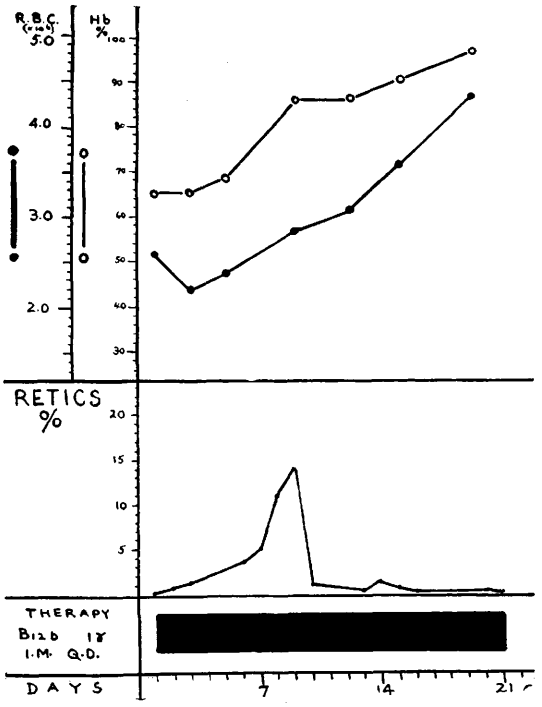

FIG. 2.
Hematological response of a patient with Addisonian pernicious anemia to vitamin B_{12b}. (Case 2).

the simultaneous addition of 150 ml of neutralized gastric juice daily for 12 days. The reticulocytes began to rise slowly on the 8th day to reach a peak of 3.4% on the 11th day. There was a concomitant red blood cell rise of 240,000 per cmm during this time, and slight but definite clinical improvement occurred. This is similar to the results obtained by Berk *et al.*(8) using vitamin B_{12}.

We observed one patient with megaloblastic anemia of pregnancy who had failed to respond to 3 weeks of therapy with 150 U.S.P. units of refined liver extract intramuscularly, and also showed no hematologic response to 1 μg daily of vitamin B_{12b} given intramuscularly in a 10 day period. The severe glossitis, unaffected by liver therapy, cleared completely on B_{12b}, and later there was a maximal hematologic response to pteroylglutamic acid. Several similar vitamin B_{12} refractory cases have been reported. The problem of different types of deficiencies occurring in megaloblastic anemias other than Addisonian pernicious anemia has been recently reviewed by Mueller *et al.*(9)

Summary. A pink fraction obtained from liver and from *Streptomyces aureofaciens* has been separated from vitamin B_{12} by silicic acid chromatography and has been shown to have different ultraviolet and visible absorption spectra. This new substance, vitamin B_{12b}, has been prepared in crystalline form and has been found to be effective parenterally in the treatment of patients with Addisonian pernicious anemia in amounts of 1 to 2 μg daily.

We are indebted to Mrs. Helen Jakubowski for performing the blood examination.

8. Berk, L., Castle, W. B., Welch, A. D., Heinle, R. W., Anker, R., and Epstein, M., *New England J. Med.*, 1948, v239, 911.

9. Mueller, J. F., Hawkins, V. R., and Vilter, R. W., *Blood*, 1949, v4, 1117.

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Sterols and Fatty Acids in the Nutrition of Entozoic Amoebae in Cultures.* (17529)

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The many excellent media which have been suggested for culture of entozoic amoebae suffer from one serious deficiency when used for quantitative studies. Statistically satisfactory reproduction of results is the exception rather

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TABLE I.
Relative Growth of *E. histolytica* with Various Serum Fractions.

Transfer	Control (LSB)	Serum fractions, % of total								
		A			B			C		
		10	5	1	10	5	1	10	5	1
1	4+	3+	2+	+	4+	3+	3+	3+	2+	2+
2	4+	3+	3+	2+	4+	4+	2+	+	+	+
3	4+	2+	2+	+—	4+	4+	3+	+	+	+
4	4+	+	+	+	3+	4+	3+	+—	+—	+—
5	4+	+—	+—	—	3+	3+	4+	—	—	—
6	4+	—	—	—	3+	3+	3+	—	—	—
7	4+	—	—	—	4+	4+	4+	—	—	—
8	4+	—	—	—	4+	4+	4+	—	—	—
9	4+	0	0	0	3+	4+	4+	0	0	0
10	4+	0	0	0	4+	4+	3+	0	0	0
11	4+	0	0	0	4+	3+	3+	0	0	0

4+ = 40+/1pf.
3+ = 30-40/1pf.
2+ = 15-30/1pf.
1+ = 1-15/1pf.
+— = less than 1/1pf.
— = No detectable growth.
0 = Series discontinued.

than the rule. A fluid medium of constant composition is an absolute requirement. For several years we have used a modification of the Cleveland and Collier(1) and Chang(2) media in conjunction with physically purified rice starch. The medium (LSB) and method of preparing the starch have been described in a previous paper.(3) A necessary component of the LSB medium was serum. Since this was necessarily obtained in the form of whole blood from an abbatoir we were faced with the tedious procedure of separation, clarification and sterilization by filtration. The final medium was also sterilized by filtration with the attendant risk of loss by contamination and of alteration of the bacterial flora of amoeba cultures when a lightly contaminated tube of medium was inadvertently used.

As Reardon and Rees(4) had shown that serum was not required in the over-lay for whole egg media and that cholesterol could substitute in part for egg yolk(5) we attempted without success to replace the serum of LSB medium with the recommended amount of cholesterol. We therefore subjected serum to fractionation.

Experimental. The serum proteins were precipitated by adding 5 vol. of ethyl alcohol at 0°C. The precipitate was exhaustively washed with cold alcohol and cold acetone. The dried precipitate was redissolved in M/30 phosphate buffer in 0.4% NaCl at pH 7.2 and brought to the volume of the original serum, (Fraction "A".) The pooled filtrates were evaporated to dryness and the residue extracted with absolute ethyl alcohol. The extract was reduced to 10 ml volume and added rapidly to a volume of buffered NaCl solution equal to that of the original serum. The alcohol was removed by boiling and the original volume restored with distilled water. The resulting emulsion was Fraction "B." The alcohol-insoluble residue was extracted with acetone and an emulsion prepared as above (Fraction "C".) The 3 fractions so obtained were added to the liver-infusion base of LSB medium in proportions corresponding to 10, 5 and 1% of whole serum. The medium containing Fraction "A" was sterilized by

1. Cleveland, L. R., and Collier, J., *Am. J. Hyg.*, 1930, v12, 606.

2. Chang, S. L., *Am. J. Trop. Med.*, 1942, v22, 471.

3. Griffin, A. M., and McCarten, W. G., *J. Parasit.*, 1949, v35, 193.

4. Reardon, Lucy V., and Rees, C. W., *J. Parasit., Suppl.*, 1939, v25, 13.

5. Rees, C. W., Bozicevich, J., Reardon, L. V., and Daft, F. S., *Am. J. Trop. Med.*, 1944, v24, 189.

filtration, the others in the autoclave for 15 minutes at 15 lbs.

All media were inoculated with the NRS strain of *Endamoeba histolytica* using the usual technic. The cultures were incubated at 37°C, examined and transferred at 48 hour intervals with the results shown in Table I. With the extracted proteins growth failed beyond the fourth or fifth transfer on repeated trials. The alcohol-insoluble, acetone-soluble fraction was even less effective. The alcohol soluble fraction, however, was apparently capable of supporting growth indefinitely. These results suggested that steroid or phospho-lipid components of serum might be the needed factor in spite of our previous failure to get persistent growth when serum was replaced with pure cholesterol. The basic liver infusion and completed medium (LSB) were analyzed for cholesterol and cholesterol esters by the method of Hess.(6) The infusion contained 0.023 mg cholesterol per ml and no detectable esters. LSB medium had 0.071 mg cholesterol and 0.130 mg cholesterol esters per ml.

We therefore substituted 0.15 mg per ml of cholesteryl oleate or palmitate for the serum of LSB medium, sterilized the media in the autoclave and tested them with the NRS strain as above. The cholesteryl oleate medium gave results at least equal to the serum medium and was perfectly clear though slightly opalescent.

As we refined our quantitative technic it became possible to assay the individual effects of components of the medium. We therefore tested the segregated action of the cholesterol and oleic acid moieties singly and in combination. Media were prepared containing cholesteryl oleate 0.15 mg per ml, cholesterol 0.09 mg per ml, oleic acid 0.06 mg per ml and the mixture of the last two. These were inoculated in duplicate with 100,000 trophic amoebae from a 48 hour culture of a strain of *Entamoeba terrapinae,* incubated for 48 hours at 30°C, and the yields determined. Subcultures were similarly inoculated where the yield was sufficient to provide 100,000 organisms, otherwise the entire sediment was transferred. *E. terrapinae* has been used for most of our quantitative studies as its growth parallels that of *E. histolytica* without being as sensitive to changes in the environment.

6. Hess, W. C., *J. Lab. and Clin. Med.*, 1947, v32, 1163.

TABLE II.
Comparison of Cholesteryl-Oleate and Cholesterol Media.
Mean yields of duplicate cultures × 10^{-4}.

	Media		
Transfer	Cholesteryl-oleate	Cholesterol	Mean
1	165	216	190
2	184	204	194
3	186	229	207
4	150	194	172
5	179	188	184
6	179	212	196
7	173	165	169
8	180	258	217
9	157	226	192
Mean	172	210	191

In the media containing oleic acid alone or mixed with cholesterol there was no growth. The initial inoculum was not recoverable and the cultures were free of amoebae after the second transfer. The results obtained with the successful media are given in Table II. Direct comparison with LSB medium was not possible since it had been abandoned in favor of the cholesteryl oleate medium. However, 40 similar cultures in LSB medium selected at random from previous work gave a mean yield of 173 x 10^4. The analysis of variance gives little reason to question the greater effectiveness of the cholesterinized medium ($F = 51.76$) and indicates that our previous failure to obtain growth with cholesterol alone was due to inadequate concentration. Transfers and interaction introduced some variability ($F = 3.66$ and 3.18 respectively) which can be traced to instability in the cholesterinized medium. The yields in cholesteryl oleate medium are notably homogeneous in successive transfers ($F = 0.86$) in contrast to those in cholesterinized medium ($F = 9.09$).

The unexpected finding that oleic acid by itself and in mixture with its equivalent of cholesterol was extremely toxic led us to a study of the quantitative nature of its action. Cholesterinized liver infusion was mixed with

TABLE III.
Effect of Oleic Acid on Growth of *E. terrapinae*. Mean yields of duplicate cultures $\times 10^{-4}$.

	Oleic acid, mg/ml						
Transfer	0.00	0.01	0.02	0.03	0.04	0.05	Mean
1	510	694	765	610	498	293	560
2	438	377	819	430	202	361	438
3	460	387	670	240	169	174	350
4	514	307	515	452	274	235	383
5	404	371	593	370	288	178	368
Mean	464	427	672	420	286	248	420

graded amounts of oleic acid up to 0.05 mg per ml and the media inoculated with 100,000 amoebae as before. The results over successive transfers are given in Table III. The highly significant effects are associated with concentrations of oleic acid ($F = 33.00$) and the comparison between the initial transfer and those following ($F = 45.01$). The slight heterogeneity existing among the second through fifth transfers and their interaction with concentration ($F = 2.64$ and 2.28 respectively) reflects the progressively poorer yields with oleic acid concentrations other than optimum.

Discussion. Our initial failure to substitute cholesterol for serum in LSB medium appears to be due to a quantitative effect. When supplied with less than some undetermined minimum amoebic growth fails. The stimulating effect of oleic acid in optimum concentration (0.02 mg per ml), together with the smaller yields obtained with cholesteryl oleate, suggests that cholesteryl oleate as such is not available to the amoebae but must be hydrolyzed to its constituent molecules.

Summary. Cholesterol in adequate amounts can be substituted for serum in liver infusion media for culture of entozoic amoebae, but the yields will be statistically heterogeneous in contrast with those obtained when cholesteryl oleate is used as a supplement. Oleic acid in optimum concentrations reinforces the action of cholesterol. It is suggested that cholesteryl oleate is not used as such but must be hydrolyzed to its constituent molecules.

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Beneficial Effects of Liver on Growth and Survival of Immature Rats Fed Lactose-Containing Diets. (17530)

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Available data indicate that the deleterious effects of rations containing high levels of lactose are dependent in considerable degree on the composition of the diet employed. Boutwell *et al.*(1) observed that rats fed a purified ration containing 48% lactose and 28% corn oil developed an unthrifty appearance and extensive diarrhea during the first 6 to 12 days of feeding. A similar ration containing 28% butter fat in place of the corn oil resulted in better growth, larger food intake and a good outward appearance. Furthermore, animals fed the butter fat ration had considerably less diarrhea than those on the corn oil diet.(2) A similar difference between animals fed butter fat and corn oil was observed by Ershoff and Deuel(3) in rats fed diets consisting solely of 70% lactose and 30% fat. Animals fed butter fat or margarine fat in conjunction with 70% lactose lived significantly longer than those fed a similar ration containing corn oil or cottonseed oil as the source of dietary fat. On a natural food ration, however, in which lactose was provided in the form of skim milk powder, no significant difference in growth was observed between rats fed butter

1. Boutwell, R. K., Geyer, R. P., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1944, v55, 153.

2. Boutwell, R. K., Geyer, R. P., Elvehjem, C. A., and Hart, E. B., *Arch. Biochem.*, 1945, v7, 143.

3. Ershoff, B. H., and Deuel, H. J., Jr., *Am. J. Physiol.*, 1947, v148, 45.