations.

1. From chemical considerations it seemed possible that MHET, if not carefully prepared, may not be biologically active. To prepare MHET the cleavage of thiamine by the Williams reaction(9) was selected as a mild and direct method. Moreover, the starting material, thiamine, contains a thiazolium moiety which has been shown to be biologically active(10,11).

2. If MHET is an intermediate metabolite derived from thiamine, then it should be active in comparable concentrations to that of thiamine. It is conceivable that higher concentrations may be harmful.

3. The method of administering MHET has to be considered. If it is an intermediate metabolite, then frequent administration of small amounts is desirable. This was accomplished by mixing the MHET in the food.

Experimental procedures. Preparation of MHET. MHET was prepared and purified by the bisulfite cleavage of thiamine as described by Williams *et al*(9). The detailed conditions were as follows: Ten mmoles of thiamine hydrochloride were dissolved in 30 ml of 2.6 M $NaHSO_3$. The temperature was raised to 50°C and the reaction allowed to proceed for one hour. The solution was quickly cooled to 5°C and the pyrimidine sulfone removed by filtration. The solution was adjusted to pH 10 and extracted 7 times with equal volumes of chloroform. The MHET was recovered from the $CHCl_3$ by extracting with water to which sufficient HCl or HCOOH had been added to give the acid salt of the compound.

Numerous tests were performed to ascertain the identity and purity of these preparations. The ultraviolet absorption spectrum and the molar absorbance were the same as described by Ruehle(12). This indicates that the extracted MHET was pure spectroscopically. The purity of the extracted MHET preparation was further ascertained by paper and column chromatography. Monophasic n-butanol/acetic acid/water (60:15:25) was a suitable solvent for separation of thiamine and MHET. One hundred μg of MHET were spotted on versene-washed Whatman No. 1 paper. The chromatogram was developed for 17 hours by the descending method. Under these conditions MHET and thiamine had R_f values of 0.83 and 0.36 respectively. Thiamine was detected by the thiochrome test as modified by Kraut and Wildemann(13). This test is sensitive to less than 1 μg of thiamine. No thiamine could be detected in 100 μg of MHET.

As a further check on purity, 2 ml of a 5% solution of the formate salt of the MHET were adjusted with NH_4OH to a pH of 5.5-6.5 and placed on a Dowex 1-8X-formate column (2 × 16 cm). The column was eluted with water at a flow rate of 8 ml per minute. The optical density of the eluate was monitored at 265 mμ in a Vanguard Automatic Ultraviolet Analyzer. Thiamine, if present, would appear almost immediately after the hold-up volume. A contamination of 0.1 mg of thiamine (0.1%) would give a readily observable peak. No thiamine was detected. After 75 to 80 ml of eluate had been collected, a very small peak, representing less than 0.1 mg of a pyrimidine or a thiazole, appeared. MHET was eluted after about 120 ml of eluate had been obtained. No other components could be detected. As a final test of identity, the MHET was crystallized by the method described by Ruehle(12). Spectrographic analysis proved the compound identical to that of Ruehle.

For animal experiments MHET was prepared as the uncrystallized formate salt and stored as a 1% solution at 5°C.

Methods for rat experiments. White male Sprague-Dawley rats, initially weighing 50-60 g, were placed in individual cages with wire-mesh screen bottoms. They were divided into groups of 4 animals each so that the average weight difference between the groups in a single experiment did not exceed 5 g. In preliminary experiments the rats were immediately fed the basal thiamine-deficient diet (Table I) to which the supplements, thiamine and MHET had been added. In later experiments the rats were fed rat pellets† until they had attained the weight of 80 g. The animals

† Purina Rat Chow.

TABLE I. Basal Thiamine Deficient Diet.

Ingredient	% of diet
Sucrose	70
Casein (vitamin-free)*	18
Alphacel	4
Salt mixture†	4
Corn oil‡	3
Choline chloride	.2
Vitamin mixture§	1
Sulfasuxidine	.5

* "Vitamin-free" casein, Nutritional Biochemicals Corp., Cleveland.
† Jones and Foster(14).
‡ Mazola, Corn Products Co., New York.
§ Composition of the vitamin mixture with the weight given as grams of the compound which were ground into 100 g of sucrose: riboflavin, 0.050; calcium pantothenate, 0.300; pyridoxine, 0.050; inositol, 0.300; nicotinic acid, 0.300; folic acid, 0.010; biotin, 0.010; menadione, 0.010.

were then fed the basal thiamine-deficient diet, supplemented with 0.25 μg thiamine/g diet, for one week before being fed experimental diets. Although the basal diet is suboptimal in the fat-soluble vitamins A, D and E, it is adequate for rats used in short term experiments (J. H. Jones, personal communication). Rats fed the basal diet, to which 1.25 μg thiamine/g diet had been added, maintained a steady growth rate throughout a 30-day experiment (Fig. 1, Curve 4) which was comparable to that of rats fed standard rat pellets.

The supplements, thiamine and MHET, were added directly to the food. The MHET was tested at concentrations ranging from 0.025 to 2.5 μg/g diet. Growth response to thiamine was tested at concentrations varying from no thiamine to 1.25 μg/g diet (Fig. 1). The minimum level of thiamine, 1.25 μg/g diet, needed to maintain continued growth agrees very well with that reported by Wostmann *et al*(15). The level of thiamine, 0.25 μg/g diet, usually incorporated into the diets was selected as one which would give a measurable response over the thiamine-deficient controls but was not sufficient to maintain growth. Sulfasuxidine was incorporated into all the diets to decrease the activity of the intestinal bacterial flora, thereby decreasing the danger of the bacteria utilizing the MHET before it could be absorbed.

All rats were furnished food and tap water *ad libitum*. They were weighed daily and results tabulated as the average weight gain for the 4 animals of a group.

Results. As shown in Fig. 1 and 2, increasing the concentration of thiamine in the diet of rats maintained on suboptimal levels of this vitamin results in both an increase in maximum weight gained and a prolongation of the time required to reach that maximum. Regardless of maximum weight gained, the rats in all thiamine-deficient groups then lost weight at a fairly constant rate with the result that the declining portions of the curves have similar slopes. Such observations are well-known in studies of vitamin deficiency.

Addition of MHET to the diet of thiamine-deficient rats results in an increase in maximum weight gained but, in contrast to the effect of thiamine, no prolongation of the time required to reach that maximum. In Fig. 2, the animals represented by Curve 1 were fed 0.005 μg thiamine/g diet. Curve 2 shows the effect of supplementing this diet with 0.25 μg MHET/g diet. Addition of MHET at this concentration caused a significantly greater weight gain than that attained by the control animals. The 4 rats in Curve 2 gained an average of 60 g as compared to an average gain of 45.5 g for the controls (Curve 1), or a 31.8% increase in weight. However, both groups of animals reached their maximum weight on the tenth day. A similar effect was obtained in the growth of rats fed a higher concentration of thiamine, 0.25 μg/g diet (Curves 3 and 4). The MHET-supplemented animals (Curve 4) gained an average of 69.5 g, 9 g or 14.4% more than their control group. Both groups of animals reached their maximum weight on the sixteenth day of the experiment. At the higher concentration of thiamine, the MHET-supplemented rats (Curve 4) lost weight much less rapidly than did their controls (Curve 3). It should be further noted that when rats were fed 0.25 μg/g diet of thiamine without MHET (Curve 3), an amount of thiamine equal to that of MHET fed to the rats in Curve 2, the weight gain in the two groups was similar. Since thiamine has

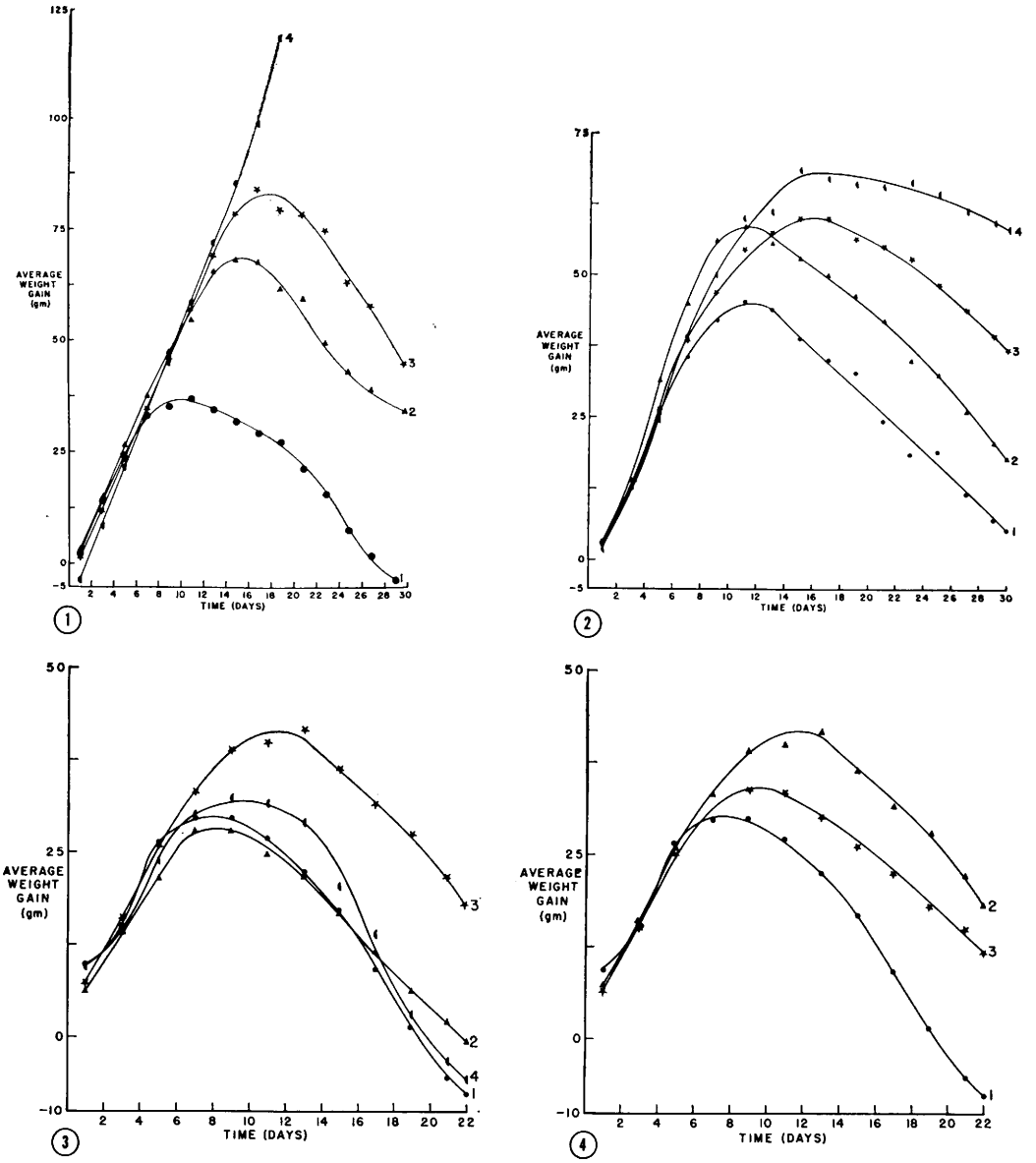

FIG. 1. Growth curves of rats showing effect of various concentrations of thiamine on growth rate. Points represent average weight gain of a group of 4 rats per day. Curve 1, thiamine-deprived; Curve 2, 0.36 μg thiamine/g diet; Curve 3, 0.5 μg thiamine/g diet; Curve 4, 1.25 μg thiamine/g diet. For brevity, Curve 4 terminated at 19 days, but growth rate remained constant for the 30-day experiment.

FIG. 2. Growth curves of rats showing stimulating effect of MHET at different concentrations of thiamine. Points represent average weight gain of a group of 4 animals per day. Curve 1, 0.005 μg thiamine/g diet (No MHET); Curve 2, 0.005 μg thiamine plus 0.25 μg MHET/g diet; Curve 3, 0.25 μg thiamine/g (No MHET); Curve 4, 0.25 μg thiamine plus 0.25 μg MHET/g diet.

FIG. 3. Growth curves of rats demonstrating effect of various concentrations of MHET on growth rate. Points represent average weight gain of a group of 4 animals per day. All groups were placed on the basal thiamine-deficient diet, containing 0.25 μg thiamine/g diet, for 8 days before being supplemented with MHET. Curve 1, thiamine-deficient controls; Curve 2, 0.025 μg MHET/g diet; Curve 3, 0.25 μg MHET/g diet; Curve 4, 2.5 μg MHET/g diet.

FIG. 4. Growth curves of rats demonstrating biological activity of MHET after column

about double the molecular weight of MHET, the activity of thiamine in producing a weight gain is greater than that of MHET. This experiment was performed twice additionally with essentially the same results; the time of maximum weight gain for Curves 1 and 2 was on the eleventh day, Curves 3 and 4 on the fourteenth day. In none of the 3 experiments did the day of maximum weight of the individual rats in Curves 1 and 2 overlap those in Curves 3 and 4. There was considerable difference in average weight of the animals but the effect of MHET on weight gain as compared to that of thiamine was within 10% of the relationship shown in Curves 2 and 3 of Fig. 2. In all instances the animals on the higher thiamine diet plus MHET lost weight more slowly than did their controls.

The effect of MHET administered at several concentrations to rats maintained on a thiamine-deficient diet is shown in Fig. 3. At this level of thiamine (0.25 μg/g diet), MHET stimulated the growth rate only at a concentration of 0.25 μg/g diet (Curve 3). Greater or lesser amounts of MHET (Curves 2 and 4) had little or no effect on growth rate of the animals. This experiment was performed 4 times. In all instances an optimal concentration of MHET was observed. Although concentrations of thiamine and MHET are the same, the growth response to MHET shown in Fig. 2, Curve 4, cannot be directly compared to that depicted in Fig. 3 and 4. In the latter 2 experiments, MHET was not added to the diet until the animals had been on the suboptimal thiamine diet (0.25 μg/g diet) for one week. The delayed addition of MHET always resulted in an extended time of maximum weight gain as compared to that of the control animals.

Discussion. In preparing MHET, certain modifications of Williams' procedure arise. In his paper Williams(9) did not take into account the effects of the variable experimental conditions on biological activity. We found that a more active preparation of MHET was obtained when conditions for the bisulfite cleavage of thiamine were 50°C for one hour rather than room temperature for 3 days. It was noted that MHET if highly concentrated or dried lost much of its activity. Our crystalline preparations had little or no activity. This might be due to the formation of thioethers which could not be utilized by the rats. The use of crystalline MHET by earlier workers might explain the essentially negative results they obtained in animal experiments.

The fact that addition of MHET to a suboptimal thiamine diet of rats resulted in an increased weight gain indicates that MHET is biologically active in the rat. It should be pointed out that the additional weight gain of the rats fed MHET was similar to those which received an equal amount of thiamine (Fig. 2, Curves 2 and 3). In low concentrations the MHET, therefore, had an activity comparable to thiamine. The possibility of thiamine contamination in the MHET preparations had to be considered very seriously. Repeated chromatography of various preparations indicated a purity greater than 99%. Fig. 4 illustrates that MHET still retains appreciable activity after purification on a resin column. The loss of activity is attributed to the fact that the MHET eluate from the column was flash-evaporated. There was no contamination of thiamine in the MHET preparation greater than 0.1% (the limit of detection). It is obvious, from data provided, that any contamination of 0.00025 μg thiamine/g diet is far below the amount required to show any effect on the growth of rats. Inasmuch as the absorption spectrum, molecular absorbance, and chromatographic partitions (both paper and ion resins) of the MHET agreed quantitatively with known values as well as the fact that its degree of activity is similar to thiamine indicates that the activity observed must be attributed to MHET.

There have been several attempts to show the synthesis of thiamine by the rat from

chromatography. Points represent average weight gain of a group of 4 rats per day. Animals were fed the basal thiamine-deficient diet, containing 0.25 μg thiamine/g diet, for 8 days prior to addition of MHET at a concentration of 0.25 μg/g diet. Curve 1, thiamine-deficient controls; Curve 2, MHET before chromatography; Curve 3, MHET following chromatography.

MHET and the pyrimidine moiety(2,3,7). The general conclusion was that the rat either cannot synthesize thiamine or, if so, only to a limited degree. Thiamine synthesized by the intestinal bacterial flora appears to be unavailable, except upon recirculation (8). Coprophagy does not appear to account for the growth response to MHET of rats on the thiamine-deficient diet. To obtain the growth response observed in our experiments, one must assume an overall efficiency of at least 60% in conversion by the intestinal flora of the fed MHET into a thiamine totally available to the rat upon recirculation. According to Barnes *et al*(16), the percentage of fecal thiamine available to the rat is remarkably low, about 35% of the total. Furthermore, if the growth response were due to bacterial conversion of MHET to thiamine, one would expect a 10-fold increase (to 2.5 μg/g diet) in the low levels of MHET fed to result in an even greater stimulation of growth rather than in the apparent toxicity which was observed (Fig. 3). If MHET were converted to thiamine by any means then it would be expected to mimic the action of thiamine in causing an increased time of growth. When MHET was incorporated initially into the diet the time of optimum weight gain was the same as the controls. This is contrary to the action of thiamine (Fig. 2, Curves 1, 2, 3, 4). In none of the experiments did the time of maximum weight gain of the individual rats, represented by Curves 1 and 2, overlap the time of those of Curves 3 and 4. There is another significant difference between MHET and thiamine. If MHET is added to the diet together with a small amount of thiamine, the rate of weight loss is less than that of an equivalent amount of thiamine. (Compare Fig. 2, Curves 3 and 4, with Fig. 1, Curve 3.) In Fig. 2, Curve 4, the rats received 0.25 μg MHET and 0.25 μg of thiamine. These rats lost weight less rapidly than those receiving 0.5 μg thiamine in Fig. 1, Curve 3. Since MHET does not act in the same manner as thiamine in these two instances, it does not seem likely that its action could be attributed to a possible conversion into thiamine.

As thiamine concentration in the diet increases, the effect of MHET decreases (Fig. 2). A 0.25 μg/g diet of MHET produced an additional weight gain of 14.5 g (31.8% increase) with no dietary thiamine, 8.8 g gain (14.4% increase) with 0.25 μg/g of thiamine in diet, and 2.5 g (3% increase) with 0.5 μg/g of thiamine in diet. Since this last weight gain is insignificant, it is not depicted in Fig. 2. In effect, thiamine in the diet abolishes the action of MHET. This indicates that dietary thiamine could be a source of MHET.

If, as our data suggest, MHET is not converted into thiamine, a possible explanation for the action of MHET is that in some manner it prevents the loss of thiamine to the animal or enables the animal better to utilize its limited supply of thiamine. Either action would effectively increase the thiamine available to the rat and hence it would seem that again the action of MHET should be to mimic thiamine. Since it does not, this does not appear to be a likely explanation.

Another possibility is that both MHET and thiamine can participate about equally well in some metabolic action. However, there is considerable difference between these two compounds. In thiamine the thiazole moiety is actually a thiazolium, the nitrogen being quaternary. In MHET the nitrogen is tertiary. A third possibility is that both thiamine and MHET are converted into the active compound. Since MHET was "biologically active" only at very low levels, these last two possibilities provide feasible explanations but the answer must await the results of enzymatic studies.

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Incorporation of Arsenic into Cock Sperm.* (29581)

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Arsenicals have been shown to stimulate growth and improve feed efficiency in broilers and to improve laying hen performance. Although Pope and Schaible(1) reported that arsanilic acid exerts a sparing action on the protein requirement of breeding hens and layers when egg production was the criterion, small amounts did not improve hatchability or fertility in breeding chickens. Excess arsenic has been shown to be toxic to chickens. The position of arsenic in the periodic table would suggest that it has some chemical properties which are similar to phosphorus, and therefore, could replace phosphorus in some compounds. Since $P^{32}O_4$ or phosphate-P^{32} is readily incorporated into the deoxyribonucleic acid (DNA) of cock sperm in amounts allowing its detection(2) it was decided to use radioactive Arsenic (As^{76}) and also stable arsenic in mineralization studies with cock sperm to determine if arsenic was incorporated into the DNA of sperm.

Experimental. Ten sexually active New Hampshire cocks selected from a group maintained for artificial insemination were each given 200 μc of radioactive $Arsenic^{76}$ as As_2O_3 subcutaneously. The semen was collected 26 hours after radionuclide administration and each 26 hours thereafter for 4 collections. Twenty-six hours was used as the collection time because it was estimated that this period plus the time required to fractionate the semen would be equivalent to one half-life of $Arsenic^{76}$ (about 26.8 hours). The radioactivity of the semen, sperm, seminal fluid, protein (TCA precipitate) and DNA fraction was determined. The DNA fraction was prepared as outlined by Borenfreund, Fitt and Bendich(3). The results are shown in Table I and demonstrate that $Arsenic^{76}$ was readily incorporated into cock sperm. Most of the $Arsenic^{76}$ was found in the diluent (seminal fluid) and fractionation of the sperm showed that on the 52 hour period the sperm contained the greatest concentration of $Arsenic^{76}$. The DNA fraction had its greatest concentration on the 78 hour period and protein fraction on the 52 hour period.

Since these data indicated that $Arsenic^{76}$ was in the DNA fraction of sperm, a further study was designed to study the incorporation of stable arsenic into the DNA of sperm. Twenty-four cocks were placed on a breeding ration containing 500 g of arsanilic acid per

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