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A Fat-Soluble Material from Plasma Having the Biological Activities of Biotin.

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It is now well established that biotin deficiency decreases the resistance of chickens and ducks to malaria parasites.[1,2] It also appears to decrease the resistance of rats to *Trypanosoma lewisi*[3] and of mice to mouse typhoid.[4] A normal level of biotin must therefore be essential to the proper functioning of some general mechanism of resistance to infection. In the course of work directed toward discovering the nature of this resistance mechanism, it was found that the plasma of a variety of animals contains a previously undescribed material which, after hydrolysis by acid or enzymes, yields a fat-soluble substance having the biological activities of biotin but differing from it chemically. This material is probably more intimately concerned in resistance to malaria parasites than is biotin itself. The changes in concentration which both it and biotin undergo in the plasma of chickens and ducks infected with malaria, and its effects on the multiplication of malaria parasites *in vitro* will be described elsewhere. It is the purpose of the present paper to present the data demonstrating the existence of the material and to relate some of its biological and chemical properties.

Of the various methods of microbiological assay for biotin, one of the most specific is that employing *Lactobacillus casei*.[5] The *L. casei* assay has therefore been used almost exclusively. The medium and method of in-

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2 Seeler, A. O., Ott, W. H., and Gundel, M. E., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 107.

3 Caldwell, F. E., and György, P., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 116.

4 Kligler, I. J., Guggenheim, K., and Herrnheiser, H., *J. Infect. Dis.*, 1946, **78**, 60.

5 Shull, G. M., Hutchings, B. L., and Peterson, W. H., *J. Biol. Chem.*, 1942, **142**, 913.

6 Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, 1942, **27**, 1086.

7 Skeggs, H. R., and Wright, L. D., *J. Biol. Chem.*, 1944, **156**, 21.

TABLE I.
Biotin Activity of Human and Duck Plasmas After Different Types of Treatment.

Plasma	Treatment	Activity as mγ biotin per ml plasma
Human	(1) Diluted in water.	2.0
	(2) Mixed with 10 X vol. of 3 N H_2SO_4, autoclaved one hr, then neutralized.	11.4
	(3) Precipitated with trichloracetic acid. Autoclaved 1 hr.	9.7
	(4) Precipitated with trichloracetic acid. Precipitate removed by centrifugation. Supernatant autoclaved 1 hr.	1.8
Duck A	(1) Diluted in sterile water. Added aseptically to assay tubes.	3.2
	(2) Autoclaved 1 hr in 10 X vol. of 3 N H_2SO_4. Neutralized.	14.6
	(3) Same as (2) but filtered before neutralization.	3.6
	(4) 24-hr treatment with takadiastase in acetate buffer, pH 4.7. Then neutralized and autoclaved.	15.7
Duck B	(1) Diluted in buffer, pH 8.0.	4.2
	(2) Same as (1) followed by ether extraction.	2.5
	(3) Autoclaved 1 hr in 10 X vol. of 3 N H_2SO_4. Brought to pH 8.2.	10.0
	(4) Same as (3) followed by ether extraction.	1.3
	(5) 24-hr treatment with takadiastase in acetate buffer, pH 4.7. Then neutralized and autoclaved.	9.8
	(6) Same as (5) followed by ether extraction.	2.3

oculation were those described by Landy and Dicken[6] except that the vitamins of the B complex were added in larger amounts.[7] pure crystalline folic acid (obtained from Lederle Laboratories through the courtesy of Dr. Y. SubbaRow) was used at 25 γ per l of double-strength medium and pyridoxamine (Merck) was added at 10 γ per l. Growth was determined by titrating the acid produced with 0.1 N sodium hydroxide. Since it soon became apparent that a substance other than biotin was being measured in terms of its biotin activity, it was found advantageous to prolong the time of incubation of the assay tubes from the usual 3 days to 4. When this was done, the results obtained with 3 different concentrations of the same sample usually agreed within 10 to 15%.

Acid hydrolysis of plasma was accomplished by mixing 0.4 ml of plasma with 4 ml of 3 N sulfuric acid and autoclaving at 15 lbs for one hour, a procedure designed to liberate bound biotin.[8] Plasma was also hydrolyzed by treatment with takadiastase,[9] 5 mg of enzyme being used to 0.5 ml of plasma diluted to 5 ml with acetate buffer of pH 4.7.

It was found that if normal plasma was hydrolyzed with sulfuric acid and filtered, either before or after neutralization, its biotin content was the same as that of plasma merely diluted in water, indicating that plasma does not contain any bound biotin. But if the hydrolyzed plasma was not filtered, a much higher biotin activity was found. Similar results were obtained after hydrolysis by takadiastase. All of the additional biotin activity resulting from the hydrolysis of plasma could be removed by shaking with ether, as well as by filtration. The shaking of unhydrolyzed plasma with ether removed a variable proportion of its already low biotin activity but did not affect the activity which appeared upon subsequent hydrolysis. Table I illustrates a few typical results out of many which have been obtained. In one series 6 mγ of pure biotin were added to duplicate 0.4 ml samples of plasma. These, and the corresponding samples without added biotin were submitted to various treatments and assayed for biotin. The % recovery of the added biotin was: 97 for the samples diluted in water, 89 for the samples autoclaved in sulfuric acid, brought to pH 7 and not

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[9] Luckey, T. D., Briggs, G. M., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **152**, 157.

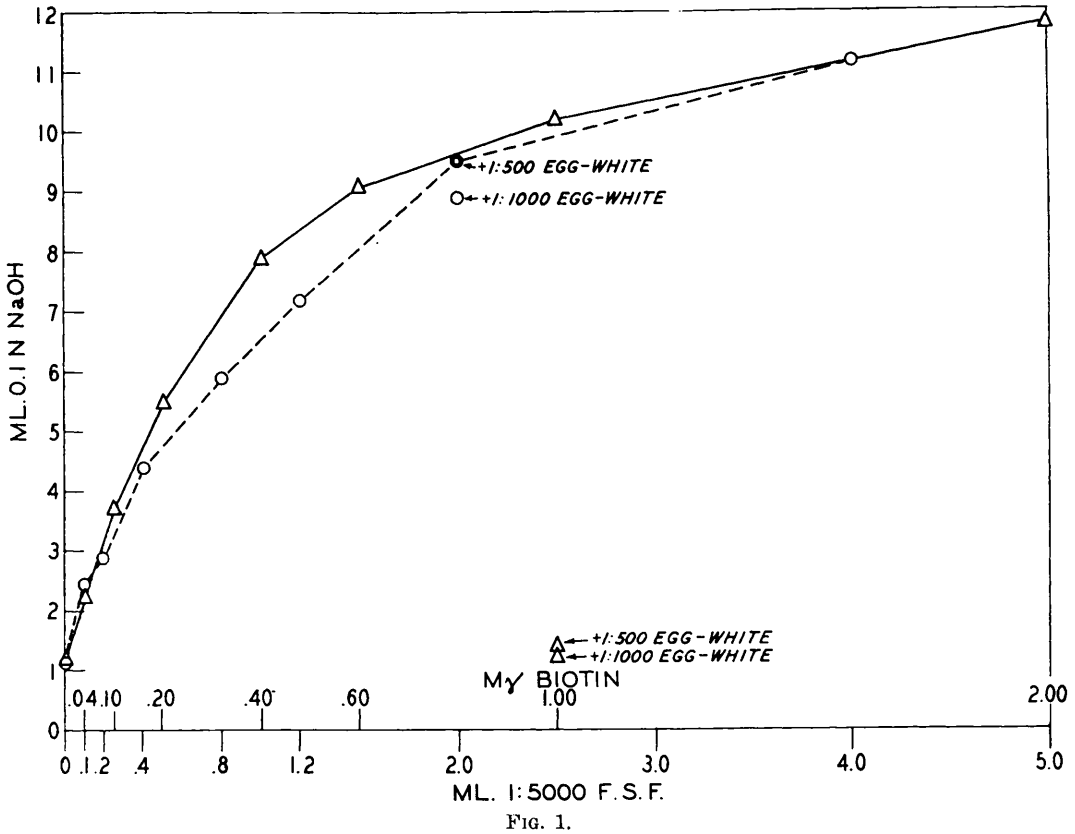

FIG. 1.

The response of *Lactobacillus casei* in an essentially biotin-free medium to the addition of different concentrations of: (1) Biotin (solid line and triangles). Amounts in mγ per 10 ml culture tube given by upper numbers on abscissa, amounts in ml of the standard biotin solution by lower numbers; (2) FSF (broken line and circles). Amounts in ml (ranging from 0.1 to 4.0) of a 1:5000 dilution of a brown oil obtained from hydrolyzed horse plasma. The indicated triangles and circles show, respectively, the growth with 1 mγ biotin plus 2 concentrations of fresh sterile egg-white, and the growth with 2 ml of the 1:5000 FSF plus 2 concentrations of egg-white.

filtered, 98 for the samples similarly treated but brought to pH 9, and 117 for samples which were filtered after being brought to pH 9. The recoveries after autoclaving in 6 N hydrochloric acid or 5 N sodium hydroxide were 79 and 62% respectively.

The biotin-active material which was removed by shaking hydrolyzed plasma with ether could be recovered in the ether extract. This fat-soluble material shall henceforth be designated as FSF. Of the various human plasma protein fractions,* fibrinogen, albumin and γ-globulin contained very little FSF, while the fractions containing the α- and β-globulins and lipids were relatively rich in it. Fractions III-0 and IV-1, 1 W had the highest FSF content, with a biotin activity after acid hydrolysis of about 300 mγ per g. A preparation of FSF from one of these fractions had a quantitatively similar effect on the growth of 3 species of lactic acid bacteria (*Lactobacillus casei, Leuconostoc mesenteroides* and *Streptococcus fecalis* R).

In order to study further the properties of FSF, 10 l of oxalated horse plasma were made 3 N with respect to sulfuric acid and autoclaved for one hour at 15 lbs. The mixture was brought to a pH of about 7.5 with 10 N sodium hydroxide and was filtered

* The plasma fractions were obtained through the kindness of Dr. L. C. Strong of the Harvard Medical School and were prepared under a contract recommended by the Committee on Medical Research between Harvard University and the Office of Scientific Research and Development.

TABLE II.

Effect of Injections of FSF or Biotin in Chicks Fed a Diet High in Egg-White and Inoculated with *Plasmodium lophurae*.

In Experiment 1 the chicks were placed on the special diets at 7 days of age, the twice weekly injections were begun at 10 days and the inoculations were done at 25 days.

In Experiment 2 the chicks were placed on the special diet at 5 days, the injections (twice weekly for biotin, 3 times weekly for FSF) were begun at 8 days, and the inoculations were done at 20 days. In both experiments the injections were intramuscular.

Exp. No.	Diet	Injection	No. chicks	Avg degree of scaliness* of feet and mouth at days 19	25	27	31	35	Avg peak No. of parasites per 10,000 red cells	No. which died within 31 days
1	Egg white 20%	None	3	—	1.8	—	—	2.7	6780	0†
	" " "	Biotin 12 γ per week	4		0	—	—	0.1	2165	0
	" " "	FSF-1 γ biotin activity per week	4	—	1.3	—	—	1.6	2505	0
	Casein 20%	None	4	—	0	—	—	0	2730	0
2	Egg white 20%	None	9	0.6	—	2.2	3.8	—	3340	5
	" " "	FSF-1.5 γ biotin activity per week	8	0.2	—	1.0	1.0	—	3175	1
	" " "	Biotin 12 γ per week	8	0.1	—	0	0.2	—	1290	0

* An arbitrary scale ranging from 0 to 6 was used to express the extent of the lesions on the feet and at the corners of the mouth.

† Two of these chicks were very weak at 35 days, when they were killed. All the other chicks in this experiment were active at this time.

through hardened filter paper on a Buchner funnel. The filtrate contained very little activity and was discarded. The black residue was shaken with several large volumes of ether and the yellow ether extracts thus obtained were concentrated *in vacuo*. When all the ether had been removed, 31 ml of a brown oil were obtained. This was liquid at room temperature but solidified to a waxy material when refrigerated. The brown oil (prepared for assay as an opalescent emulsion made by diluting an aliquot with alcohol and then with water or buffer) had a biotin activity of 2.5 γ per ml, and represented a 70% recovery of the total activity of the hydrolyzed plasma. This material was used to demonstrate the ability of *L. casei* to dispense with biotin if adequate amounts of FSF are present. A dilute washed suspension of *L. casei* was inoculated into tubes of biotin assay medium free from added biotin but containing an adequate concentration of FSF. Twenty-four hours later, a loopful of the growth which had occurred was inoculated to 2 similar tubes. Such subinoculation was repeated twice more. The 24-hour growth in the 4th transfer was washed and made into a dilute suspension in the usual manner.[6] This was used to inoculate 2 series of tubes, one containing graded concentrations of biotin, the other graded concentrations of FSF. In each series there were included tubes with adequate biotin or FSF plus more than enough fresh sterile egg-white to inactivate the biotin activity. As is evident from Fig. 1, very similar curves were obtained relating the extent of growth to the relative concentrations of the 2 growth factors. However, the addition of egg-white completely inhibited growth in the tubes with biotin, but had no effect on growth in the presence of FSF. This result indicates that FSF is not merely a stimulatory substance, but can replace biotin in the growth of *L. casei*. All traces of biotin which may have been present in the culture medium must be considered to have been inactivated by the excess of fresh egg-white.

Although the amounts and concentrations of FSF so far available have not permitted a complete test of its effectiveness against egg-white injury in animals, sufficient data have been obtained to show that it prevents the dermatitis produced in chickens by a diet high in egg-white. It also acts like biotin in preventing the increased susceptibility of chickens to infection with the malarial parasite *Plasmodium lophurae,* which otherwise occurs when the animals are maintained on an egg-white diet. The results of 2 experiments are shown in Table II. The diets used consisted of 80% of a chick-starting mash plus 20% of either powdered egg albumin or casein mixed with riboflavin at the rate of 5 mg per 100 g casein. In both experiments, FSF was administered in the breast muscle as the brown oil prepared from hydrolyzed horse plasma. The maximum amount of this which could be injected at one time was 0.2 ml, representing a biotin activity of 0.5 γ. In Exp. 1, the material was injected twice weekly and was reasonably well absorbed between injections. In Exp. 2, where 3 injections per week were given, pockets of the oil formed in the breast muscle. In both experiments the total dosage of FSF, in terms of microbiological biotin activity, was such that an equivalent amount of biotin also would not have given complete protection from biotin deficiency.[10]

All preparations of FSF so far examined have been found to be hemolytic for both duck and sheep red blood cells. The hemolysis is prevented by normal duck plasma. The biotin-like growth activity and the hemolytic activity have gone together through the following preliminary fractionation of the brown oil prepared from hydrolyzed horse plasma. A fraction which was difficultly soluble in alcohol but readily soluble in chloroform had little activity, as did a second fraction soluble in alcohol at room temperature but giving a copious white precipitate from cold alcohol. The active material was soluble in cold alcohol and was non-saponifiable. In the crude state it was soluble in acetone, but after its separation from the inactive saponifiable fraction, it was insoluble in acetone. It is interesting that the addition of 3 parts of acetone to 1 part of an alcoholic solution of the non-saponifiable fraction resulted in a quantitatively equivalent partition of both the growth and the hemolytic activities between the precipitate and the filtrate. Resaponification of the acetone insoluble material again yielded all the activity in the non-saponifiable fraction. The minimum concentration of the various fractions which gave complete hemolysis was equivalent to a biotin activity of 0.2 to 0.3 mγ per ml, when 0.1 ml of 5% red cells was added to 0.9 ml of buffered mixture, incubated ½ hour at 37°C and held overnight in a refrigerator. It is worthy of note that a crude preparation of FSF from a human plasma protein fraction gave complete hemolysis at a concentration with a biotin activity of 0.3 mγ per ml and slight hemolysis at a concentration of 0.15 mγ per ml.

It is apparent from the few properties of FSF thus far known that it cannot be biotin itself, which is not readily extracted in organic solvents[11] and which is inactivated by avidin.[12] FSF also does not correspond to any of the hitherto described analogues or vitamers of biotin, since all of these are either inactive in the growth of *L. casei* and against egg-white injury in animals or, if active, are like biotin itself inactivated by avidin.[13-19]

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A number of growth-stimulating effects on *L. casei* by naturally occurring fat-soluble substances have been described.[20,21] These effects have been shown to be due to certain fatty acids[22-24] such as oleic acid, which had earlier been identified as a factor essential for the rapid growth of *Corynebacterium diphtheriae*.[25] Oleic acid and related compounds which stimulate the growth of *L. casei* in the presence of suboptimal concentrations of riboflavin or pantothenate do not permit growth in media lacking these vitamins.[22,23] The situation with respect to oleic acid and biotin seems to be rather different.[26,27] *L. casei* is evidently capable of continuous growth in a medium containing only traces of biotin but supplied with an adequate amount of oleic acid and adjusted to an initial pH of 5.6.[27] At first thought one might conclude that the activity of FSF is due to oleic acid, but a close inspection of the available facts makes such a conclusion untenable. Oleic acid in the absence of added biotin produced a maximal growth effect in a medium with an initial pH (before autoclaving) of 5.6. If the pH was 6.5 or higher there was no growth. All the experiments with FSF were routinely done with a medium of pH 6.7-6.8 before autoclaving and 6.2-6.3 after autoclaving. Moreover, in a special experiment, the pH of the assay tubes was adjusted aseptically after autoclaving to 6.8-6.9. Fractions containing FSF had the same biotin activity under these conditions as at a pH of 6.2-6.3. FSF was fully active for *L. casei* even when all traces of biotin in the medium were rendered unavailable by an excess of avidin, a condition which has not been tested with oleic acid. FSF was also active against egg-while injury in chicks, an activity concerning which nothing has been reported for oleic acid. Finally the FSF activity was non-saponifiable and insoluble in acetone, and hence could not have been due to an ordinary fatty acid. It would seem likely, however, that there may be some relation between FSF and oleic acid, and that both are related to the utilization and function of biotin. A knowledge of the chemical nature of FSF may give a new insight into the mode of action of biotin.

Summary. The plasma of various species of animals yields, after hydrolysis with acids or enzymes, a fat-soluble material capable of replacing biotin in the growth of *Lactobacillus casei* and other lactic acid bacteria but not inactivated by avidin. When injected in chickens the material protected them from the injurious effects of a diet high in egg-white. Preparations containing the active material were found to be hemolytic, and in preliminary fractionations the growth and the hemolytic activities have gone together. The properties of the material do not correspond to those of oleic acid or of any previously described vitamers of biotin.

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