response to D_2 appears, while response to I is absent. The absence of response to I cannot be due to the absence of muscle fibers available for stimulation, since response to D_2 is present. After 10 to 20 minutes of stimulation with D_1, response to I begins to reappear and to increase gradually in size, but usually remains somewhat smaller than the D_2 response. After "fatigue" (*i.e.*, approach to the original resting length) the responses to both I and D_2 are very marked and may approach the height of initial contraction. If at this stage continued stimulation through D_2 or I is superimposed on the uninterrupted D_1 stimulation, a large tetanic contraction occurs, which however returns to the baseline much faster than it would have had the muscle been stimulated solely through these electrodes. If the stimulation of D_2 or I is interrupted at this moment, while D_1 stimulation continues, the response to D_2 or I stimulation recovers.

When continued stimulation is applied through D_2, the responses both to D_1 and I stimulation appear within a short time and are present throughout the development of fatigue.

The continued stimulation through D_1 is accompanied by an increase in muscle resistance, as indicated by the oscilloscope studies. This increased resistance is related in time of appearance to the return of response to indirect stimulation. The effect of D_2 stimulation was less marked. Investigations by means of crossed D_1 and D_2 electrodes revealed that the difference between continued D_1 and D_2 stimulation is due to the lower D_1 electrode inserted into the tendon.

The results described above could be reproduced in all experiments with only one exception. Experiments on cats were confirmatory. It appears doubtful whether the diminishing response to continued direct stimulation may be regarded as a simple fatigue phenomenon. Rather it appears more likely to be due to some kind of rectification or polarization in the tissues, however, not to polarization of the electrodes themselves.

The more intimate mechanism of these effects is being investigated. The effects themselves appear to have some practical significance in connection with the therapeutic use of alternating currents in muscle stimulation. They may also be of importance in relation to fundamental aspects of excitability and fatigue in striated muscle.

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"Folic Acid" Active Compounds in the Nutrition of the Monkey.*

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Two years ago we reported[1] that the norite eluate concentrate of Hutchings *et al.*,[2] a rich source of "folic acid," was active in producing normal growth and preventing leucopenia in monkeys given a synthetic ration containing all the B vitamins available in pure form. Hutchings *et al.*[3] had shown previously that this concentrate contained factors necessary for the normal development of chicks. More recently Briggs *et al.*[4] concluded that the norite eluate contains at least 3 factors: folic acid necessary for the growth of *Lactobacillus casei;* vitamin B_{10}, necessary for normal feathering; and vitamin B_{11}, necessary for normal growth in chicks. They[5] further demonstrated that fractions high in vitamin B_{10} and

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B_{11} activity but low in folic acid can promote excellent growth and feathering in the chick. Pfiffner *et al.*[6] isolated a crystalline compound from liver which they called vitamin B_c and found that it has both growth-promoting and hemopoietic properties for the chick. Since vitamin B_c also has growth-promoting properties for *Lactobacillus casei,* these workers suggested that vitamin B_c, Hutchings' norite eluate factor, and Williams' folic acid[7] are probably identical. Binkley and coworkers[8] isolated from yeast a B_c conjugate which has vitamin B_c activity for the chick but no activity for the bacterium. However, upon enzymatic digestion the B_c conjugate becomes active as a source of B_c for the *Lactobacillus casei.* Day and coworkers[9] reported that highly purified *Lactobacillus casei* factor was effective in treating vitamin M-deficient monkeys. In this paper we wish to report the relative effectiveness of vitamin B_{10} and B_{11} concentrates, vitamin B_c, vitamin B_c conjugate and the synthetic *Lactobacillus casei* factor when used in place of the norite eluate concentrate in our purified rations for the monkey.

Experimental. The method of handling the monkeys has been described previously.[10] The M-2 basal diet consisting of sucrose 73, Labco casein 18, salts IV 4, corn oil 2, cod liver oil 3 was fed *ad libitum* and supplemented with adequate amounts of ascorbic acid, thiamine, riboflavin, nicotinic acid, pyridoxine, choline, pantothenic acid, inositol, *p*-aminobenzoic acid, and biotin.

A liver fraction high in B_{10} and B_{11} activity but low in "folic acid" activity was prepared according to the directions of Briggs *et al.*[5] In brief this method consists of dialysing a concentrated superfiltrol eluate[2] in a cellophane bag against cold water for 96 hours. The residue is saved and placed in a Visking cellulose casing and dialysed against hot distilled water. The dialysate is saved and concentrated so that 1 cc of the final solution is equivalent to 10 g of the original solubilized liver extract (fraction L).

The dialysate was assayed for B_{10} and B_{11} activity using chicks and for "folic acid" using the microbiological method. Using as a standard (100%) the growth and feathering of chicks fed a ration containing 2% solubilized liver (fraction L), one cc of the dialysate showed 95% B_{10} and B_{11} activity. Assay with *Streptococcus lactis* showed that 1 cc of the dialysate contained less than 10 γ folic acid using crystalline vitamin B_c as the standard.

Studies with B_{10} and B_{11} Concentrates. Three monkeys, No. 217, 218, and 219, were placed on the basal ration and given daily 1 cc of the concentrated dialysate. The monkeys grew at a slow rate for 8 weeks after which they began to lose weight. The supplement was then increased to 2 cc per day equivalent to 20 g of the original solubilized liver. This too proved to be inadequate and within two weeks the monkeys became cachetic and developed anorexia. Monkeys 217 and 219 were

Hutchings of Lederle Laboratories, Inc., Pearl River, N.Y., for the synthetic *Lactobacillus casei* factor; to Mrs. Edith Jones for assistance in the differential leucocyte counts; to Mr. T. D. Luckey for the B_{10} and B_{11} assays; and to Mrs. Lillian Alberty for the folic acid assays.

[1] Waisman, H. A., and Elvehjem, C. A., *J. Nutrition,* 1943, **26**, 361.

[2] Hutchings, B. L. Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, 1941, **141**, 521.

[3] Hutchings, B. L., Bohonos, N., Hegsted, D. M., Elvehjem, C. A., and Peterson, W. H., *J. Biol. Chem.*, 1941, **140**, 681.

[4] Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1943, **148**, 163.

[5] Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1945, **158**, 303.

[6] Pfiffner, J. J., Binkley, S. B., Bloom, E. S., Brown, R. A., Bird, O. D., Emmett, A. D., Hogan, A. G., and O'Dell, B. L., *Science,* 1943, **97**, 404.

[7] Mitchell, H. K., Snell, E. E., and Williams, R. J., *J. Am. Chem. Soc.*, 1941, **63**, 2284.

[8] Binkley, S. B., Bird, O. D., Bloom, E. S., Brown, R. A., Calkins, D. G., Campbell, C. J., Emmett, A. D., and Pfiffner, J. J., *Science,* 1944, **100**, 36.

[9] Day, P. L., Mims, V., Totter, J. R., Stokstad, E. L. R., Hutchings, B. L., and Sloane, N. H., *J. Biol. Chem.*, 1945, **157**, 423.

[10] Waisman, H. A., Rasmussen, A. F., Jr., Elvehjem, C. A., and Clark, P. F., *J. Nutrition,* 1943, **26**, 205.

given 2 cc of the crude norite eluate concentrate daily equivalent to 10 g of the original solubilized liver in place of the dialysate, and within a week they began to gain weight rapidly, and the cachexia soon disappeared. Four weeks later the norite eluate concentrate level was reduced to 1 cc per day equivalent to 5 g solubilized liver, and the monkeys continued to grow at the same rate.

Monkey 218 developed a severe diarrhea and refused to eat the ration. Since folic acid-deficient monkeys occasionally develop dysentery, this monkey was given 1 g of sulfasuxidine per day. In addition the M-2 basal was supplemented with 3% whole liver powder which was fed together with the sulfasuxidine by stomach tube. After a week the diarrhea disappeared, and the monkey regained its appetite so that the tube feeding was no longer necessary. Shortly thereafter a sharp increase in weight occurred, and the monkey continued to gain at a rapid rate. Apparently a concentrate showing high vitamin B_{10} and B_{11} activity is inadequate as a source of the essential factor for the monkey. On the other hand the crude norite eluate concentrate carries the active factor since it caused a resumption of growth when fed to the deficient monkeys.

Studies with Crystalline B_c, B_c *Conjugate, and Synthetic* Lactobacillus casei *Factor.* The same monkeys (No. 217, 218, and 219) were used in this study and were returned to the basal ration after having been on the folic acid complete ration for 3 months. In the case of monkeys 217 and 219 the norite eluate concentrate was discontinued while the whole liver powder was removed from the diet of monkey 218. After 10 weeks on the basal ration the growth rates of these 3 monkeys decreased considerably. At this point monkey 217 had a white blood cell count of 8,000 per cmm; monkey 218 5,000 per cmm; and monkey 219 5,000 per cmm. These low counts and the reduced growth rates were good indications that a characteristic "folic acid" deficiency existed.[1] A week later the animals began to lose weight, and accordingly therapy with B_c and B_c conjugate was instituted. 100 γ of vitamin B_c per day was added to the ration of monkey 217, and 200 γ B_c conjugate to the ration of monkeys 218 and 219. White blood cell counts were made 3 times a week.

Preliminary results showed that the free vitamin B_c caused a more rapid response than an equivalent amount of the conjugate. However, it soon became apparent that a complicated deficiency existed in two of the monkeys and so individual case histories are given for purposes of clarification.

No. 219. After 2 weeks of supplementation with 200 γ B_c conjugate the white blood cell count rose from 5,000 to 11,000 per cmm and the animal maintained its weight. During the next week the monkey showed a small increase in weight. The B_c conjugate level was then increased to 300 γ per day, but this change had no additional effect either on the white blood cell level or weight. Two weeks later the monkey started to lose weight but the white blood cell count did not fall. 100 γ B_c per day was substituted for the B_c conjugate but the monkey continued to lose weight during the next 2 weeks. Substituting 150 γ of the synthetic *Lactobacillus casei* factor for the B_c for one week also proved ineffective in checking the decline in weight. Hemoglobin determinations showed that the monkey was anemic, the values averaging 8 g of hemoglobin per 100 cc of blood. Differential white cell counts indicated a definite reversal in the neutrophile-lymphocyte ratio. These changes are typical symptoms of a deficiency of the monkey anti-anemia factor.[11,12] Since the leucocyte count was 16,000 per cmm it seemed unlikely that a concomitant folic acid deficiency existed. Whole liver powder, a good source of the monkey anti-anemia factor,[13] was then fed at a level of 3% of the ration. The monkey rapidly increased in weight, gaining 600 g in one week, and the hemoglobin level rose to 12.2 g per hundred cc. The neutrophile-lymphocyte ratio returned to normal within 2 weeks. Evidently the B_c conjugate, B_c, and the synthetic *Lactobacillus casei* factor were effective in combatting the

[11] Cooperman, J. M., Waisman, H. A., McCall, K. B., and Elvehjem, C. A., *J. Nutrition,* 1945, **30**, 45.

[12] McCall, K. B., Waisman, H. A., Elvehjem, C. A., and Jones, E. S., *J. Nutrition,* in press.

[13] Cooperman, J. M., McCall, K. B., and Elvehjem, C. A., *Science,* 1945, **102**, 645.

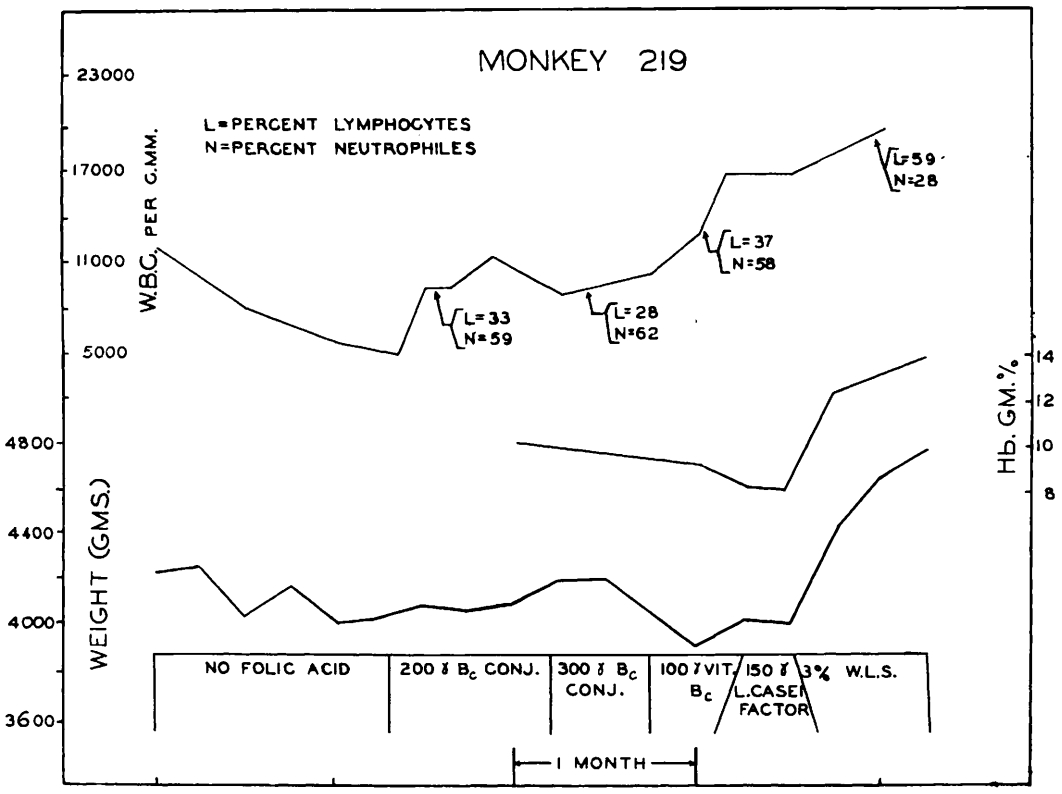

FIG. 1.

The effect of B_c conjugate, vitamin B_c, the synthetic *L. casei* factor and whole liver powder on the growth, leucocytes, and hemoglobin of a monkey with a complicated folic acid deficiency. The levels of the various supplements were fed daily for the period of time indicated on the graph.

γ B_c conj. = μg of B_c conjugate.

γ Vit. B_c = μg of vitamin B_c.

γ *L. casei* factor = μg of synthetic *Lactobacillus casei* factor.

W.L.S. = whole liver substance (whole liver powder).

folic acid deficiency but whole liver powder was necessary in addition for the monkey to recover from the monkey anti-anemia factor deficiency which was precipitated. The data are summarized in Fig. 1.

No. 218. The ration of this animal was supplemented with 200 γ B_c conjugate. During the next 10 days the monkey continued to lose weight, and the leucocyte count did not rise significantly. Hemoglobin determinations showed that the animal was not anemic since the values were in the range of 14.3 g per hundred cc. The monkey was then given 100 γ B_c per day instead of the conjugate, and within a week the monkey increased in weight and the leucocyte count increased to 18,000 per cmm. The weight gain continued as did the rise in leucocytes, and after 4 weeks the synthetic *Lactobacillus casei* factor was substituted for the B_c at a level of 100 γ per day. The weight increase continued at the same rate and the leucocyte count eventually reached 26,000 per cmm. Differential leucocyte counts showed a normal neutrophile-lymphocyte ratio. In this case there appeared to be no complicating deficiencies. The B_c conjugate at a level of 200 γ per day proved to be insufficient in correcting the folic acid deficiency; on the other hand 100 γ B_c per day was quite effective as was the synthetic *Lactobacillus casei* factor. Since this monkey had been given a ration containing 3% whole

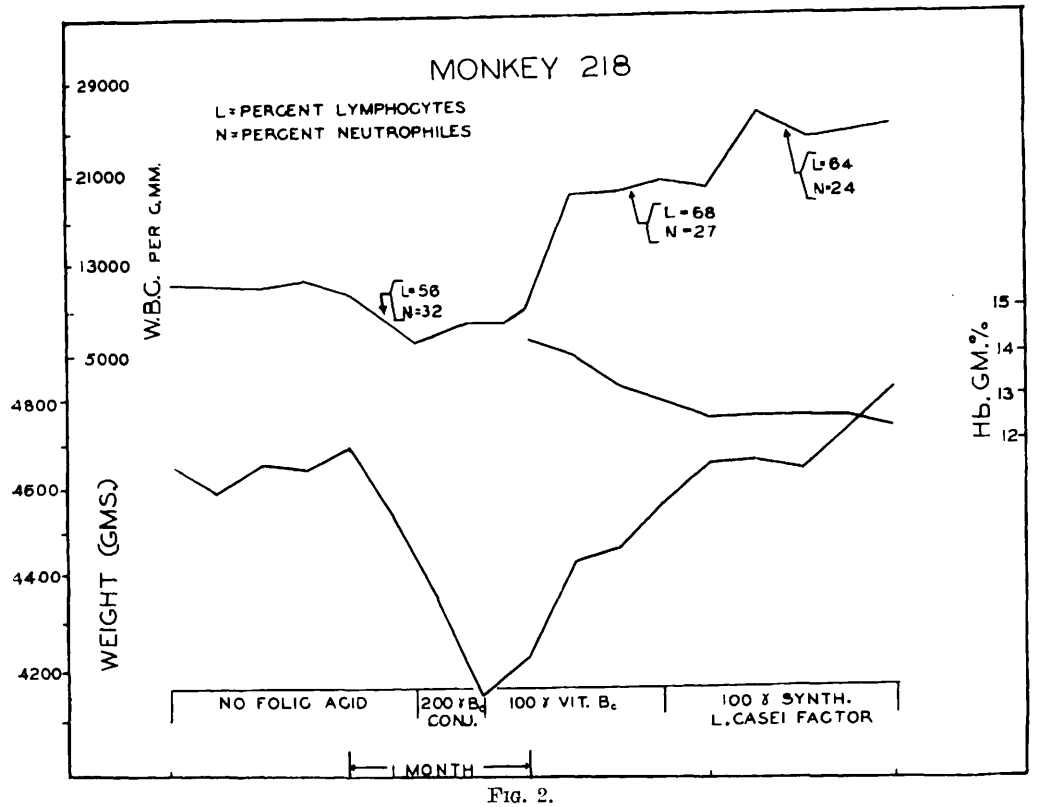

FIG. 2.

The effect of B_c conjugate, vitamin B_c, and the synthetic *L. casei* factor on the growth, leucocytes, and hemoglobin of a monkey with an uncomplicated folic acid deficiency.

The levels of the various supplements were fed daily for the period of time indicateed on the graph.

γ B_c conj. = μg of B_c conjugate.

γ Vit. B_c = μg of vitamin B_c.

γ *L. casei* factor = μg of synthetic *Lactobacillus casei* factor.

liver powder for 3 months before it was placed on the M-2 basal, it is very likely that it had an adequate store of the monkey anti-anemia factor and developed an uncomplicated folic acid deficiency. The data for this animal are summarized in Fig. 2.

No. 217. The weight loss in this monkey was arrested after the animal received 100 γ of B_c per day for 1 week and the leucocyte count rose to 13,000 per cmm. During the next 3 weeks the leucocytes remained at a level of 14,000 per cmm but there was only a slight increase in weight. The level of B_c was increased to 150 γ per day and during the next 10 days the weight increased at a slightly greater rate. At this time the leucocyte count was 20,000 per cmm. 150 γ of the synthetic *Lactobacillus casei* factor per day was then substituted for the B_c and the monkey continued to grow at the same rate and maintained its leucocyte count. Hemoglobin values ranged between 11 and 12 g per 100 cc for this monkey and differential counts showed a reversed neutrophile-lymphocyte ratio. These together with the poor growth rate indicated the existence of a complicating deficiency of the monkey anti-anemia factor. The leucocyte count on the other hand responded to the B_c and *Lactobacillus casei* factor.

Discussion. The crude norite eluate concentrate has been used in our earlier work as a source of folic acid in our purified monkey ration. Since it is desirable to substitute as soon as possible, crystalline compounds for crude concentrates in nutritional studies, folic

acid-like compounds were tested for activity. Vitamin B_{10} and B_{11} concentrates which are prepared from the crude norite eluate apparently do not possess activity for the monkey but do contain compounds which are available for the chick. Crystalline vitamin B_c or the synthetic *Lactobacillus casei* factor can replace the norite eluate concentrate in the ration of the monkey each possessing about equal activity. Evidently the B_c conjugate is less active than either of these two compounds.

A deficiency in the monkey anti-anemia factor complicated the folic acid deficiency; similar conditions have been observed in riboflavin,[11] B_6 and pantothenic acid[42] deficiency in the monkey. This deficiency is characterized by a suboptimal hemoglobin level, lack of growth and a reversal in the neutrophile-lymphocyte ratio. In the normal monkey the lymphocytes constitute 60-70% of the total leucocytes and the neutrophiles 25-35%. However, in monkeys deficient in the monkey anti-anemia factor the ratio is reversed and the neutrophiles rise to 60-70% of the total leucocytes and the lymphocytes fall to 25-35% while the total leucocyte count is not appreciably reduced. There is, therefore, an actual increase in the number of neutrophiles and a decrease in the number of lymphocytes. Therapy with whole liver corrects this reversal, and produces a normal hemoglobin level and a resumption of growth.

The folic acid deficiency evidently precipitated a deficiency of this factor in the monkeys which had never received a ration containing whole liver. Thus in monkey 219 the low leucocyte count due to folic acid deficiency was corrected by vitamin B_c but whole liver was necessary to correct the neutrophile-lymphocyte ratio reversal, the low hemoglobin levels and the loss in weight. On the other hand monkey 218 was fed 3% whole liver powder in the ration for 3 months and consequently had an adequate store of this factor when the folic acid deficiency developed. This animal showed the best response to the various crystalline supplements since the folic acid deficiency was uncomplicated. Monkey 217, like monkey 219, showed a good leucocyte response to therapeutic doses of vitamin B_c. The weight response on the other hand was poor and blood studies showed that a dyscrasia existed typical of a deficiency of the monkey anti-anemia factor.

Summary. Vitamin B_{10} and B_{11} concentrates were inactive as a source of folic acid for the monkey.

Crystalline vitamin B_c and the synthetic *Lactobacillus casei* factor were active as a source of folic acid for the monkey when fed at a level of 100 γ per day. The B_c conjugate had less activity when fed at levels of 200 and 300 γ per day.

Folic acid deficiency precipitates a deficiency for the monkey anti-anemia factor which is characterized by lack of growth, suboptimal hemoglobin levels and a reversal in the lymphocyte-neutrophile ratio.

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A Differential, Microbiological Assay for O-Heterobiotin.*

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O-Heterobiotin (an analog of biotin in which the sulfur atom has been replaced by an oxygen atom) has biotin-like activity for *L. casei*,[1,2,3] *S. cerevisiæ*,[1,2,3] *R. trifolii*,[4] *L. arabinosus*,[3,5] chicks,[3] and for counteracting egg white toxicity in rats.[3,5] Hofmann *et al.* have developed an assay for O-heterobiotin with *S. cerevisiæ* after the biotin in the sample

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