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FRACTIONATION OF GROWTH-STIMULATING FACTOR IN LIVER*

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In a previous paper (1) results were presented to show that an alcoholic extract of liver produced a pronounced growth stimulation in rats fed a natural diet. The results showed that the activity was not due to any of the known vitamins and that yeast did not possess similar activity.

Bosshardt and collaborators have described a growth-stimulating effect of liver in rats (2), while Zucker and Zucker (3) described a syndrome involving kidney lesions in rats which was cured with liver. Liver has been shown to contain factors essential for the nutrition of the monkey (4) and for the growth stimulation of *Streptococcus faecalis* (5).

The present investigation was undertaken to improve the assay procedure with rats and to prepare liver fractions which would show higher activity

EXPERIMENTAL

All the experiments were made with male Sprague-Dawley rats 35 to 45 gm. in weight. Each animal was kept in a separate cage, and food and water were given *ad libitum.*

The ration used consisted of whole ground yellow corn 46.35 per cent, commercial soy bean meal 46.35 per cent, corn oil (Mazola) 5 per cent, cystine 0.3 per cent, $CaHPO_4$ 0.92 per cent, $CaCO_3$ 0.60 per cent, NaCl (iodized) 0.44 per cent, $MnSO_4 \cdot 4H_2O$ 0.04 per cent.

To each kilo of this ration the following amounts of vitamins were added: thiamine 3 mg., calcium pantothenate 20 mg., pyridoxine hydrochloride 2 mg., choline hydrochloride 1 gm., nicotinic acid 20 mg., folic acid 0.25 mg., biotin 0.1 mg., inositol 100 mg., *p*-aminobenzoic acid 250 mg. 1 drop of oleum percomorphum diluted with 3 parts of corn oil was fed once weekly

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by dropper. The level of nicotinic acid was high enough to counteract the growth-inhibiting effect of corn found by Krehl *et al.* (6).

A comparison of the growth responses of rats on this ration and on a synthetic ration with and without liver supplementation is given in Table I. The synthetic ration contained sucrose 73, casein (Smaco) 18, corn oil (Mazola) 5, Salts IV 4 per cent, and the same vitamin supplements as used in the natural diet. The rate of growth for the animals on the supplemented natural ration was greater than for those on the supplemented synthetic ration, although the rate of growth for the rats on the two unsupplemented diets was about the same. Consequently, the corn-soy bean meal diet was used in all of the assays.

Extracts of fresh liver were prepared as follows: 1 kilo of fresh beef liver was ground in a meat grinder and mixed with 2 liters of 95 per cent alcohol.

TABLE I

Comparison of Growth Response of Rats to Liver Extract When Fed Synthetic Ration Or Soy Bean Meal-Corn Ration*

Group	No. of animals	Diet	Supplement	Average weekly weight gain in 5 wks.
A	6	Synthetic		25.8
B	6	"	Liver extract	30.0
C	12	Soy meal-corn ration		23.5
D	12	" " " "	Liver extract	33.1

* 3 drops of an alcoholic extract of fresh liver equivalent to 0.45 gm. of dried fresh liver were fed daily by dropper.

The mixture was filtered through cheese-cloth and the material remaining in the cloth was pressed in a filter press. The residue was treated twice with 1 liter of 60 per cent alcohol, filtered, and pressed. The combined filtrates were distilled under reduced pressure to a volume of 100 ml., extracted three times with ether, and filtered. Traces of ether were removed by distillation under reduced pressure. 1 ml. of this extract was equivalent to 10 gm. of fresh liver. In the preliminary experiments, each rat received 3 drops of this preparation daily by dropper. This intake was equal to 1.5 gm. of fresh liver or 0.45 gm. of dried fresh liver per day.

Extracts of the dry liver powders were made by mixing 1 part of the powder with 4 parts of water and adding sufficient 95 per cent alcohol to give a 60 per cent alcohol solution. The mixture was stirred for 1 hour and placed in the cold room overnight. The insoluble material was filtered off and reextracted with 60 per cent alcohol. The filtrates were combined, distilled under a vacuum to a volume of 100 ml., extracted three times

with ether, and filtered. The final filtrate was concentrated under a vacuum to remove the ether, and the volume adjusted so that a few drops could be used as the daily supplement. All extracts were neutralized with 5 N NaOH before feeding.

Alcoholic extracts of fresh liver, lyophilized liver (Squibb), and defatted whole liver powder (VioBin) produced definite growth responses in rats when fed at daily levels equivalent to 0.11 to 0.45 gm. of dry liver (Table II, Groups 2, 3, 13, 14, 16).

A concentration of the active material contained in these extracts was attempted by adsorption on norit (Darco G-60). 100 ml. of the alcoholic extract were adjusted to pH 2 with concentrated HCl, filtered, and treated with 20 gm. of norit for 1 hour with stirring. The filtrate from the norit was treated with an additional 20 gm. of norit and the residues were combined. Most of the activity was removed, since the supernatant from the adsorption gave little growth, even when fed at 4 times the original level (Table II, Group 4). The elution was carried out by treating the norit with 150 ml. of a 50 per cent methanol solution containing 10 per cent ammonia. Since the first eluate contained little activity (Table II, Group 5), the elution procedure was repeated ten times. The more extensive elution method removed much of the activity from the norit (Table II, Groups 6, 17, 18). The most active preparation which produced a growth response in rats supplied approximately 2 mg. of dry matter per day per rat.

Several commercial liver preparations, which were found to be inactive when fed to rats at lower levels, showed good activity when concentrated by this procedure. Among these preparations was a Lederle liver powder, No. 1432, which was used in subsequent work.

Two groups of rats received an alcoholic extract of fresh liver $\cong$ 0.45 gm. of dried fresh liver per day, but the supplement of one group was heated in a boiling water bath for 15 minutes prior to feeding. Both extracts were equally active in stimulating growth at the levels fed (Table II, Groups 2 and 7).

In order to determine the minimum amount of alcoholic liver extract necessary to give growth stimulation in rats on the mixed diet, the original preparation was diluted 1:2 and 1:4. 3 drops daily of an extract, 1 ml. of which corresponded to 5 gm. of fresh liver, gave a maximum response, while a preparation equivalent to 2.5 gm. of liver per ml. produced nearly maximum response (Groups 13 and 14). The amount of this extract fed daily corresponded to 0.11 gm. of dry liver. Some of the activity remained in the residue after the alcoholic extraction, as shown in Group 15, Table II.

Bosshardt and associates (2) described experiments in which they observed improved growth and utilization of food protein in rats on a casein diet supplemented with 1:20 liver powder or a butanol extract of this pow-

TABLE II

Growth Response in Rats Fed Different Liver Preparations and Other Materials

Series No.	Group No.	No. of animals	Supplement	Level	Average gain per wk. (5 wks.)
					gm.
I	1	6	None		24
	2	4	Alcoholic extract of fresh liver	≅ 0.45 gm. dry liver per day	32
	3	6	Alcoholic extract of lyophilized liver (Squibb)	≅ 0.3 gm. dry liver per day	33
	4	6	Supernatant from norit adsorption of lyophilized liver	≅ 1.2 gm. dry liver per day	28
	5	4	First norit eluate of lyophilized liver	≅ 0.3 gm. dry liver per day	25
	6	6	Norit eluate from lyophilized liver	≅ 0.3 gm. dry liver per day	30
	7	4	Alcoholic extract of fresh liver boiled for 15 minutes	≅ 0.45 gm. dry liver per day	33
	8	4	Butanol extract of Lederle No. 1432 liver preparation	≅ 1.2 gm. dry liver per day	26
	9	4	Sharp and Dohme liver extract, No. 2505	≅ 3 drops per day	33
	10	4	Sharp and Dohme liver extract, No. 2505 injected	≅ 0.35 ml. per wk.	33
II	12	4	None		28
	13	4	Alcoholic extract of fresh liver	≅ 0.22 gm. dry liver per day	35
	14	4	Alcoholic extract of fresh liver	≅ 0.11 gm. dry liver per day	34
	15	4	Residue from alcoholic extract of fresh liver	≅ 3%	33
	16	4	Alcoholic extract of defatted whole liver powder (VioBin)	≅ 0.3 gm. dry liver per day	34
	17	4	Norit eluate of fresh liver	≅ 0.45 gm. dry liver per day	32
	18	4	" " " " defatted whole liver powder (VioBin)	≅ 0.3 gm. dry liver per day	34
	19	4	Butanol extract from alcoholic extract of fresh liver	≅ 0.45 gm. dry liver per day	28
	20	4	Residue from butanol extract	≅ 0.45 gm. dry liver per day	36
	21	4	Reticulogen, No. 360 (Lilly)	≅ 3 drops daily	36
	22	4	Fish press water	≅ 0.3 gm. dry material per day	33
	23	4	Norit eluate from alcoholic extract of fresh liver stored 6 wks.*	≅ 0.9 gm. dry liver per day	28

* This solution had been highly active at the time of its preparation.

der. We prepared a butanol extract of a powdered liver preparation (Lederle, No. 1432) which had been shown to be active in stimulating growth when concentrated by norit adsorption. This butanol extract was inactive in our assay when fed at a level corresponding to the amount of liver which had been used for the preparation of the active norit eluate (Table II, Group 8). In another experiment our alcoholic extract of fresh liver was extracted seven times with butanol, the butanol was evaporated, and the residue dissolved in water and freed from traces of butanol. This extract was brought to the same concentration as the residue solution with respect to fresh liver, and both solutions were fed to rats at a level of 0.45 gm. of dry weight of fresh liver per day. Only the residue solution showed growth-stimulating activity (Table II, Groups 19 and 20).

Two commercial anti-pernicious anemia liver preparations were tested; Sharp and Dohme, No. 2505 (15 U. S. P. units per ml.) and Lilly reticulogen, No. 360 (20 U. S. P. units per ml.). Both preparations showed full activity when given at a level of 3 drops daily. When given parenterally three times weekly, the Sharp and Dohme extract was equally active in the amount of 0.35 ml. per week (Table II, Groups 9, 10, 21). The possible relation of the unidentified factor stimulating rat growth and the pernicious anemia factor will be tested further.

Finally, a group of rats received the basal diet supplemented with fish press water, since this is known to cause stimulated growth in chicks when fed in addition to a soy bean-corn ration (7, 8). 0.3 gm. of fish press water was fed daily by dropper and a growth effect similar to that caused by liver supplements was observed (Table II, Group 22).

DISCUSSION

The experiments reported in the present paper confirm our earlier finding that the growth of rats fed a diet consisting mainly of whole yellow corn and soy bean meal and supplemented with all the known vitamins is increased when an extract of fresh liver is given. The growth difference between the controls and the liver-treated animals averaged 4 gm. per week on the purified ration and 7 to 10 gm. per week when the mixed ration was used. Nevertheless, there was no significant difference in the growth of the rats in the control groups. This differential effect may be due to the presence of small amounts of growth factors in the "vitamin-free" casein or to variations in the direct and indirect requirement of the animals fed the two diets. Spitzer and Phillips (9) have emphasized the difference between purified and soy bean rations for growth, reproduction, and lactation. Similar differences have been observed in the case of growing chicks (Patton *et al.* (10), and unpublished data).

The active factor in the alcoholic extract of liver is readily adsorbed on

norit, but the elution is difficult and probably incomplete. The first eluate from the norit contained much of the total dry matter adsorbed, but showed very little biological activity, whereas the subsequent treatments removed approximately 50 per cent of the activity from the norit.

It was found that the liver extract retained its activity when heated in a boiling water bath for 10 minutes. However, it must be pointed out that in this experiment an excess of the active factor was fed, since it was later established that, when an alcoholic extract equivalent to 0.11 gm. of dried fresh liver was fed daily to rats on the corn-soy bean diet, more than half maximum growth resulted under the conditions of the experiment. Spontaneous inactivation of some of the alcoholic liver extracts (Group 23), norit eluates, and fresh liver was observed in several instances during storage in the dark and at freezing temperature for relatively short periods of time.

Since no activity was detectable in the butanol-soluble fraction of liver preparations while the extracted residue retained activity, the active factor is probably different from that of Bosshardt *et al.* The fact that commercial concentrated anti-pernicious anemia liver preparations showed activity in rat growth warrants further investigations. The absence of growth-stimulating activity in the butanol extract is of significance in this connection, since Cohn, McMeekin, and Minot reported the anti-pernicious principle soluble in this solvent (11).

Fish press water, which stimulates growth of chicks when fed a natural ration (7, 8), was active when tested with rats. The possibility that this material contains a factor or factors similar to the active principle in liver will be tested in further experiments.

SUMMARY

The growth-stimulating effect of liver extracts has been studied in rats fed a synthetic and a corn-soy bean meal ration containing ten B vitamins and cystine. The effect of liver was more pronounced on the latter diet.

It has been shown that the active material can be adsorbed on and eluated from norit, and that it is soluble in ethanol but not in butanol. Commercial anti-pernicious anemia liver preparations showed considerable activity. A spontaneous inactivation of liver extracts was observed after storage in the cold. Activity was retained after short periods of heating. Fish press water showed a growth-stimulating activity in rats similar to that of liver.

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