in Fig. 2 that the 5 treated dogs all showed higher serum cholinesterase activities after the 5th day following D.F.P., than the 2 untreated control animals. The differences in serum cholinesterase prevailing between treated and untreated animals on the 8th day after D.F.P. ranged from 11 to 32%.

Discussion. The D.F.P. was administered intramuscularly in aqueous solution to 2 of the animals (open dots and top dashed line, Fig. 2). We found by experience that the D.F.P. lost potency rapidly when diluted in water, and for this reason we diluted our next sample in olive oil and injected the remainder of the animals intramuscularly with the oil solution. The 2 dogs injected with aqueous solution of D.F.P. showed recovery of their serum cholinesterase activities to normal in 15 and 21 days (for treated and untreated animals, respectively) but the other animals were not observed beyond the 8th day. From the 2nd to 4th days the smallest dog, which had received the low dose of 0.6 mg of D.F.P. showed the greatest regeneration of enzyme activity. It therefore seemed wise to give the other dogs a single dose of 1 mg irrespective of body weight. Variation in body weights do not account for any differences in rapidity of cholinesterase regeneration, as far as we can ascertain.

Liver extract or folic acid alone were given to particular animals at the start of the experiment shown in Fig. 2. Since no rapid changes were produced thereby, we commenced giving both preparations to all experimental dogs, except one, on the 3rd experimental day. The average amounts given each dog were 2 mg daily of folic acid (pteroyl glutamic acid) and 5 units of liver extract.

If proof should come forth that the cholinesterase potentiating actions of liver and folic acid are the important actions of these substances in the treatment of certain human anemias, then it would seem likely that liver preparations could be assayed by one of the methods reported in this paper.

Summary and Conclusions. The incubation of liver extracts or pteroyl glutamic acid with certain dog blood sera caused an increase in the cholinesterase activity of the sera. From such experiments it was estimated that 1 mg of pteroyl glutamic acid was roughly equal to 1 ± 0.5 U.S.P. unit of the liver extracts examined, in cholinesterase activity potentiating power.

The administration of liver extracts and pteroyl glutamic acid to dogs in which the serum cholinesterase had been greatly depleted by diisopropyl-fluorophosphate injections, caused more rapid regeneration of this enzyme activity than was observed in untreated control animals.

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Effect of Riboflavin and Other B Complex Vitamins on Work of Perfused Frog Muscles.

N. W. SHOCK* AND W. H. SEBRELL.

From the Division of Physiology, National Institute of Health, United States Public Health Service, Bethesda, Md., and the Baltimore City Hospitals, Baltimore, Md.

Previous experiments have shown that when appropriate concentrations of thiamine,[1,2] cocarboxylase,[2] pantothenic acid,[3] or pyridoxine,[4] were added to the perfusion fluid; the work output of the frog gastrocnemius muscles was significantly increased. The fol-

* With the technical assistance of Frances S. Berg.

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2 Shock, N. W., and Sebrell, W. H., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 212.

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TABLE I.
Effect of Riboflavin on Total Work Output (Averages of 10 Animals for Each Concentration).

Conc. mM/L	Avg work done: Control leg g-cm	Exp. leg g-cm	Mn diff.* exp.-control leg g-cm	C.R.†	% incr.
.0000	1697	1829	132 ± 70.3	1.9	7.8
.0000001	1716	1790	74 ± 95.3	0.8	4.3
.0000005	1916	2003	87 ± 110.8	0.8	4.6
.000001	1999	2202	203 ± 76.3	2.7	10.1
.000005	1516	1790	274 ± 140.0	2.0	18.1
.00001	2097	2380	283 ± 82.9	3.4	13.5
.0001	2163	2395	232 ± 58.4	4.0	10.7
.0005	1993	2245	252 ± 31.9	7.9	12.6
.001	1864	2179	315 ± 84.4	3.7	16.9
.005	1896	2257	361 ± 73.9	4.9	19.0
.01	2085	2281	196 ± 62.3	3.1	9.4
.05	1811	1945	133 ± 111.0	1.2	7.4
.1	1583	1727	144 ± 92.6	1.6	9.1

* Mean difference ± 1 standard error of the mean.

$$\text{† Critical ratio} = \frac{\text{Mean difference}}{\sigma_{\text{Mn. diff.}}}$$

lowing report presents the results of similar tests made with the addition of riboflavin alone, as well as the simultaneous addition of all the vitamins of the B complex previously tested.

Experimental. Fatigue curves were recorded graphically for each gastrocnemius muscle of the frog under the same conditions of perfusion, loading, stimulation, and recording as previously described.[1,3] After recording the fatigue curve on the first gastrocnemius muscle, riboflavin was added in known concentrations to the buffered glucose-Ringer's solution used for perfusion of the frog while the fatigue curve was recorded on the second gastrocnemius muscle. Ten animals were tested under each experimental condition. Individual work curves for the control and vitamin-treated muscles were plotted for each animal, and the total work done was estimated from the areas under the curves as measured with a planimeter. Standard errors of all average values were calculated using formulae for small numbers.

Results. Effects of Increasing Concentrations of Riboflavin. The average total work output of control and experimental muscles exposed to increasing concentrations of riboflavin in the perfusion fluid is shown in Table I. A statistically significant increase in work output was observed when the concentration of riboflavin in the perfusion fluid was maintained between 0.00001 and 0.01 mM per liter. Concentrations of riboflavin greater or less than these limits failed to show a significant improvement in work output.

Effect of Increasing Concentrations of Riboflavin on the Distribution of Work Output. The average height of contraction at each minute of the work period was calculated for the control and riboflavin-treated muscles. The average difference between the control and vitamin-treated muscles at each minute was calculated in millimeters and also as the percentage of the height of contraction of the control muscle at that time. From these calculations, the maximum absolute differences and the maximum percentage differences shown for each riboflavin concentration were tested for statistical significance as shown in Table II. From this table, it may be seen that the average height of contraction of the vitamin-treated muscles is significantly greater than that of the untreated muscles after 9-17 minutes of exercise when the perfusion fluid contains riboflavin in contractions of 0.000005 to 0.01 mM per liter. At riboflavin concentrations of 0.05 or 0.1 mM per liter, no significant effect was observed.

TABLE II.
Effect of Riboflavin on Extent of Contraction of Perfused Frog Muscles.

Riboflavin conc. mM/L	Avg height of contraction of muscle mean difference (exp.-control leg) (Mean of 10 animals at each concentration)							
	At max. absolute difference				At max. percentage* difference			
	Min. of exercise	Mean absolute diff.†	C.R.‡	% diff.§	Min. of exercise	Mean absolute diff.†	C.R.‡	% diff.§
.0000000	10	1.0 ± 0.8	1.3	3.0	13	0.8 ± 0.6	1.3	3.0
.0000001	9	1.0 ± 0.4	2.5	5.6	16	0.5 ± 0.4	1.0	5.0
.0000005	9	4.3 ± 2.9	1.5	17.7	12	2.8 ± 1.8	1.6	20.3
.000001	10	8.0 ± 2.0	4.0	25.9	17	4.5 ± 1.5	3.0	43.3
.000005	9	11.0 ± 4.1	2.7	32.1	16	5.1 ± 1.8	2.8	54.7
.00001	8	12.3 ± 2.1	5.9	45.3	11	7.1 ± 1.8	3.8	57.9
.0001	9	8.2 ± 1.5	5.3	39.4	13	5.0 ± 1.2	4.1	59.4
.0005	11	10.0 ± 1.4	7.1	48.3	18	6.8 ± 1.1	6.2	60.0
.001	8	10.2 ± 1.9	5.4	42.8	13	6.1 ± 1.1	5.5	73.0
.005	10	12.1 ± 2.3	5.3	55.0	15	6.9 ± 1.3	5.3	82.1
.01	11	7.7 ± 1.6	4.8	46.4	17	4.4 ± 1.1	4.0	71.0
.05	17	6.0 ± 2.0	3.0	32.1	12	5.1 ± 1.8	2.8	38.0
.1	11	5.1 ± 2.9	1.8	39.8	14	3.9 ± 1.7	2.3	51.3

* Calculated as $\frac{\text{Ht. of experimental curve} - \text{Ht. of control curve}}{\text{Ht. of control curve}} \times 100.$

† Mean ± 1 standard error of the mean.

‡ C.R. $= \frac{\text{Mean difference}}{\sigma_{\text{Mn. diff.}}}$

§ Calculated as

$$\frac{\text{ht. of experimental curve} - \text{ht. of control curve}}{\text{ht. of control curve}} \times 100$$

TABLE III.
Effect of Mixture of Vitamins of the B Complex on Total Work Output of Perfused Frog Muscles.

Exp. conditions	Avg work done				
	Control leg g-cm	Exp. leg g-cm	Mn. diff. Exp.-Control leg g cm	C.R.	% incr.
Vit. B mixture*	1811	2201	390 ± 75.2	5.2	21.5
Ringer's + 20 units insulin per L.	1663	1681	18 ± 70.6	0.2	1.1
Vit. B mixture* + 20 units insulin per L.	1639	2022	383 ± 62.5	4.1	23.3

* See text for composition of vitamin mixture.

Effect of Simultaneous Administration of Vitamins of the B Complex on Total Work Output. Experiments were performed in which the second leg of the frog was perfused with buffered glucose-Ringer's solution to which the following vitamins of the B complex were added simultaneously: 0.005 mM thiamine hydrochloride per liter, 0.01 mM calcium pantothenate per liter, 0.1 mM nicotinic acid amide per liter, 0.0001 mM pyridoxine hydrochloride per liter, and 0.0005 mM riboflavin per liter. The first line of Table III shows that the work output was significantly increased by the addition of vitamins to the perfusion fluid.

Discussion. The experiments with riboflavin show that there is an optimum concentration at which improvement in total work output of perfused frog muscles is observed. For riboflavin, this optimum concentration

is 0.0005 mM per liter. As shown before for thiamine hydrochloride, calcium pantothenate and pyridoxine hydrochloride, at increased concentrations of the vitamin, the improvement in work output is no longer significant.

The experiments with the mixture of vitamins of the B complex give no evidence that the improvement in work output observed with the vitamins individually is additive, since the degree of improvement observed with the mixture was of the same order of magnitude as that observed with the individual vitamins. Thus, if it is assumed that the improvement in work output observed with the addition of vitamins to the perfusion fluid results from metabolic changes, there must be some other substance or substances essential to the recovery process which impose a ceiling on the energy available for muscle contraction. Such substances might conceivably be oxygen or glucose. Although ample glucose was present in the perfusion fluid, it seemed possible that the frog was unable to supply sufficient insulin for its metabolic use. Hence, additional experiments were performed to test this hypothesis. Control experiments were first run, in which 20 units of insulin were added to each liter of perfusion fluid used for the second leg. The second line of Table III shows that no significant improvement in work output resulted. This finding is in accordance with similar tests made by Eddy.[5] Insulin (20 units per liter) was then added to the vitamin mixture which was used in perfusing the second leg. The results shown in line 3 of Table III give no support to the hypothesis that utilization of glucose is the limiting factor in determining the improvement in work output following the addition of vitamins of the B complex to the perfusion fluid, since the improvement was no greater than that observed without the addition of excess insulin.

Summary. The work output of the gastrocnemius muscle of the perfused frog has been determined following the addition of riboflavin to the perfusion fluid in concentrations ranging from 0.0000001 to 0.1 mM per liter.

The total work output was significantly increased by the addition of 0.00001 mM riboflavin per liter. Concentrations below this level were ineffective. A significant improvement in total work output was observed with riboflavin concentrations up to and including 0.01 mM per liter; with concentrations of 0.05 and 0.1 mM per liter, the improvement was not statistically significant.

The extent of muscle contraction after 8-11 minutes of work was significantly increased by the addition of 0.00001 to 0.01 mM of riboflavin per liter of perfusion fluid.

When a mixture of thiamine hydrochloride, calcium pantothenate, nicotinic acid amide, pyridoxine hydrochloride and riboflavin, each at optimal concentrations is added to the perfusion fluid; the improvement in total work output is no greater than that observed with the most effective vitamins singly (thiamine hydrochloride, calcium pantothenate, and pyridoxine hydrochloride).

The addition of 20 units of insulin per liter of perfusion fluid along with the vitamin mixture did not result in any greater increase in work output than that observed with the vitamins alone.

[5] Eddy, N. B., *Am. J. Physiol.*, 1924, **69**, 432.