TABLE III. Reticulocyte Response to B_{12}. (Reticulocytes/100 red cells.)

Days after B_{12}	Basal		Basal + dimethylaminoethanol	
0	1.5	2.8	2.0	1.9
4	2.9	5.5	4.8	4.4
6	6.4	8.5	5.8	5.0
8	9.9	10.5	7.8	10.0
10	8.4	12.1	5.2	8.7
13	6.1	6.5	3.3	3.8

Summary. The effect of a B_{12} deficiency on transmethylation from methionine to choline has been studied. The deficiency did not influence the level of choline in the liver nor the excretion of urinary choline. Histological examination of livers and kidneys did not reveal the fatty infiltration or renal damage characteristic of a choline deficiency. From this study it appears that vit. B_{12} is not involved in this direct transmethylation.

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Histochemical Changes in Succinic Dehydrogenase Activity in Rat Kidney Following Administration of Mercurial Diuretics.* (20864)

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It is a well known fact that the sulfhydryl-containing enzymes are inhibited by compounds of heavy metals such as mercury(1). Consequently, succinic dehydrogenase activity has been shown to be inhibited by mercurial diuretic agents *in vitro* in the heart (2) and *in vivo* in the kidney(3). Such enzymatic inhibition is concomitant with the diuretic action of mercurials and may be related to the depression of tubular reabsorption, since both enzymatic inhibition and diuresis can be prevented by the administration of 2,3 dimercaptopropanol (BAL)(4).

It is generally assumed that mercurial diuretics act on some specific portion of the kidney tubule, although there is no satisfactory physiological evidence on this point (5). Recently, Mustakallio and Telkkä(6) using a histochemical method, reported that the "distal segment" of the tubule is inhibited by mercuhydrin. Since pathological and other evidence is not in agreement with their conclusions, we restudied the problem by an improved histochemical method and, in addition, investigated the activity of BAL, cysteine, and glutathione in suppressing the mercurial inhibition.

Methods. Nearly all of our work has been done on young female rats (175-200 g) of the Holtzman strain although kidneys of a few adult males and immature animals of this strain also have been studied. The animals were killed by decapitation, a kidney removed immediately, and sectioned at 50 μ on the freezing microtome. Sections were placed in physiological saline solution and within a period of 30 minutes transferred to small,

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covered stender dishes containing 20 ml of the following incubation medium: 0.075 M sodium pyrophosphate buffer (pH 8.2) containing 0.1% neotetrazolium chloride and 0.15 M sodium succinate. The tetrazolium and succinate were added to the buffer shortly before use and the freshly prepared medium placed in an incubator. All sections were incubated at 37°C for 45 minutes and then transferred to distilled water. After brief rinsing, they were placed in 10% formalin overnight, washed in water and mounted in glycerine jelly. For comparative purposes we have used also the method described by Seligman and Rutenburg(7). Altogether, over 100 rats have been used in these studies. Kidneys from 10 untreated animals formed the control series while the remaining animals were given injections of either mercuhydrin alone (2-160 mg Hg/kg) or mercuhydrin and one of the following mercurial antagonists: BAL, l-cysteine, or glutathione. The injections were given subcutaneously, except in a very few cases in which the mercuhydrin was given intraperitoneally. BAL (in peanut oil) was administered in a molar ratio of 1:1 of Hg, l-cysteine in molar ratios of 1:1 and 50:1, and glutathione in molar ratios of 1:1 and 10:1. Most of the treated animals have been killed 48 hours after the injection of mercuhydrin since early work indicated that the effect of mercuhydrin was a marked and highly predictable one after this period of time. In addition, the activity of mercuhydrin alone has been studied at the following intervals after its administration: 2, 3, 6, and 24 hours and 2, 3, 4, 6, 7, 10, 11, and 17 days. Finally in a few experiments the *in vitro* effect of mercuhydrin and the mercurial antagonists on sections of normal kidneys was studied.

Histochemical method. We have used neotetrazolium chloride in preference to any of the other tetrazolium salts since its superiority in histochemistry was thoroughly demonstrated by Shelton and Schneider(8). The concentration of pyrophosphate used in this method with *intact cells* does not inhibit the succinoxidase. The method will work equally well, moreover, with 0.1 M phosphate buffer or without any buffer, provided the pH of the medium is properly adjusted prior to incubation. We have been able to show that the effects of mercuhydrin treatment were the same, regardless of whether a phosphate or a pyrophosphate-buffered medium was used. Following the addition of the neotetrazolium and sodium succinate, the pH of the pyrophosphate-buffered medium is approximately 7.3.

Our results with the original method of Seligman and Rutenburg(7) were technically so poor, that we were unable, by their method, to localize the site of mercurial action on the kidney tubule. We used 50 μ sections, as this thickness withstands the necessary manipulations without undue folding, matting, or fragmenting. In such preparations the topographical relationships can be visualized readily and intact tubules can be followed for considerable distances. Sections much thicker than 50 μ are too dense for microscopic study and with sections as thick as 100 μ the method is no longer specific since some coloration occurs in the absence of added substrate. The specificity of the method we have used is shown by the fact that no color develops when succinate is omitted from the medium. Also, we failed to obtain any coloration when sodium malate was substituted for succinate as substrate. Finally, the reaction with added succinate can be inhibited by sodium malonate.

Normal distribution of enzymatic activity. When sections of normal rat kidneys are placed in the described incubation medium, coloration begins in a few minutes and increases progressively for about 30 minutes. The color is pink to purplish, depending on the amount of enzymatic activity, and it results from the intracellular deposits of formazan in the kidney tubules. The glomeruli are essentially uncolored, as are also the collecting ducts. The medulla exhibits three zones: a) an outer, lightly stained area through which pass the descending limbs of Henle's loops, b) an intermediate zone composed of heavily stained parallel limbs of Henle's loops which alternate with radiating unstained columns of collecting ducts and blood capillaries, and c) an inner, essentially unstained, zone which occupies approximately the inner one-half of the medullary area; this is composed of col-

lecting ducts and feebly stained thin segments of Henle's loops (Fig. 1). Our explanation of the sharp boundary which is present between the intermediate and inner zones is not in accord with other interpretations in the recent literature and therefore requires further comment. Mustakallio and Telkkä(6) considered that at this sharp boundary "the dark blue loops of Henle bent to reenter the kidney cortex." In explaining the same phenomenon, Padykula(9) states "the heavily stained mid-region of the medulla contains both loops of Henle and collecting ducts. The staining reaction in the collecting ducts terminates abruptly, forming the innermost mass of pyramid of very faintly stained collecting ducts". We find that neither interpretation is correct—that the sharp boundary results instead from the abrupt transition from thin segments to darkly stained, thick (ascending) limbs of Henle's loops. It is this abrupt transition in the tubules of the juxtamedullary generation of glomeruli which produces the striking zonal boundary as seen in Fig. 5. Padykula's explanation implies that the collecting ducts are stained in the intermediate "mid-region" of the medulla, which is not the case. In our Fig. 1 and 3, unstained columns of collecting ducts are especially well shown in this zone, alternating as they do with areas of heavily stained loops of Henle. The same is clearly illustrated in Fig. 2 of a recent paper by Malaty and Bourne(10). Even in Padykula's own figures (1-4) the collecting ducts in the "mid-region" of the medulla are obviously unstained, although since she presents transverse sections of the kidney, the radiating columns of collecting ducts appear as unstained circular or oval areas. The explanation offered by Mustakallio and Telkkä(6) ignores the presence of any portion of Henle's loops in approximately the inner one-half of the medulla. Since loops can be seen by classical methods in the inner medulla, their explanation seems untenable. Furthermore, during the course of our studies, evidence was obtained from the kidneys of younger animals which clearly shows that the loops of Henle descend deeper into the pyramid than the conspicuous boundary referred to. Thus, in rats younger than 10 days of age, the kidney tubules are relatively undifferentiated and give a uniform coloration in their course through the medulla (Fig. 6). Such loops can readily be observed to approach very near to the apex of the papilla and it is their progressive differentiation during the next 10 days of life which results in the zonal pattern previously described. The convoluted tubules in the cortex are darkly colored in the kidneys of normal animals. This holds for both the proximal and distal convolutions, although the distal tubule is normally somewhat darker than the proximal. Very little variation in the pattern or intensity of coloration in the kidneys of the control series of animals has been observed. As already mentioned, this is in contrast to the extremely inconsistent results we have obtained on a limited number of kidneys using the original method of Seligman and Rutenburg(7).

Mercurial inhibition. Within 2-3 hours following a single, subcutaneous injection of 40 mg Hg/kg of mercuhydrin there is a marked inhibition of the succinic dehydrogenase activity in the *proximal* convoluted tubules. After incubation, the cortex in such sections appears pale on gross examination, and on microscopic examination shows the distal convoluted tubules intensely colored; whereas, the proximal convoluted tubules are now only feebly colored. Even when our highest dose was used (160 mg Hg/kg) neither the complete inhibition of activity, nor a marked inhibition of activity in the thick limb of Henle's loops, as reported by Mustakallio and Telkkä, could be observed. We believe that these differences are due to the inadequacy of their method to demonstrate experimentally induced changes in enzymatic activity. The maximal inhibition of activity observed by us occurred at 24-48 hours following the injection of mercuhydrin (Fig. 2). At this time tubular damage is already apparent, whereas extensive tubular necrosis and cystic degeneration generally result within 3-7 days after the administration of 20 mg Hg/kg (Fig. 4). Higher dosage levels are invariably lethal in the first week after injection, and even a dose of 10 mg Hg/kg is sufficient to cause extensive tubular damage together with marked enzymatic inhibition for at least a week following

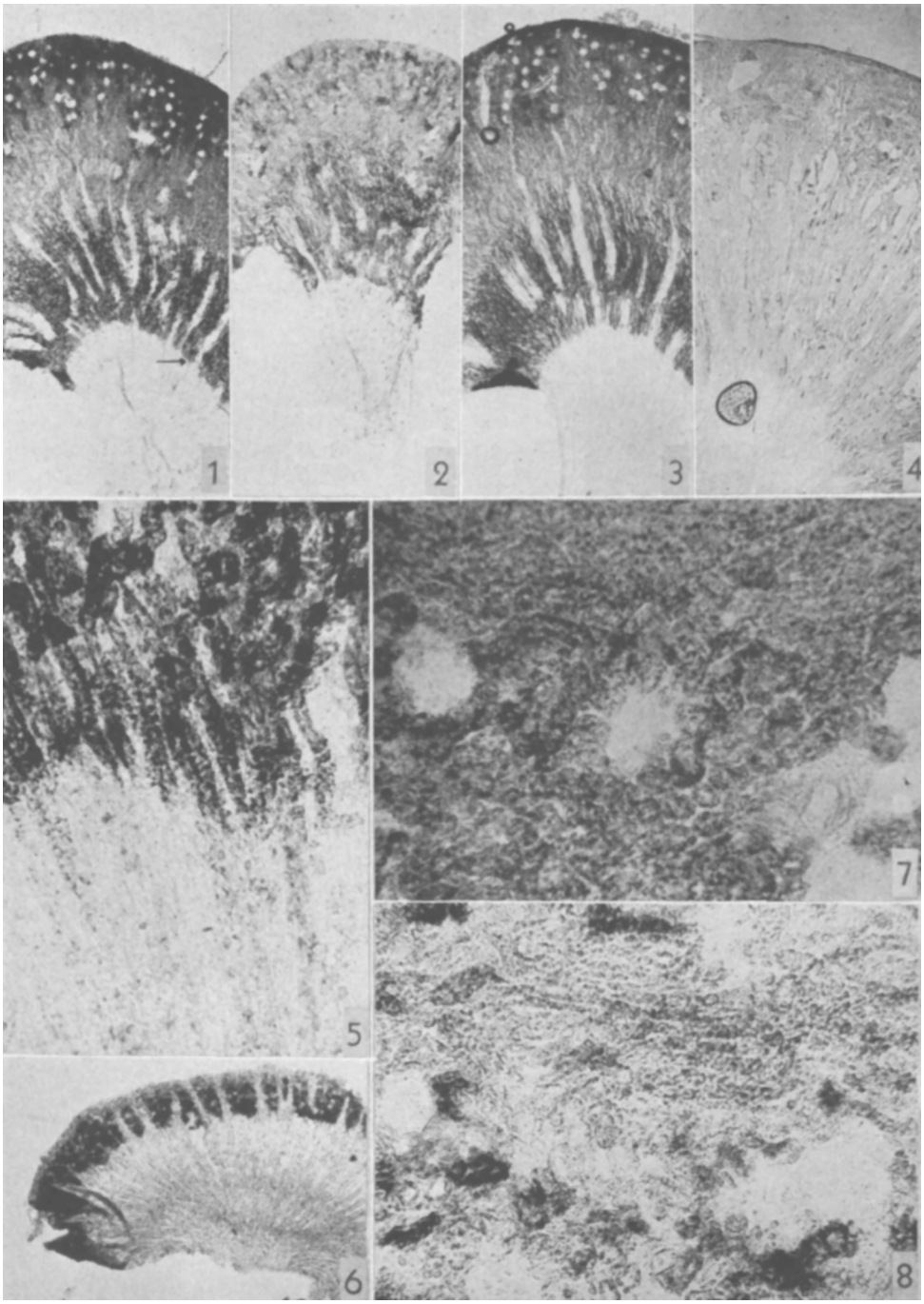

FIG. 1. Section of normal rat kidney showing sites of succinic dehydrogenase activity. Arrow indicates prominent boundary discussed in text. ×9.

FIG. 2. Section of kidney removed 48 hr after subcutaneous injection of 20 mg Hg/kg mercuhydrin. ×9.

FIG. 3. Section of kidney after treatment as described for Fig. 2 except that an equimolar amount of BAL was given simultaneously. ×9.

FIG. 4. Section of kidney removed 7 days after subcutaneous injection of 20 mg Hg/kg of mercuhydrin. (Black circle in lower left is an artifact due to air bubble in preparation.) ×9.

FIG. 5. Higher magnification of area indicated by arrow in Fig. 1. This is the area of abrupt transition from thin segments to thick limbs. ×100.

FIG. 6. Section of 6-day-old normal rat kidney showing undifferentiated tubules. ×12.

FIG. 7. Higher magnification of small area of cortex shown in Fig. 1. ×100.

FIG. 8. Higher magnification of small area of cortex shown in Fig. 2. Reaction is almost completely inhibited in proximal convoluted tubules. ×100.

the injection. We cannot, therefore, agree with Mustakallio and Telkkä in their findings that "The changes after doses of 10-40 mg Hg/kg were reversible within 3 days." We do believe however, that it is unlikely that the very early enzymatic inhibition is due to cellular damage *per se*. It seems more probable that cell injury and necrosis may result from the reduced metabolic capacity of cells which have their sulfhydryl enzymes inactivated. Once tubular necrosis is pronounced, the almost total lack of enzymatic activity must be considered as a secondary result of the cellular damage. Further attempts to correlate the degree of enzymatic inhibition with the histopathology of the kidney after the administration of mercurial agents are now under study.

Up to now we have been unable to demonstrate a marked inhibition of succinic dehydrogenase activity in the kidney with a dose lower than 10 mg Hg/kg. Even with this dosage the effect is not pronounced until some 24-48 hours after the injection. Since larger doses will produce the same effect in a shorter period of time, it seems likely that the effect is dependent on the selective concentration of the mercury in the cells of the proximal convoluted tubules. Virtually the entire amount of mercury contained in a single dose of diuretic is excreted in the urine within 24 to 36 hours(11). This explains the inhibition of succinic dehydrogenase activity *in vivo* only in the kidneys, but not in the heart or liver(3). Actually, there is some reason to believe that mercury is removed from the blood by tubular excretion(12) and if this is the case, one might expect that its passage through the tubular epithelium would result in a selective enzymatic inhibition in that portion of the tubule through which it passes. Pathologists have known for some time that the proximal convoluted tubule is the site of principal damage in cases of mercury poisoning and in experimentally induced mercury damage(13). It is not surprising therefore, to find that this is also the site of maximal enzymatic inhibition. We believe that this observation is of functional significance, inasmuch as we have found that the addition of mercuhydrin (1×10^{-5} M) to the incubation medium results in the total inhibition of the coloration of sections of normal kidneys. Reduction of the titre of mercuhydrin gives progressively less reduction in coloration but without any *differential* effect upon the tubules such as occurs when the mercuhydrin is administered to the animal. This means that the results of enzyme studies on cell homogenates and even on tissue sections cannot be easily interpreted in terms of what happens in the intact organ.

Counteraction of enzyme inhibition. The injection of BAL (in equimolar amounts) simultaneously with an effective dose of mercuhydrin will completely suppress the inhibition of succinic dehydrogenase activity. Sections of kidneys of animals treated in this manner exhibit a normal pattern of enzymatic activity at 48 hours after the injections (Fig. 3). The injection of l-cysteine and glutathione in equimolar and in much larger amounts does not counteract the inhibition when injected simultaneously with mercuhydrin. These two compounds also fail to counteract the diuretic action of mersalyl(4). As in previously reported studies *in vitro*(2), the addition of l-cysteine or glutathione in equimolar amounts, as well as BAL, completely reverses the inhibition. It seems likely that, when injected, monothiols may be oxidized before reaching the kidney.

Summary and conclusions. Mercuhydrin, when administered to rats by subcutaneous injection causes a marked depression of succinic dehydrogenase activity in the proximal convoluted portion of the kidney tubules, while other portions of the nephron are little affected. This enzymatic inhibition is concomitant with the known time of diuresis and both can be prevented by the administration of BAL. This suggests that the enzymatic

inhibition is, in fact, related to the diuresis. This places the locus of mercurial action on the proximal convoluted tubule—most certainly as regards its inhibition of succinic dehydrogenase activity and by implication also its diuretic action.

ADDENDUM: After manuscript was submitted a note appeared in *Science* describing a similar study by Wachstein and Meisel (*Science*, v119, 100). Their findings on localization of mercurial action are in essential agreement with those reported in the present paper.

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Relation of Dietary Protein Levels to Pancreatic Damage in the Rat. (20865)

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Extensive degenerative changes in the pancreas brought about by ethionine, a presumed methionine antagonist(1-3) have focused attention on the influence of dietary factors essential for the structural integrity of the pancreas. While the effects of obstruction of the pancreatic ducts as well as of other mechanical factors which are thought to be of importance in the etiology of human pancreatic necrosis have been extensively investigated(4), few workers have paid attention to the influence of nutrition on the pancreas. The feeding of diets low in protein and/or of poor quality and often high in fat was found to lead to pancreatic damage in the dog(5) and rat(6-9). In ducklings, a synthetic diet rich in sucrose often leads to pancreatic fibrosis(10).

It is the purpose of the present communication to describe briefly the changes that occur in the pancreas of the rat on a synthetic diet devoid of any protein as well as the influence of various protein levels and of methionine in such a synthetic diet on the microscopic structure of the pancreas.

Material and method. Young male rats of the Wistar strain weighing approximately 150 g were used. Sixty animals were given a basic protein free diet. Groups of 15 rats were fed the same diet supplemented with 2, 4, and 7.5% vitamin free test casein (Nutritional Biochemical Corp.) and 1% dl-methionine respectively. The basic diet contained 70% corn starch, 10% vegetable oil, 15% cellulose, 1% cod-liver oil and 4% salt mixture U.S.P. No. XIV. The following vitamin supplements were added per kg of food: Vitamin A concentrate 20,000 units, Viosterol 11,000 units, inositol 110 mg, alpha tocopherol 110 mg, choline chloride 1.65 g, Menadione 49 mg, para-amino benzoic acid 110 mg, niacin 100 mg, riboflavin 22 mg, pyridoxine 22 mg, thiamine 22 mg, calcium pantothenate 66 mg, biotin 0.44 mg, folic acid 2 mg, vit. B_{12} 0.03 mg. Animals on the basic protein free diet were allowed to live up to 75 days in order to study the development of the pancreatic lesions. Rats were sacrificed on the 5th, 7th,