UNIDENTIFIED GROWTH FACTORS IN CHICK AND POULT NUTRITION

By

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Thesis submitted to the Faculty of the Graduate School
of the University of Maryland in partial
fulfillment of the requirements for the
degree of Doctor of Philosophy

1952

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ACKNOWLEDGMENTS

I wish to express my gratitude to Dr. Gerald F. Combs for his invaluable suggestions during the course of the work described herein. Acknowledgment is made to Dr. Fletcher P. Veitch for his advice concerning the initial phases of the fractionation procedures. I also want to thank Dr. Morley A. Jull and Dr. Mary S. Shorb for their interest in my problem. I am indebted to Dr. Peng-Tung Hsu for contributing the data showing the alkali-stability of the liver factor. My thanks are given to Miss Josephine A. T. Blair and Mr. James E. Sizemore for their technical assistance in some phases of the work presented here. Finally, I am deeply grateful to my wife, Ruth Gold Menge, for her many sacrifices that have made it possible for me to pursue the problem disclosed within this manuscript.

We are indebted to Merck and Company, Rahway, New Jersey, for crystalline vitamins, orotic acid, lyxoflavin, and the crude concentrates of vitamin B_{12}; Allied Mills, Inc., Portsmouth, Virginia, for soybean oil; Abbott Laboratories, North Chicago, Illinois, for halivor oil; Lederle Laboratories, Pearl River, New York, for synthetic folic acid; and U. S. Industrial Chemicals, Inc., for DL-methionine. The liver fraction "L" was furnished by Wilson and Company Laboratories, Chicago, Illinois, and the Bioper fraction was obtained through the courtesy of Armour and Company Laboratories, Chicago, Illinois. The Protamone and a sample of "grass juice factor concentrate" were presented by the Cerophyl Laboratories, Inc., Kansas City, Missouri. Crystalline vitamin B_t and crude concentrates of this vitamin were supplied through the courtesy of Dr. H. R. Carter, Department of Biochemistry, University of Illinois, Urbana, Illinois.

This work was supported in part by a Public Health Service research grant.

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INTRODUCTION

The attention of research workers has for many years been focused on the study and identification of growth factors found in certain crude feedstuffs. The term "animal protein factor" came into general usage as the name of the factor, or factors, found, for the most part, in products of animal origin. After the isolation of vitamin B_{12} in 1948 and reports that the pure vitamin could support growth equivalent to that obtained with crude animal protein supplements, it appeared that vitamin B_{12} was identical with the growth-stimulating factor, or factors, present in these crude materials. However, a number of recent reports have shown that the so-called "animal protein factor" is a complex consisting of vitamin B_{12} and one or more unidentified factors. It is apparent that both chicks and poults require an unidentified factor, or factors, for rapid growth.

The work presented here is a further study of the growth factor, or factors, required by the chick and the poult. Procedures for the fractionation and concentration of one of these factors are also presented. The methods were developed to prepare a highly potent concentrate of the unidentified factor as a basis for final isolation and identification.

REVIEW OF LITERATURE

A. Studies with Chicks

The isolation of vitamin B_{12} by Rickes, Brink, Koniuszy, Wood, and Folkers, (1948) and Smith (1948) has made possible a more thorough and complete study of unidentified dietary factors required for rapid growth of chicks and poults. Ott, Rickes, and Wood (1948) found that under their experimental conditions crystalline vitamin B_{12} produced a growth response comparable with that obtained with various crude supplements of animal origin. Lillie, Denton, and Bird (1948) confirmed these observations and also obtained a similar response from concentrates of the cow manure factor as described by Rubin and Bird (1946). Later, Nichol, Dietrich, Cravens, and Elvehjem (1949) reported that vitamin B_{12} was as effective in promoting chick growth as condensed fish solubles or injectable liver preparations. Thus much of the early work involving the growth-stimulating activity of certain animal protein feedstuffs can be attributed to the presence of vitamin B_{12} in these materials.

However, it is difficult to explain entirely on the basis of their vitamin B_{12} content the growth responses obtained with dried whey (Berry, Carrick, Roberts, and Hauge, 1943; Hill, Scott, Norris, and Heuser, 1944; and Hill, 1948), distillers dried solubles (Synold, Carrick, Roberts, and Hauge, 1943; Hill, Scott, Norris and Heuser, 1944; and Novak, Hauge, and Carrick, 1947), or dried brewers yeast (Schumaker, Heuser, and Norris, 1940; and Hill, Scott, Norris and Heuser, 1944).

Moreover, unidentified chick growth activity has been demonstrated for dried whey and certain liver preparations by Menge, Combs, and Shorb (1949), and for fish meal and dried brewers' yeast by Carlson, Miller, Peeler, Norris, and Heuser (1949) after adequate amounts of vitamin B_{12} were supplied. Furthermore, studies conducted by Combs, Carlson, Miller, Peeler, Norris and Heuser (1950) have revealed the presence of two growth-promoting substances in fractions prepared from a refined liver paste dialysate which are not identical with vitamin B_{12} or any of the known required nutrients. These workers obtained some evidence of a mutual supplementary effect by feeding combinations of fractions containing these unknown growth substances.

Sunde, Cravens, Elvehjem, and Halpin (1950a) presented evidence which suggested that corn, dextrin, or a combination of yeast and whey are good sources of a chick growth factor, or factors. These workers modified the synthetic type diet used by Carlson, Miller, Peeler, Norris, and Heuser (1949) by substituting sucrose for glucose and found that this basal ration would not support growth or livability of chicks even when such supplements as whole liver powder, fish meal, condensed fish solubles, dried whey, refermented distillers solubles, alfalfa leaf meal, soybean meal (10 percent), or APF concentrates containing aureomycin and streptomycin residues were added. However, a normal rate of chick growth was obtained when the basal ration was supplemented with either of the following feedstuffs: 10 percent wheat middlings, 10 percent wheat bran, 20 percent ground yellow corn, 10 percent distillers solubles, 5 percent yeast

extract (Difco), or a combination of 10 percent torula yeast plus 10 percent dried whey. Substitution of autoclaved corn starch (dextrin) for sucrose in the basal ration also resulted in satisfactory growth and no mortality. These observations suggest, therefore, (1) that corn, wheat bran, or middlings, autoclaved corn starch, or yeast plus whey contain a growth factor different from that supplied by such materials as liver products, fish meal and fish solubles, or (2) that the type of carbohydrate supplied in the ration exerted a favorable influence on the bacterial synthesis of important nutrients in the intestinal tract.

Hill and Briggs (1950), using a purified type ration, have obtained somewhat similar results. The observations of these workers indicate that corn meal and soybean oil meal contain an unidentified chick growth factor when fed at levels of 10 to 25 percent. Since increased chick growth was obtained with corn meal, ground corn cobs, as well as levulinic acid and xylose, it was suggested that the factor may be associated with carbohydrate fractions. Whole liver substance and dried brewers' yeast also appeared to possess growth activity for the chick. An ethanol-soluble, heat and alkali-stable component which promoted growth of chicks was extracted from dextrin by Dietrich, Monson, and Elvehjem (1951). These workers used a purified diet containing sucrose as the source of carbohydrate. Growth activity was also obtained from the addition of yeast, whole liver powder, bran, casein, and dried whey. Savage, O'Dell, Kempster, and Hogan (1950) also using a synthetic type diet, found that a liver preparation

stimulated growth of chicks. These workers reported evidence to indicate that soybean oil meal contains the same growth-promoting factor as the liver fraction used in their studies. Cravens, Bruins, Sunde, and Snell (1951) using an alpha protein, purified diet, have presented evidence for two different unidentified chick growth factors. One of these was found in torula yeast while the other was present in wheat bran. Animal products tested were not good sources of either factor.

Using a practical type chick starter, Sunde, Cravens, Elvehjem, and Halpin (1950b) obtained evidence which indicated the presence of an unidentified chick growth factor in certain liver products and fish solubles. Johnson (1950) found that the addition of dried whey to a practical type chick starter containing an adequate level of vitamin B_{12} promoted the growth of chicks when the diet contained 37.5 percent soybean oil meal. However, no response was evident when the meal was increased to a level of 70 percent. These results are indicative of the presence of a chick growth factor in certain soybean oil meals and are in agreement with the observations of Hill (1948). Reed and Couch (1950), Hill and Branion (1950), and Reed, Atkinson, and Couch (1951) have also reported results which indicated that dried whey contains an unidentified chick growth factor. Arscott and Combs (1950) found that when either fish meal, or butyl molasses solubles were added singly at graded levels to a corn-soybean meal chick starter, the growth responses obtained were suboptimal. However, a combination of these materials elicited responses that were significantly greater than when either supplement was used alone.

Dried whey, and dried brewers yeast also stimulated increased growth, but the combination of either of these substances with butyl molasses solubles did not result in more rapid chick growth. These observations indicate the presence of two different unidentified growth factors, one of which is present in fish meal and the other in butyl molasses solubles, dried whey, and dried brewers' yeast.

The presence of a substance in green leaves of alfalfa and cereal grasses possessing chick growth activity was reported by Kohler and Graham (1950). These workers found whole liver, liver fraction "L," whole pork liver, and dried brewers' yeast to be good sources of the factor, whereas, dried whey, dried distillers' solubles, and fermentation solubles were found to be deficient in the factor. Evidence was also presented which indicated that condensed fish press water contains an additional unidentified chick growth factor which is not present in the "grass juice factor" concentrate or fish meal.

Novak, Hauge, and Carrick (1947) reported distillers dried solubles to contain an unidentified chick growth factor. A method of obtaining this factor (named Vitamin B_{13}) from distillers dried solubles, rice polishings concentrate, and liver extract in a highly purified state was described by Novak and Hauge (1948a), and (1948b). Later, Austin and Boruff (1949) prepared a vitamin B_{13} concentrate from dried distillers solubles and found it to stimulate a growth response in chicks equivalent to that of the original material.

Stokstad, Jukes, Pierce, Page, and Franklin (1949) reported the presence of an unidentified chick growth factor in a fermentation product of bacterial origin. Later, Stokstad and Jukes (1950a)

noted this growth stimulation to be due, at least in part, to aureomycin. McGinnis, Stephenson, Levadis, Carver, Garibaldi, Ijichi, Snell, and Lewis (1949) also demonstrated that APF concentrates from certain fermentation residues contain a growth factor for poults and chicks which is different from vitamin B_{12}. Since these products contained antibiotics, the growth responses obtained may thus be explained largely on the basis of their antibiotic content. The mode of action of antibiotics within the intestine of the animal may be concerned with the establishment of a"desirable" type of microflora which, in turn, may exert an indirect effect on the growth of the animal through the synthesis of important nutrients. The fact that antibiotics may exert a definite influence on the intestinal flora was discussed by Groschke (1950), who also presented evidence to suggest that the "whey factor" may be one of the factors synthesized by the so-called "desirable types." The further addition of high levels of five B-complex vitamins augmented intestinal synthesis of this factor. These results were substantiated through observations noted by Jones and Combs (1951). Corn-soybean meal rations adequate in all known required nutrients containing streptomycin, or procaine penicillin G, were found to support growth of chicks similar to that obtained when fish meal, dried brewers' yeast, or butyl molasses solubles were also included. These supplements, however, did improve the growth when added to rations which did not contain an antibiotic. These results clearly show that antibiotic supplementation reduces the requirement of the chick for

unidentified growth factors present in these feedstuffs. Romoser, Shorb, Jones, Combs, and Pelczar (1951) also presented evidence which suggested that bacterial synthesis of an unidentified growth factor(s) is responsible in part for the improved chick growth as a result of antibiotic feeding.

A similar review of literature concerning unidentified growth factors for chicks was presented at the 1951 World Poultry Congress (Combs, 1951a).

B. Studies with Poults

The ability of various crude animal protein materials to supplement diets containing corn and soybean oil meal as the major source of protein has not been investigated with turkeys as thoroughly as with chickens. However, the requirements of the turkey poult for the most nutrients bear a fairly direct relationship to those of the chick. The requirements of the turkey in most instances are higher than those of the chick approximately in proportion to the increased rate of growth of the turkey over that of the chick.

Hammond and Marsden (1939) found that the growth of poults was improved when the diet contained at least 7.5 percent crude protein from animal sources. Later, Bird, Marsden, and Kellogg (1948) reported that fish meal was more effective than meat meal for growth of poults during the first 6 weeks of life. During the later stages of growth, either fish or meat meal was effective in meeting the requirements of the poult for vitamin B_{12}. Lillie, Marsden, Groschke, and Bird (1949) stated that fish meal was superior to meat meal because it contained a larger amount of vitamin B_{12} and that meat meal was satisfactory for the latter stages of growth because the poults required less of the vitamin at this time. However, Hammond, Haynes, Marsden, and Titus (1944) reported that diets which contained soybean meal or a combination of soybean and peanut meal supported as rapid growth of poults as a control mash which contained 7 percent meat scrap and 14 percent fish meal. Fritz, Halpin, and Hooper (1947) also found that poults which received soybean meal diets grew as

rapidly as those which received diets containing substantial quantities of animal protein.

The addition of fish solubles to a diet which contained milo maize, soybean meal, and 4 percent sardine meal was found by German, Schweigert, Pearson, and Sherwood (1948) to improve the rate of growth of poults. Later, Richardson and Blaylock (1950) reported that the response of poults and growing turkeys to the addition of fish meal, fish solubles, or an APF concentrate to a milo-soybean meal diet was variable. Fish meal, fish solubles, and an APF concentrate exhibited a marked growth response in some trials and no response in others. Johnson (1950) found that poults reared on litter that was changed infrequently did not show a growth response to fish solubles or dried buttermilk. It is conceivable that fish solubles or other animal protein supplements may have given a growth response had the poults been raised on wire floors and not had access to droppings.

McGinnis, Stephenson, Levadie, Carver, Garibaldi, Ijichi, Snell, and Lewis (1949), Singsen and Matterson (1949), Stokstad and Jukes (1950b), Atkinson and Couch (1951), and Patrick (1951) have reported that crystalline vitamin B_{12} or vitamin B_{12} concentrates did not supplement an all vegetable protein turkey starter. However, a crude "animal protein factor" supplement prepared from aureomycin mash (McGinnis, Stephenson, Levadie, Carver, Garibaldi, Ijichi, Snell, and Lewis, 1949; Singsen and Matterson, 1949; Stokstad, Jukes, Pierce, Page, and Franklin, 1949), and crystalline aureomycin hydrochloride (Stokstad and Jukes, 1950c)

was found to produce growth responses in poults in excess of the responses produced by vitamin B_{12}. These results were confirmed and extended by Almquist and Merritt (1951) who obtained similar results with a different basal diet, a different variety of turkeys, and with separate sexes.

Combs and Shaffner (1950) reported that poults require an unidentified factor, or factors, for rapid growth. Dried brewers' yeast, menhaden fish meal, and crab meal were found to be good sources of this growth factor. The vitamin B_{12} supplements used in these studies were of no value in stimulating growth. Patrick (1951) also found that turkey poults did not respond to supplements of synthetic vitamin B_{12}. The poults did, however, exhibit a growth response to supplements of fish meal, fish solubles, and methanol solubles fraction of fish solubles. It was concluded that the requirements of the poult for vitamin B_{12} was much less than for chicks, and that the response of poults to crude fractions containing vitamin B_{12} suggests that the poult requires an unidentified factor(s) for adequate nutrition.

Aureomycin, streptomycin, an APF concentrate containing aureomycin, liver "L," fish meal, and fish solubles were found by Atkinson and Couch (1951) to be effective in stimulating growth of poults. The APF concentrate, liver "L," fish meal, and fish solubles were postulated to contain unidentified growth factors for the poult. McGinnis, Berg, Stern, and Carver (1951a) reported that the addition of the antibiotics, penicillin, terramycin, and aureomycin to a diet for starting poults containing fish meal resulted in a marked increase

in weight gain over that exhibited by the poults receiving the corn-soybean meal basal diet alone, or in combination with either of the antibiotics or fish meal. These results indicated that certain crude products contain an unidentified growth factor.

Scott, Heuser, and Norris (1948) presented results showing that turkey poults receiving a practical-type, high energy ration fortified with known vitamins and animal protein supplements required, in addition, an unidentified growth factor found to be present in crude casein. Since this factor appears to have properties similar to those of factor S, it was assumed that these factors are identical. Factor S was found by Scott, Norris, and Heuser (1947) to possess growth activity for the chick. Later Scott (1951) demonstrated that poults require an unidentified factor or factors present in grass and alfalfa juice, dried skim milk, dried distillers' solubles, and dried brewers' yeast. Animal products, including liver, were shown to be poor sources of the factor. This would indicate that this factor is not a member of the animal protein factor complex. However, the finding that grass and alfalfa juices are excellent sources of this growth factor for poults indicates that the factor may be identical with the "grass juice factor" discovered by Kohler, Elvehjem, and Hart (1936). The results of Sizemore and Combs as presented by Combs (1951b) show a consistent growth response by poults to a factor, or factors, present in fish meal, dried brewers' yeast, dried whey, butyl molasses solubles, butyl grain solubles, and liver fractions. Combinations of these materials used failed to elicit an increased rate of growth.

The work presented in the present paper is the result of a study of the requirement of the chick and the poult for an unidentified growth factor, or factors. The development of fractionation methods and procedures is also shown. These procedures were developed to prepare a highly potent concentrate of the unidentified factor, or factors, as a foundation for its ultimate isolation and identification.

EXPERIMENTAL PROCEDURE

A. Studies with Chicks

Day-old New Hampshire chicks of both sexes were used in all chick studies. These chicks were obtained from the breeding flock maintained at the University of Maryland. The chicks used in experiments 1, 8, and 10 through 14, were progeny of hens maintained on raised wire floors and fed a ration containing no animal protein supplement. Chicks from dams kept on litter and fed a complete breeder ration were used in experiments 2 through 7, and experiment 9. These hens had been in production for 10 to 12 months at the time eggs were collected for hatching. The chicks used in experiment 8 were from two different sources. Some of the chicks were progeny of pullets in their fourth month of production while others were progeny of hens in their second year of production. The chicks used in the countercurrent distribution studies (experiments 18, and 19) were progeny of hens in their second year of production. These dams were housed on wire floors and fed a soybean meal ration containing no animal protein supplement.

The purified-type basal rations used in most of the chick studies are shown in Table I. An isolated soybean protein (alpha protein, Glidden and Company, Chicago, Illinois) was used as the source of protein in diets R-123, R-124, and R-126. Diet R-123 was employed in experiments 11 through 13. The protein level of this ration is calculated to be 35.7 percent. Diet R-124 which was used in

experiments 1 through 5, and also in experiments 9, 10, and 14 differed from R-123 primarily in that the protein level was lowered to approximately 21.9 percent by reducing the amount of alpha protein. The wheat gluten protein diet R-125 was employed in experiments 6 and 7. This ration was calculated to contain approximately 22.5 percent protein. Diet R-126, a modification of R-123, was used in experiment 8 and in one of the countercurrent distribution studies (experiment 18). This diet, R-126, was calculated to contain 37.6 percent protein.

Mineral mix 185 as described by Briggs (1946) contains the following minerals per 100 grams of diet: calcium carbonate 1.5 gm., tricalcium phosphate 1.3 gm., dibasic potassium phosphate 0.9 gm., dibasic sodium phosphate 73 gm., sodium chloride 0.88 gm., magnesium sulfate 0.50 gm., ferric citrate 0.14 gm., manganous sulfate (monohydrate) 41 mg., potassium iodide 4 mg., zinc chloride 2 mg., copper sulfate (anhydrous) 1.3 mg., boric acid 0.9 mg., and cobalt sulfate 0.1 mg. Since the chick feces were quite "watery," this mineral supplement was modified to contain 0.50 gm. sodium chloride and 0.25 gm. of magnesium sulfate instead of the amounts prescribed.

Preliminary studies have revealed that additional thiamin is required in chick diets containing alpha protein. This is necessary since this protein isolated from soybeans contains a small amount of sodium sulfite, which inactivates thiamin through cleavage. In addition to supplying 1 mg. of thiamin per 100 gm. of diet, every precaution was taken to keep the diet as dry as possible.

A premix for each trial was made containing the minimum amount of each ingredient common to all diets used. Water solutions of thiamin, riboflavin, calcium pantothenate, niacin, pyridoxine, and

TABLE I

COMPOSITION OF PURIFIED DIETS USED IN CHICK STUDIES

Dietary ingredient	R-123 %	R-124 %	R-125 %	R-126 %
Alpha protein	40.00	23.00	-	40.00
Wheat gluten	-	-	22.00	-
Glucose (Cerelose)	45.00	65.02	68.08	49.55
Ruffex	5.00	-	-	-
Soybean oil	3.00	4.00	-	-
Corn oil	-	-	1.50	1.50
*A-D oil (3000A)(400D)	0.50	0.50	0.50	0.50
**Mineral mix 1 M	6.00	6.00	6.00	6.00
DL-methionine	0.50	0.30	0.40	1.00
L-cystine	-	0.30	-	-
Glycine	-	1.00	-	1.40
L-arginine-HCl	-	-	0.60	-
DL-lysine-HCl	-	-	1.50	-
L-leucine	-	0.10	-	-
Protamone	0.03	0.03	0.05	0.03
Phosphoric acid	-	0.0058	0.0058	0.0058
	(milligrams per 100grams of diet)			
Thiamin HCl	1.00	1.00	0.50	1.00***
Vitamin B_{12}	(as indicated)	.003	.002	.002
Riboflavin	1.00	1.00	1.00	1.00
Calcium pantothenate	2.00	2.00	2.00	2.00
Pyridoxine HCl	0.60	0.60	0.60	0.60
Niacin	5.00	5.00	5.00	5.00
Folic acid	0.30	0.30	0.30	0.30
p-Aminobenzoic acid	0.20	0.20	0.20	0.20
Menadione (vitamin K)	0.50	0.50	0.50	0.50
Biotin	0.02	0.02	0.02	0.02
Choline chloride	200.00	200.00	200.00	200.00
i-Inositol	100.00	100.00	100.00	100.00
*alpha-Tocopherol acetate	0.50	0.50	0.50	0.50
Crude protein (calc. %)	35.70	21.90	22.50	37.60

* 1200 I.U. vitamin A, 170AOAC units vitamin D and 0.5 mg. of alpha-Tocopherol acetate were administered by dropper, weekly, in addition to that supplied in the diet.

** Mineral mix 1 M (Briggs,1946) is described in Experimental procedure.

***Additional thiamin was also given each week by dropper at the rate of 0.5 mg. per 100 gm. feed consumed.

choline chloride, alcoholic solutions of biotin, folic acid, p-amino benzoic acid, and Menadione, and water solutions of the trace minerals were mixed in the premix. Soybean oil, or corn oil (Mazola), and alpha-tocopherol acetate were also mixed as part of the premix. The remaining amounts of each ingredient including the prescribed supplements were then mixed with a proportionate amount of premix to give each complete experimental ration. The diets were prepared a few days before their intended use and kept refrigerated to minimize vitamin destruction. The alpha protein was not included in the preparation of the premix. In this manner, it was possible to add the alpha protein to a thoroughly mixed and comparatively dry premix, thus minimizing the possibility of a reaction between the sodium sulfite and the thiamin.

Vitamin A (1200 I.U.) and alpha-tocopherol acetate (0.50 mg.) was also administered by dropper to each chick at weekly intervals in addition to that contained in the ration. This practice was designed to insure an adequate supply of these vitamins, and to compensate for probable losses that may occur as the feed remains in the hoppers prior to consumption. A special effort was made to keep the feed in the hoppers as fresh as possible by placing only enough feed before the chicks that could be consumed in a short period of time. The chicks in experiments 8 and 18 which received the high level alpha protein diet (R-126) were also given additional thiamin orally at weekly intervals during the course of each trial.

A practical-type soybean meal chick starter was used in experiment 19. This diet as shown in Table II is calculated to contain 22.5 percent protein.

TABLE II

COMPOSITION OF PRACTICAL TYPE DIETS USED IN CHICK AND POULT STUDIES

Dietary ingredient	Chick starter %	Poult starter %
Gr. yellow corn	61.12	45.35
Soybean oil meal (50%)	34.00	48.00
Limestone	2.00	-
Multifos (32% Ca - 18.5% P)	-	3.75
Calcium carbonate	-	1.50
Dicalcium phosphate	2.000	-
Manganese sulfate (mono-hydrate)	0.025	0.05
NaCl (iodized)	0.500	0.50
Cod liver oil (3000A, 400D)	0.250	0.75
DL-methionine	0.075	-
Protamone (iodinated casein)	0.030	0.05
	(milligrams per kilo of diet)	
Biotin	-	0.154
Menadione (vitamin K)	0.44	0.660
Folic acid	-	1.100
Pyridoxine-HCl	-	3.300
Riboflavin	3.52	4.400
alpha-Tocopherol acetate	-	4.950
Calcium pantothenate (dextro)	4.40	11.000
Nicotinic acid	22.00	33.000
Choline chloride	440.00	1540.000
APF Supplement #3	-	2200.000*
Crystalline vitamin B_{12}	0.02	-
Crude protein (calc. %)	22.50	28.00

*Calculated to supply 60 micrograms vitamin B_{12} per kilogram of diet.

Each of the basal rations used in these studies were considered adequate in all known required nutrients. Substitutions in all of the diets were made at the expense of the carbohydrate (purified diets) and the corn (practical-type starter) with appropriate corrections for the total protein content of the ration.

The experimental periods employed in these studies varied from 17 days to 5 weeks as indicated in the tables. A preliminary period of depletion was also used in some trials. The duration varied from 1 to 2 weeks as indicated. During this time, the chicks received the unsupplemented basal diet. At the start of each experimental period the chicks were weighed individually, wing-banded, and distributed into uniform groups on the basis of body weight and general appearance.

The chicks were reared in electrically heated batteries with raised wire floors for the entire experimental period. Feed and water were made available to the chicks at all times. The batteries used were of the single unit type placed in tiers of five. Every attempt was made to minimize experimental error due to position in the batteries. Individual weights were taken at weekly intervals.

The tables show the average gains in grams for each group for the designated experimental period. The percentage increased gain attained by the supplemented groups in excess of that exhibited by the negative control groups is given in the tables. The tables also show the percentage total solids contributed to the basal diet by each of the prepared fractions and the original material.

Liver fraction "L," liver fraction Biopar "C," dried whey, dried brewers' yeast, and butyl molasses solubles (Paco 250) were used as

sources of unidentified growth factors. Liver fraction "L" was obtained from Wilson and Company, Chicago, Illinois, and is the 70% alcohol-insoluble portion of the total aqueous extract of raw liver subjected to enzymatic action. Liver fraction Hiopar "C" was obtained from Armour and Company, Chicago, Illinois, and is the 70% alcohol-insoluble portion of a hot water extract of liver. The alkali-treated liver fraction "L" used in experiment 1 was prepared by adjusting an aqueous solution of liver "L" to pH 11.5 with sodium hydroxide, steaming at 100°C. for 30 minutes, followed by neutralization with hydrochloric acid. The excess moisture was then removed by evaporation.

The dried whey used in experiments 1, 2, 5, and 7 was a dried whey-product with whey fermentation solubles (Ribolac) obtained from Western Condensing Company, Appleton, Wisconsin. A commercial dried whey was used in experiments 3, 4, and 6. Two different samples of delactosed whey were used in experiment 8. One of these was obtained from National Dairy Company, Oakdale, Long Island, New York and is designated as delactosed whey "D," while the other was supplied by Western Condensing Company and is designated as delactosed whey "C." Neither of these samples contained whey fermentation solubles. Delactosed whey "C" was also used in experiment 18 as a source of the "whey factor."

The crystalline vitamin B_{12} was obtained from Merck and Company, Inc., Rahway, New Jersey. The Protamone (iodinated casein) used in all studies was provided by the Cerophyl Laboratories, Inc., Kansas City, Missouri. This material has a potency of approximately 3% thyroxine as determined by the manufacturer. The "Ruffex" used in diet 7-123

is a roughage material distributed by the Fisher Scientific Company, Pittsburgh, Pennsylvania. It is processed from rice hulls and contains 70% alpha cellulose, the balance being simple and hydrocelluloses. It is reported to contain neither proteins, fats, nor vitamins and gives an ash value of less than 1 percent.

B. Deutectomy

A simplified technique for the removal of the yolk from day-old chicks was developed in this laboratory. This procedure which was designed to render the chicks more nearly deficient in the nutrients transmitted from the dam, was used in experiments 9 through 13. The yolk-removal technique (Menge et al., 1951b) follows: The chick is grasped by the legs and placed on its back with the umbilicus toward the operator. It is held in this position with its legs well forward during the entire operation. A solution of 0.5% phenol in 95% alcohol is used to saturate a small area about the umbilicus. This tends to sterilize the locus and to keep the down in place during the operation. The solution is also used to clean the instruments at regular intervals. A small pair of tweezers is inserted into the umbilicus to enlarge the opening, and an incision of approximately one-eighth inch is made in the anterior portion with small, sharply-pointed scissors. The entire opening, including the umbilicus, should not be over one-fourth inch in length. If the incision is too long, considerable difficulty will be experienced in keeping the intestines within the body cavity especially after the yolk sac has been removed. On the other hand, if the opening is too small, it may be quite difficult to tease the yolk sac through the incision without tearing. Extreme care must be exercised in making the incision so that only the skin, the muscular wall, and the peritoneum are cut, while the thin membrane of the yolk sac is left intact. The yolk membrane with its contents is usually visible after this incision is made, for it will tend to force its way through the opening after the peritoneum has been severed. The yolk is easily recognized

by its yellow color. Every attempt is made to stimulate the chick to chirp, since this creates an internal pressure which forces the yolk through the opening.

The yolk sac is grasped by the fingers and carefully teased through the opening until the yolk stalk, which joins the yolk sac to the intestine, becomes visible. Instruments have been used with little success in the actual removal of the yolk because the yolk membrane itself is very delicate and is easily ruptured. A certain amount of tearing will be experienced, but in such cases the membrane can be removed with as much of its contents as possible and the remainder of the yolk material forced out of the body cavity by application of gentle pressure to the abdomen of the chick. A small spatula is placed over the cavity and at the juncture of the yolk stalk and intestines. The yolk stalk is then severed with a cauterizing needle. The spatula blade as used above serves to prevent the intestine from being forced through the body cavity and also shields the intestine from the heat of the cauterizing instrument. The incision is then closed with a metal suture.

Immediately following the operation, the chicks should be provided a good source of heat in order to lessen the effect of shock. Most of the chicks exhibit no signs of discomfort within an hour after deutectomy.

The fact that the yolk sac is removed through a natural opening contributes to the success of the operation. There appears to be less chance of tearing the yolk membrane and the skin by utilizing the umbilicus in this fashion, for it is here that the muscles have been

separated naturally to allow the yolk sac to enter the body cavity of the chick. This type of incision can also be easily closed. After a certain amount of practice, from two to four minutes were found to be required to deutectomize each chick. The mortality observed throughout was only 4% in excess of that noted in unoperated chicks.

Two preliminary experiments were conducted to study the effect of deutectomy on early nutritional requirements of the day-old chick (Table VIII).

Diet R-124 was used in these trials (experiments 9 and 10). The chicks used in experiment 9 were the progeny of hens receiving a ration containing 4% fish meal. Six uniform groups, each containing 20 day-old chicks, were employed. One-half of the chicks were deutectomized, while the other half used as controls in duplicate groups were not deutectomized. The negative control groups in each case did not receive supplementary vitamin B_{12}. The total amount of vitamin B_{12} (mcg.) that each chick of the supplemented groups received by intramuscular injection is given in the table. This amount was administered as follows: 0.6 mcg. on the first and eighth day and 1.2 mcg. on the 15th, 22nd, and 29th day of the experiment.

The chicks employed in experiment 10 were from hens receiving a ration which contained no animal protein concentrates. Seventy-five chicks, 50 of which were deutectomized, were fed the vitamin B_{12}-deficient diet for a 2-week preliminary period. During this time each chick received 0.6 mcg. of vitamin B_{12} by intramuscular injection on the first and eighth day. At the end of this time, one group of unoperated chicks and two groups of deutectomized chicks were selected.

Each group consisted of 16 chicks, comparable on the basis of general appearance and body weight. The total amount of vitamin B_{12} (mcg.) administered by intramuscular injection is given in the table. The chicks given 4 mcg. of vitamin B_{12} received injections of this vitamin as follows: 1.2 mcg. on the first and eigth day and 1.6 mcg. on the 15th day of the 3-week experimental period. The chicks given 8 mcg. of vitamin B_{12} received twice the amount of the vitamin at each injection.

Three experiments were conducted with deutectomized chicks (Table IX). Diet R-123 was used in experiments 11 through 13. The chicks were the progeny of hens maintained on raised wire floors and fed a ration containing no animal protein supplement. A preliminary period of 1 week followed by an experimental period of 2 weeks was employed in experiments 11 and 12, whereas, a preliminary period of 2 weeks with an experimental period of 17 days was used in experiment 13. The chicks in experiments 11 and 12 each received 0.6 mcg. vitamin B_{12} by intramuscular injection at hatching time and at weekly intervals during the course of each trial. Vitamin B_{12} at the rate of 30 mcg. per kilo of diet was given in experiment 13.

C. Studies with Poults

The day-old poults used in experiments 15, 16, and 17 were the progeny of Beltsville White matings and matings between Bronze and Beltsville White. Each mating was equally represented in all experimental groups. The poults were from hens maintained on a complete turkey breeder ration at the University of Maryland. The basal ration used in these studies is shown in Table II. This diet is calculated to contain 28 percent protein. Vitamin B_{12} was supplied by Merck's AFF supplement #3 (60 micrograms per kilogram of diet).

A 4-week experimental period was utilized in experiments 15 and 17, and a 3-week experimental period in experiment 16. The poults were maintained in electrically heated batteries with raised wire floors during the experimental period. Feed and water was made available at all times. The batteries used were of the single unit type placed in tiers of five. Every attempt was made to minimize experimental error due to position in the batteries. Individual weights were taken at weekly intervals. Table XI shows the average gains in grams for each group during the experimental period. The percentage increased gain attained by the supplemented groups in excess of that exhibited by the negative control groups is also given. The tables also show the percentage total solids contributed to the basal diet by each of the prepared liver fractions and the original material.

Liver fraction Biopar "O" was used as the source of the unidentified growth factor in these trials. This material is the 70%

alcohol-insoluble portion of a hot water extract of liver. The preparation of the liver fractions used in both the chick and the poult studies is presented in the following section.

D. Preparation of Fractions

1. Dialysis (fraction 1). Four hundred grams of liver "L" or Biopar "C" were stirred to a thick paste after the addition of 600 ml. of distilled water. This mixture was then placed in a cellophane sausage casing that had been previously soaked in distilled water. The casing and its contents were then suspended in four liters of distilled water at room temperature. Toluene was used to cover the surface of the dialysate to keep bacterial infection to a minimum. The water was changed three times at 24-hour intervals, filtered, and concentrated *in vacuo* to a convenient volume separately before being combined and concentrated to 400 ml. by vacuum distillation on a steam bath.

2. Phenol, and water soluble fractions. Four hundred ml. of dialysis (1) were placed in a separatory funnel containing an equal quantity of a phenol-water solution (72% phenol, 28% water) and shaken. This mixture formed a two-phase solubility system of phenol and water after reaching equilibrium. The water layer (a) was removed and the residue (b) washed three times with equivalent amounts of water. These washings were combined with the water layer (a) and washed twice with equivalent quantities of a phenol-water solution. The resulting phenol-water washings were combined with residue (b) and labeled phenol soluble of (1). The water layer (a) was designated phenol insoluble of (1). Each of these fractions were washed with ether to remove traces of phenol and then concentrated *in vacuo* on a steam bath to a convenient volume. These preparations were used in experiments 11 and 12, Table IX.

The method used throughout to remove the phenol from prepared fractions was as follows: A volume of distilled water equivalent to one-half the fraction was added, after which a quantity of ether equal to the resulting total amount was shaken with each of the fractions containing phenol. The ether layer was then removed and washed with small quantities of water until the washings were of a light straw color. The remaining ether layer was then discarded. The water washings were then combined with the original water layer and extracted several times (usually five times) with small quantities of ether until the last ether washing yielded a negative Millon's test. The ether washings were combined and washed with very small volumes of water until the washings were colorless. The ether layer was discarded. The water washings were then combined and washed with small amounts of ether until the last ether wash exhibited the characteristic red color when subjected to the Millon's reaction. In the above-described procedure, the phenol was dissolved by the ethyl ether and consequently discarded, whereas the active substance was retained in the water. The residual ether was removed from the solution containing the active material by vacuum distillation on a steam bath.

3. Ethanol fractions of phenolic extracts (fraction 2). The ethanol fractions used in experiment 13 (Table IX) were prepared by direct extraction of liver "L" with phenol. Crystalline phenol was melted and added to liver "L" in the ratio of 1 part liver fraction to 4 parts phenol. This mixture was stirred for 2 hours on a steam bath and then filtered through a steam-jacketed sintered glass filter using suction. The phenol soluble and insoluble portions were then

placed separately into a soxhlet apparatus and extracted with alcohol for 5 hours, renewing the alcohol four times during this period. The alcohol was removed by vacuum distillation and the phenol was removed by ether extraction.

4. Precipitation with ammonium sulfate. The preparations used in experiment 14 (Table X) were fractions of a liver "L" dialysate (1) soluble and insoluble in a 75% saturated ammonium sulfate solution at pH 3.0. Ammonium sulfate was removed from each fraction through precipitation with barium hydroxide.

5. Precipitation of impurities with 80% ethanol (fraction 3 and 4). Four hundred ml. of a Bioper "C" dialysis (1) were made to a concentration of 80% ethanol by the addition of 95% alcohol. This mixture was shaken several times and then placed in a cold room (32°-34° F) for one or two days. The supernatant was then filtered and the residue washed with 250 ml. of cold 80% ethanol. This washing was combined with the supernatant which was then concentrated *in vacuo* to 160 ml. This constituted the 80% ethanol soluble fraction (3). The residue was dissolved in water, the alcohol driven off by vacuum distillation, and the remaining solution concentrated to 400 ml. *in vacuo*. This portion was designated 80% ethanol insoluble fraction (4). These preparations were used in experiment 15 (Table XI) and also served as a preliminary fractionation procedure for subsequent studies.

6. Seperation within phenol-water two-phase solubility system (fraction 5, 6, and 7). Phenol dissolves in water and forms a homogenous liquid if its concentration is less than 8 percent. However,

if the quantity of phenol is in excess of 8 percent, it ceases to be dissolved and a second liquid phase is formed. This second phase consists of the excess phenol and water, or a solution of water in phenol. The solution of phenol in water at 25°C will contain approximately 8 percent phenol and 92 percent water, whereas the solution of water in phenol will consist of approximately 72 percent phenol and 28 percent water. The phenol-water two-phase solubility systems used in these studies were prepared as follows: Five hundred grams of crystalline phenol were added to 1500 ml. of distilled water in a large round-bottomed flask. Twenty-five milligrams of sodium chloride were then added and the mixture shaken thoroughly. The sodium chloride was added to eliminate the formation of an emulsion, and to hasten the formation of the two layers of the solubility system.

Two hundred ml. of the 80% ethanol soluble fraction (3) representing 500 grams of original Biopar "C" were placed in a separatory funnel. Four hundred fifty ml. of the phenol layer (saturated with water) of a two-phase solubility system composed of phenol and water were then added and the mixture shaken. After reaching equilibrium, the dark lower layer was removed. This layer constitutes the phenol layer of the two-phase system and contains most of the liver factor activity. One hundred ml. of the water layer (saturated with phenol) of the solubility system described above were then added to the dark lower layer, the mixture shaken, and then allowed to attain equilibrium. The dark lower layer was again removed and the separation repeated until ten different extractions with the water layer (saturated with phenol) had been completed. The dark lower layer was designated fraction (5) or phenol soluble of (3).

The light upper layers were combined and extracted five times in the same manner as that above using 100 ml. of the phenol layer (saturated with water) of the two-phase solubility system. The light upper layer remaining was designated fraction (6) or water extract of (5). The extracts were combined and labeled fraction (7) or phenol extract of (6). Each fraction was extracted with ether according to the method previously described and then concentrated to 200 ml. (representing 500 grams of original Biopar "C". These fractions were used in experiment 16 (Table XI) and also as initial steps for further fractionation procedures in subsequent studies.

7. Separation within 25% phenol-butanol-water two-phase solubility system (fraction 8 and 9). The 25% phenol-butanol-water system was prepared as follows: A quantity of distilled water containing 3% glacial acetic acid was brought to pH 2.0 with HCl after which a solution of n-butyl alcohol containing 25% crystalline phenol was added. The mixture was shaken thoroughly and then allowed to come to equilibrium.

Two hundred ml. of fraction (5) which is the phenol soluble fraction of (3) were placed in a separatory funnel. This quantity represents 500 grams of original Biopar "C." Four hundred ml. of the upper layer (phenol-butanol saturated with water) and 200 ml. of the lower layer (water saturated with phenol-butanol) of the 25% phenol-butanol-water system were added to the preparation in the funnel. The mixture was shaken and then allowed to attain equilibrium. The upper layer of the resulting two-phase solubility system was labeled fraction (8) or 25% phenol-butanol soluble of (5), and the lower layer as fraction

(9) or water-phenol soluble of (5). Fraction (8) was concentrated to 75 ml. and fraction (9) to 100 ml. after removal of the butanol and the phenol by ether extraction. These fractions were used in experiment 17 (Table XI), and also as preliminary steps in later fractionation procedures.

8. Countercurrent distribution. Countercurrent distribution procedures were selected for use in further purification of the unidentified growth factor, or factors, under consideration in these studies. This method, described by Craig (1944), is suitable for use in the fractionation or the characterization of unknown compounds. This procedure provides a method of removing a large portion of the impurities from the substance to be purified by means of a distribution of the compounds involved throughout a solvent system composed of two immiscible layers. The substance to be purified must be soluble in both of the immiscible layers of the solvent system to approximately the same extent for the successful application of this procedure.

Craig (1944) has also described a special apparatus for use in countercurrent distribution procedures. This apparatus consists of a series of tightly fitted, super-imposed chambers contained in a frame, the upper half of which may be rotated. The super-imposed chambers are fitted to form a closed container or tube. By revolving the upper part of the frame slightly, the upper half of each tube may be moved over the lower half of the adjacent tube. Two immiscible solvent phases are placed in the tubes so that the meniscus is at the center of each tube. The two phases in each tube are separated and the upper phase is transferred to the adjacent tube by revolving the frame containing the upper half of each tube.

Separatory funnels of one liter capacity were used as tubes in the studies described in this report. This was necessary to permit preparation of sufficient quantities for use in the chick assay. The distribution was accomplished by transferring the lower layer of the solution from each of the funnels to the adjacent funnel on the right. In this manner, a number of plates or series of equilibriums were performed so that the same distribution occurred as would have been effected if the special apparatus described by Craig (1944) had been employed. After each transfer, the two liquid phases were shaken manually for two minutes at an approximate rate of 180 shakes per minute. This period of shaking was found to be adequate to establish equilibrium.

The countercurrent distribution studies reported here involved eight plates or series of equilibriums. An eight plate distribution requires nine separatory funnels (tubes) numbering from 0 to 8 inclusive. A solvent system composed of an n-butyl alcohol layer containing 25 gm. of phenol per 100 ml. and a water layer consisting of 3 percent glacial acetic acid and sufficient hydrochloric acid to bring to pH 2.0 was used as the solvent system in the first two countercurrent distribution procedures. The countercurrent distribution fractions were prepared as follows:

Two liters of distilled water containing 3 percent glacial acetic acid were made to pH 2.0 with hydrochloric acid. The acetic acid was necessary to prevent the formation of an emulsion. Four hundred grams of crystalline phenol were then dissolved in 1600 ml. of n-butyl alcohol. This solution was added to the water and the mixture thoroughly shaken. The two layers formed were then used in the separatory funnels in the countercurrent work.

Three hundred ml. of the alcohol phase (upper layer) were placed in each of the funnels, or tubes. One hundred fifty ml. of fraction (8), representing 1000 gm. of original Biopar "C," and 150 ml. of the water layer (lower layer of the solvent system) were then added to funnel number 0 which contained 300 ml. of the alcohol phase. Four ml. of glacial acetic acid and 10 mg. of sodium chloride were also added to the funnel to prevent the formation of an emulsion and to facilitate separation. The contents of this funnel were shaken for 2 minutes and then allowed to stand until the two phases separated. The lower phase (water layer) was removed and transferred to the adjacent funnel (#1). This completed one plate. Three hundred ml. of the water layer of the prepared solvent system were then added to funnel #0, and both funnels, #0, and #1 were shaken. After the two layers had formed, the lower layer was removed from each separatory funnel and placed in the next funnel to the right (arranged in order of numbers). This completed the second plate. The procedure was repeated until all of the 9 funnels (0 to 8) were involved, adding a fresh 300 ml. portion of the water layer to funnel #0 each time. This completed the eight plate countercurrent distribution of the material throughout the solvent system.

After the eight plate distribution was completed, the contents of each tube were extracted with ether to remove the phenol and butanol. The ether was removed by vacuum distillation with steam, and the remaining solutions concentrated to a convenient volume and added singly to the diet fed the chicks in experiments 18 and 19 (Table XII).

EXPERIMENTAL RESULTS

A. Studies with Chicks

1. Preliminary Studies. The data presented here are the results of experiments concerned with the study of unidentified growth factors required in chick nutrition. This work is a continuation of studies which have shown that at least one unidentified factor, which is not identical with vitamin B_{12}, is required for rapid chick growth. The unidentified growth factor was shown to be presented in liver and dried whey (Menge et al. 1949, 1951a).

The results of experiment 1 (Table III) demonstrate the presence of an unidentified growth factor in the liver "L" fraction which is stable to mild alkaline hydrolysis. The addition of 3 percent dried whey to this diet (R-124) also produced a marked growth response. Dried whey stimulated a further growth increase when added in combination with either 2 percent liver "L" or 2 percent alkali-treated liver "L." Since previous studies have clearly demonstrated that a 2 percent supplement of liver is equally as effective as a 4 percent supplement on the basis of growth stimulation, these results indicate that two different unidentified growth factors are involved.

The marked growth responses exhibited by the chicks in this study (experiment 1) to the crude supplements is believed to be due principally to the fact that these chicks were progeny of dams maintained on wire floors and fed a ration containing no animal protein supplements for a 9-month period. The chicks were apparently more deficient with respect to the liver factor than were chicks obtained from these dams earlier in the year. Chicks from dams housed on litter and fed a

TABLE III

GROWTH RESPONSE OF CHICKS TO VARIOUS CRUDE SUPPLEMENTS

(Diet R-124)

Experiment No.	Group No.	Supplement	Average gain (gm.)	Percent increased gain
1	1	None	157	-
	2	2% liver "L" (No.1)	215	37
	3	2% alkali-treated liver "L"	210	34
	4	3% dried whey-product	225	43
	5	As group 2+3% dried whey-product	267	70
	6	As group 3+3% dried whey-product	261	66
2	1	None	221	-
	2	4% Biopar "C" (No.1)	209	-6
	3	As group 2+6% dried whey-product	262	19
	4	As group 2+7.5% dried brewers yeast	209	-6
	5	As group 2+2% butyl molasses solubles.	192	-13
3	1	None	190	-
	2	2% Biopar "C" (No.2)	199	5
	3	4% Biopar "C" (No.2)	195	3
	4	0.5% dried whey	203	7
	5	1.5% dried whey	226	19
	6	3.0% dried whey	225	18
	7	As group 2+0.5% dried whey	220	16

An experimental period of 4 weeks was used in experiments 1 & 2. A 2-week preliminary period followed by an experimental period of 3 weeks was employed in experiment 3.

Each experiment contained 15 chicks per group at the start of the experimental period.

complete breeder ration were used in experiments 2 through 7. These dams had been in production for 10 to 12 months at the time eggs were collected for hatching. It will be noted from the results of experiments 2 through 5 that the chicks from these dams did not show a response to the liver supplements, although consistent responses were obtained with dried whey. These results clearly show the effect of maternal depletion on the growth response of the progeny to unidentified growth factors.

When the liver fraction Bioper "C" was used alone or in combination with dried brewers' yeast or butyl molasses solubles (experiments 2 and 3, Table III) there was no evidence of growth activity in these substances. On the other hand, the addition of dried whey alone or in combination with the liver supplement exhibited a growth response that would indicate the presence of a growth factor which was apparently absent in the liver fraction Bioper "C."

Table IV presents a summary of the results obtained in experiment 4 in which three different samples of liver substance were used. The slight growth responses obtained with these liver fractions demonstrate their similarity to each other and also show the 2 percent supplement to stimulate growth equivalent to a 4 percent supplement. Additions of dried whey alone or in combination with Bioper "C" exhibited growth responses which clearly demonstrated the presence of an unidentified growth factor.

The results of experiment 5 (Table V) present more evidence for the presence of an unidentified growth factor in dried whey. These data also suggest the existence of two unidentified growth factors,

TABLE IV

GROWTH RESPONSE OF CHICKS TO CRUDE LIVER AND WHEY SUPPLEMENTS

(Diet R-124)

Experiment No.	Group No.	Supplement	Average gain (gm.)	Percent increased gain
	1	None	210	-
	2	2% Biopar "C" (No.2)	220	5
	3	4% Biopar "C" (No.2)	219	4
	4	4% Biopar "C" (No.1)	217	3
4	5	4% liver "L" (No. 1)	216	3
	6	1.5% dried whey	225	21
	7	3.0% dried whey	234	11
	8	6.0% dried whey	248	18
	9	As group 2 + 3% dried whey	247	18

A preliminary period of 2 weeks followed by an experimental period of 3 weeks was used in this experiment.

Each group contained 14 chicks at the start of the experimental period.

one of which is present in the liver fraction and dried brewers' yeast, and the other in dried whey. The chicks in group 2 which received the liver supplement apparently lacked the growth factor supplied by dried whey, and conversely, the chicks in group 3 which received the whey supplement were somewhat deficient in the liver factor. However, the combination of dried whey and the liver fraction stimulated a marked increase in growth (group 5). Since the combination of Biopar "C" and dried brewers' yeast (group 6) gave only a slight growth response over that exhibited by either supplement alone, it would appear that each contains a like growth factor. Moreover, a combination of dried brewers' yeast and dried whey (group 7) gave a growth response that was similar to the combination of liver and dried whey (group 5), further substantiating the similarity of the growth factor in liver and dried brewers' yeast.

The growth stimulations obtained from the supplements of dried whey are believed due to the presence of a growth factor not found in the liver substances used in these trials. This is clearly shown in the results of experiments 3 and 4. In these trials, three different samples of liver substance were used, one of which was fed at two levels. These results show that dried whey supplied a growth factor which was not furnished by the liver fractions. The addition of 4 percent Biopar "C" produced only a very slight growth response, while the addition of dried whey alone or in combination with Biopar "C" produced a marked increase in growth. Since these liver preparations

TABLE V

GROWTH RESPONSE OF CHICKS TO CERTAIN CRUDE SUPPLEMENTS

(Diet R-124)

Experiment No.	Group No.	Supplement	Average gain (gm.)	Percent increased gain
5	1	None	301	-
	2	4% Biopar "G" (No.2)	309	2
	3	6% dried whey-product	347	15
	4	5% dried brewers yeast	324	8
	5	As group 2+6% dried whey-product	387	29
	6	As group 2+5% dried brewers yeast	317	5
	7	As group 3+5% dried brewers yeast	367	22
	8	As group 3+5% dried brewers yeast +4% Biopar "G"(No.2)	350	16

A preliminary period of 2 weeks followed by an experimental period of 3 weeks was used in this experiment.

Each group contained 15 chicks at the start of the experimental period.

had previously been found to promote chick growth when more deficient chicks were used, it follows that two different unidentified growth factors are required for rapid chick growth.

Table VI presents a summary of the results obtained in experiments 6 and 7 in which basal diet S-125 was employed. In experiment 6, the addition of 3 percent dried whey alone (group 3) did not promote a maximum growth response. However, the combination of 3 percent dried whey and 2 percent Biopar "C" (group 5) did produce a growth response equivalent to that obtained from a supplement of 6 percent dried whey. Since a 2 percent supplement of Biopar "C" has been found to supply an adequate amount of the liver factor, these results together with those of the preceding trials indicate that whey contains a growth factor not supplied by Biopar "C," and strongly suggest that whey contains a small quantity of the liver factor.

The results of experiment 7 (Table VI) further demonstrate the supplementary effect of whey when fed in combination with Biopar "C" or dried brewers' yeast. These results also show the similarity of the unidentified growth-stimulatory factor present in liver and dried brewers' yeast, since the combination of both elicited a growth response that was comparable with that exhibited by either supplement alone. However, when whey was fed in combination with either the liver fraction or yeast, the chicks grew more rapidly than those given supplements of dried whey, Biopar "C," or yeast. The addition of Biopar "C," dried whey, and dried brewers' yeast in combination (group 8) did not further improve chick growth. Hence, these observations lend support to the results of the preceding trials in which the growth activity of two factors was suggested.

TABLE VI

CHICK GROWTH RESPONSES TO VARIOUS DIETARY SUPPLEMENTS

(Diet R-125)

Experiment No.	Group No.	Supplement	Average gain (gm.)	Percent increased gain
6	1	None	133	-
	2	4% Biopar "C" (No.2)	156	17
	3	3% dried whey	168	26
	4	6% dried whey	191	44
	5	2% Biopar "C" + 3% dr. whey	196	47
7	1	None	172	-
	2	4% Biopar "C" (No.2)	193	12
	3	6% dried whey-product	278	62
	4	5% dried brewers yeast	213	24
	5	As group 2+6% dried whey-product	281	63
	6	As group 2+5% dr. brewers yeast	209	22
	7	As group 3+5% dr. brewers yeast	285	66
	8	As group 2+5% dr. brewers yeast +6% dr. whey-product	257	49

An experimental period of 3 weeks in experiment 6, and an experimental period of 5 weeks in experiment 7 was employed.

Experiments 6 and 7 contained 15 and 16 chicks per group, respectively, at the start of the experimental period.

Table VII presents a summary of the results obtained in experiment 8. This study was designed to study the effect of maternal depletion on the response of progeny to unidentified growth factors. One-half of the chicks used in this trial were progeny of hens in their second year of production, and the other half of the chicks were progeny of pullets in their fourth month of production. Both groups of dams had been housed on wire floors and fed a ration containing no animal protein supplements since coming into production. The breeder ration used contained an adequate level of vitamin B_{12} and all other known dietary essentials. The hens in their second year of production had received this ration for an 18-month period. It is evident from the results obtained with these progeny that a considerable difference exists in the amount of carry-over of the factor present in the liver fraction. The growth response obtained from the Bioper "C" supplement to the ration fed the progeny of the pullets was very slight, whereas, a definite growth response to this substance was exhibited by the progeny of the hens. Although the differences in the responses of these groups to the factor supplied by either sample of dried whey were less marked, the results again indicate that the progeny of the hens was somewhat more deficient at hatching time. However, excellent growth responses were obtained in both groups of progeny when a combination of dried whey and liver were supplied. These results indicate that the length of the depletion period for the breeders has a definite influence on the response of the progeny to unidentified growth factors.

TABLE VII

INFLUENCE OF MATERNAL DEPLETION ON RESPONSE OF PROGENY TO UNIDENTIFIED GROWTH FACTORS

(Experiment 8)

Group No.	Supplements to diet R-126	Average gain (gm.) during first 4 weeks	
		Progeny of hens*	Progeny of pullets**
1	None	231	264
2	2% Biopar "C" (No.2)	288	276
3	2% delactosed whey "C"	298	309
4	2% delactosed whey "D"	283	292
5	As group 2+2% delactosed whey "C"	390	377

* Progeny of hens in their second year of production, housed on wire floors, fed a ration deficient in animal protein for 18 months.

** Progeny of pullets in their fourth month of production, housed on wire floors, and fed a ration deficient in animal protein for 4 months.

Each group contained 10 chicks at the start of the experimental period.

2. Deutectomy. Several investigators have found a positive relationship between early chick growth and the diet of the dam. It is evident, therefore, that nutritional studies involving the chick are limited by the extent to which the chick can be depleted of the nutrients under consideration which have been transmitted from the dam through the egg. Studies concerning vitamin B_{12} and unidentified chick growth factors conducted in this laboratory (Menge et al. 1949, 1951a) have involved the use of chicks from dams fed rations containing no animal protein supplements and maintained on raised wire floors. Nevertheless, a preliminary period of from 1 to 2 weeks was usually necessary to further deplete the chicks and to standardize the effect of carry-over of the nutrient being investigated.

Parker (1929), and later Sloan (1936) developed a technique for the removal of the yolk from day-old chicks. Sloan termed the operation "deutectomy" and postulated that this procedure could be very well adapted to nutritional studies. A simplified technique for the removal of the yolk from day-old chicks was developed, therefore, to render them more nearly deficient in the nutrients transmitted from the dam. The technique is described under Experimental Procedure.

The results of experiments 9 and 10 are presented in Table VIII. These trials were designed to study the effect of deutectomy on the response of chicks to vitamin B_{12} and the unidentified growth factor in liver. In experiment 1, the deutectomized chicks which received the vitamin B_{12}-deficient diet alone grew less rapidly than did the unoperated chicks fed the same diet. However, when vitamin B_{12} was administered to deutectomized and unoperated chicks fed the same diet,

TABLE VIII

EFFECT OF DEUTECTOMY ON THE NUTRITIONAL REQUIREMENTS OF CHICKS

Experiment No.	Supplements to diet P-124	Average gain*(gm)during 5-week exptl. period	
		Deutectomized chicks	Unoperated chicks
9	None	239(17)**	316(19)
	4.8 mcg. vitamin B_{12}	322(18)	321(18)
	4.8 mcg. vitamin B_{12}+4% Biopar "C"	317(20)	344(20)
		3-week exptl. period	
10	4.0 mcg. vitamin B_{12}	205(13)	261(12)
	8.0 mcg. vitamin B_{12}	248(14)	—

* The average gains shown were adjusted for sex differences.

** Numbers in parenthesis() refer to number of surviving chicks.

An experimental period of 5 weeks was used in experiment 9. A preliminary period of 2 weeks followed by an experimental period of 3 weeks was used in experiment 10.

Experiments 9 and 10 contained 20 and 16 chicks per group, respectively, at the start of the experimental period.

The total amount of vitamin B_{12} (mcg.) injected per chick during the experimental period is shown in the table.

no difference in average gain was obtained. The deutectomized chicks which received Biopar "C" in combination with vitamin B_{12} did not show a more rapid growth response than that exhibited by the deutectomized chicks which received vitamin B_{12} alone. It is evident, therefore, that removing the yolk from day-old chicks resulted in the removal of vitamin B_{12} or another substance possessing vitamin B_{12} activity, but that, under the conditions of this experiment, these chicks did not exhibit a growth response to Biopar "C." However, the response observed with the unoperated chicks which received vitamin B_{12} in combination with Biopar "C" suggests the presence of an unidentified growth factor in the crude supplement.

The results of experiment 10 (Table VIII) confirm the observations noted above since the deutectomized chicks required twice as much supplemental vitamin B_{12} as did unoperated chicks in order to support a similar rate of growth. These data are in agreement with Yacowitz et al. (1950) and Milligan and Combs (1950) who have showed that vitamin B_{12} activity is transmitted from the dam through the yolk of the egg. These workers also demonstrated that the diet of the dam had a direct influence on the amount of vitamin B_{12} activity in the egg yolk.

The growth response of deutectomized chicks as compared with that of unoperated chicks in both experiments demonstrates that the yolk-removal operation had little or no effect on growth when the diet of the chick was adequately supplemented. Although no evidence was obtained in these trials to demonstrate the removal by deutectomy of nutrients other than vitamin B_{12}, this procedure is considered to be of value as a means of reducing the initial carry-over of nutrients

from the dam in day-old chicks. The technique was employed in experiments 11 through 13 (Table IX).

3. Fractionation Studies. Tables IX and X present a summary of the chick growth responses obtained from prepared fractions of liver "L." Since an adequate amount of vitamin B_{12} was supplied to a diet which contained sufficient quantities of all known required nutrients, the growth responses exhibited by the groups which received the crude liver supplement are indicative of the presence of an unidentified growth factor in this material. This is, of course, in accord with the preceding experiments which have repeatedly indicated that liver contains such an unidentified growth factor. The growth responses observed in the groups which received the prepared fractions indicate that the factor is dialyzable (experiments 11 and 12). Furthermore, the dialyzable factor appears to be distributed equally in both layers of a phenol-water, two-phase system. The apparent growth depression noted in group 6 as compared with group 5 (experiment 12) which received a smaller quantity of the phenol fraction is believed due to the presence of phenols.

The fractions used in experiment 13 were prepared by direct extraction of liver "L" with phenol followed by a continuous extraction with 95 percent ethanol. The growth response exhibited by the groups which received the alcohol soluble fraction of the phenol soluble portion (group 3) indicates that the chick growth factor was readily soluble in phenol and alcohol. The growth-stimulating substance was apparently concentrated in the phenol soluble portion of liver "L" through solution. The substance was then removed from the phenol by

TABLE IX

CHICK GROWTH RESPONSES TO PREPARED FRACTIONS OF LIVER "L"

(Diet R-123)

Experiment No.	Group No.	Supplement	Percent solids added	Average gain (gm.)	Percent increased gain
11*	1	None	-	130	-
	2	4% liver "L"	3.600	156	20
	3	(1)Dialysate≃4% liver"L"	0.918	152	17
	4	Phenol sol. of (1)≃4%liver"L"	0.392	146	12
12*	1	None	-	126	-
	2	4% liver "L"	3.600	166	32
	3	Phenol sol. of (1)≃2%liver"L"	0.160	141	12
	4	Phenol sol. of (1)≃4%liver"L"	0.320	141	12
	5	Phenol insol. of (1) ≃ 2% liver "L"	0.436	138	10
	6	Phenol insol. of (1) ≃ 4% liver "L"	0.872	128	2
13**	1	None	-	186	-
	2	4% liver "L"	3.600	210	13
	3	(2)EtOH sol. of phenol extr. ≃ 4% liver "L"	0.336	215	16
	4	EtOH insol. of extr. ≃ 4% liver "L"	0.408	158	-15
	5	EtOH insol. of phenol residue ≃ 4% liver"L"	0.340	172	-7

* Each chick received 0.6 mcg. vitamin B_{12} by injection at hatching time and at weekly intervals during the trial periods.

** Vitamin B_{12} incorporated within diet (30mcg./kilo).

Experiments 11, 12, and 13 contained 15, 8, and 14 chicks per group, respectively, at the start of the experimental period.

The chicks were deutectomized at one day of age.

A preliminary period of 1 week followed by an experimental period of 2 weeks was employed in experiments 11 and 12. A preliminary period of 2 weeks with an experimental period of 17 days was used in experiment 13.

continuous extraction with alcohol. It can be noted from the tables that the percentage solids contributed to the ration by the phenol soluble liver fractions were considerably less than that of the phenol insoluble or water layer fractions. Thus, the ability of phenol to remove the active material from a water solution and retain a smaller amount of inactive solids was used as an important step towards the concentration of the growth factor in later studies.

The growth activity of fractions prepared by precipitation of a liver "L" dialysate in a 75 percent saturated ammonium sulfate solution at pH 3 was studied in experiment 14 (Table X). The results of this experiment suggest that the factor is precipitated in a 75 percent saturated ammonium sulfate solution. This procedure was abandoned in favor of separation in a phenol-water, two-phase system because it was much less effective than phenol in removing the factor without an undue amount of inactive material.

TABLE X

CHICK GROWTH RESPONSES TO AMMONIUM SULFATE FRACTIONS

(Experiment 14)

Group No.	Supplements to Diet R-124	Percent solids added	Average gain (gm.)	Percent increased gain
1	None	-	221	-
2	4% liver "L"	3.600	243	10
3	75% ammonium sulfate sol. of (1) ≃ 4% liver "L"	0.804	250	13
4	75% ammonium sulfate insol. of (1) ≃ 4% liver "L"	0.968	233	5

Each group contained 15 chicks at the start of the 4-week experimental period.

Crystalline vitamin B_{12} incorporated within diet (30mcg./kilo).

B. Studies with Poults

Since the growth response of chicks to unidentified factors was usually small, although consistent, and preliminary studies with poults revealed a greater growth response, poults were used in the following trials (experiments 15 through 17).

The growth responses exhibited by poults which received Biopar "C" and fractions prepared from this substance are presented in Table XI. Observations of the growth activity shown by the poults fed the Biopar supplements in the presence of an adequate amount of vitamin B_{12} (60 mcg. per kilo of diet) show that this liver preparation contains a growth factor not identical with vitamin B_{12}. A comparison of the growth obtained with the 2 percent and the 4 percent liver supplement shows clearly that the lower level of the liver substance was sufficient to meet the requirements of the poults under these experimental conditions. It is apparent, therefore, that the poult as well as the chick requires an unidentified factor which is present in liver for rapid growth.

The growth responses obtained with the prepared Biopar dialysate fractions in experiment 15 (group 5) show that the factor is dialyzable and indicate that it is readily soluble in 80 percent alcohol. This method of separation which involved dialysis and precipitation of much inactive material was employed as initial steps in further fractionation and concentration of the active material. The growth activity exhibited by the phenol soluble fraction (5) in experiment 16 (group 4) confirms previous work with chicks and shows that the growth factor present in liver is preferentially soluble in phenol. The results of this trial

(experiment 16) suggest the presence of two growth-promoting substances, one of which was retained in the phenol layer and was not removed by treatment with water saturated with phenol, while the other was removed and left in the water layer. Subsequent treatment of the water extract with phenol saturated with water failed to remove the material from this fraction. It then appears that the poults in groups 4 and 5 responded to different forms of the same biological growth factor rather than to different factors since both groups grew as well as the group which received the 4 percent Bioper "C" supplement (group 3). The growth response obtained with the phenol soluble fraction (5) can be compared favorably with the group which received a complete turkey starter (group 7). On the basis of percentage solids, fraction (5) represents a 14-fold concentration of the growth factor.

The phenol soluble fraction (5) which represented the most active concentrate of the growth factor was then subjected to further separation into the two layers of a two-phase solubility system composed of 25 percent phenol in n-butanol and water. This procedure was designed to investigate the possibility of the use of this solubility system in countercurrent distribution studies. The growth responses obtained with the resulting fractions are also given in Table XI (experiment 17). These results confirm previous fractionation studies and indicate that this solubility system is quite suitable for the countercurrent distribution process. Calculations based on the percentage solids contained in fraction (8) show that this preparation represents an approximate 75-fold concentration of the growth factor when compared with the crude liver supplement. On this basis, 26 mg. per 100 gm. of diet gave a marked growth stimulation.

TABLE XI

GROWTH RESPONSES OF POULTS TO PREPARED FRACTIONS OF BIOPAR "C"

Experiment No.	Group No.	Supplement	Percent solids added	Average gain (gm)	Percent increased gain
15	1	None	-	212	-
	2	1% Biopar "C"	0.952	271	28
	3	2% Biopar "C"	1.904	370	74
	4	4% Biopar "C"	3.808	347	64
	5	(3)80% EtOH sol. of (1) ≃ 4% Biopar "C"	0.410	362	71
	6	(4)80% EtOH insol. of (1) ≃ 4% Biopar "C"	0.620	296	40
16	1	None	-	195	-
	2	2% Biopar "C"	1.904	221	13
	3	4% Biopar "C"	3.808	228	18
	4	(5)Phenol sol. of (3) ≃ 8% Biopar "C"	0.256	247	27
	5	(6)Water extr. of (5) ≃ 8% Biopar "C"	0.684	226	16
	6	(7)Phenol extr. of (6) ≃ 8% Biopar "C"	0.208	199	2
	7	Complete turkey starter	-	250	28
17	1	None	-	365	-
	2	2% Biopar "C"	1.904	446	22
	3	4% Biopar "C"	3.808	405	11
	4	(8)Phenol-ButOH layer of (5) ≃ 8%Biopar "C"	0.026	425*	16
	5	(9)Water-Phenol layer of (5) ≃ 8% Biopar "C"	0.128	424	16

Each consecutive experiment contained 8, 8, and 10 poults per group at the start of the experimental period.

An experimental period of 4 weeks in experiments 15 and 17, and an experimental period of 5 weeks in experiment 16 was employed.

*This fraction represents an approximate 75-fold concentration of the growth factor as compared with the crude liver supplement.

Considerable difficulty was experienced with a swollen hock condition similar to that described by Scott (1950). Observations on the incidence of this syndrome indicate that the growth factor required by the poult is not identical with the substance necessary for the prevention of the swollen hock disorder. The liver preparations studied have promoted growth of poults but have been ineffective in preventing the swollen hock condition which appears between the second and fourth weeks after hatching. However, the crude liver supplements have been very effective in promoting growth and in the prevention of the hock disorder.

C. Countercurrent Distribution Studies

A laboratory procedure described by Craig (1944) was selected for use in the further purification of the active growth principle under consideration in these studies. This procedure, termed countercurrent distribution, is suitable for use in fractionation and characterization of unknown compounds.

Fraction (8), which represented the most active concentrate of the growth factor as determined by the response of poults (experiment 17, group 4), was subjected to further fractionation through an eight-plate countercurrent distribution process. The same two-phase solubility system (25 percent phenol in n-butanol and water) employed in the preparation of fraction (8) was used in the countercurrent distribution as the solvent. The growth responses obtained when the contents of each tube were added singly to a synthetic diet (R-126) are given in Table XII. Delactosed whey "C" (0.5 percent) was included in the diets as the source of one unidentified factor which, according to the preceding studies appears to be necessary for a complete response to the liver factor by chicks at this stage of depletion and fed a synthetic diet. The contents of each tube were added to the diet in an amount calculated to be equivalent to Biopar "C" at a level of 15 percent of the diet. The results of this trial show two definite peaks of growth activity. Although the magnitude of the responses are not great, tubes 0, 4, 5, and 6 appear to contain concentrations of the growth factor(s).

Observations obtained through a repetition of the preceding trial are also given in Table XII (experiment 19). The soybean oil meal basal diet as shown in Table II was used. Essentially the same results were

TABLE XII

CHICK GROWTH RESPONSES TO LIVER FRACTION (8) SUBJECTED TO EIGHT-PLATE COUNTERCURRENT DISTRIBUTION

Group No.	Supplements	Experiment 18 Diet R-126 plus 0.5% whey* Av. gain (gm)	Experiment 18 % increased gain	Experiment 19 Soybean meal basal Av. gain (gm)	Experiment 19 % increased gain
1	None	200	-	206	-
2	2% Biopar "C"	237	19	233	13
3	4% Biopar "C"	-	-	236	15
4	Tube 0 ≃ 15% Biopar "C"	232	16	217	5
5	Tube 1 ≃ 15% Biopar "C"	217	9	232	13
6	Tube 2 ≃ 15% Biopar "C"	206	3	220	7
7	Tube 3 ≃ 15% Biopar "C"	196	-2	209	1
8	Tube 4 ≃ 15% Biopar "C"	239	20	209	1
9	Tube 5 ≃ 15% Biopar "C"	230	15	229	11
10	Tube 6 ≃ 15% Biopar "C"	222	11	230	12
11	Tube 7 ≃ 15% Biopar "C"	207	4	227	10
12	Tube 8 ≃ 15% Biopar "C"	183	-8	195	-5

* Delactosed whey "C"

Experiments 18 and 19 contained 15 and 12 chicks per group, respectively, at the start of the experimental period.

An experimental period of 3 weeks was employed in each study.

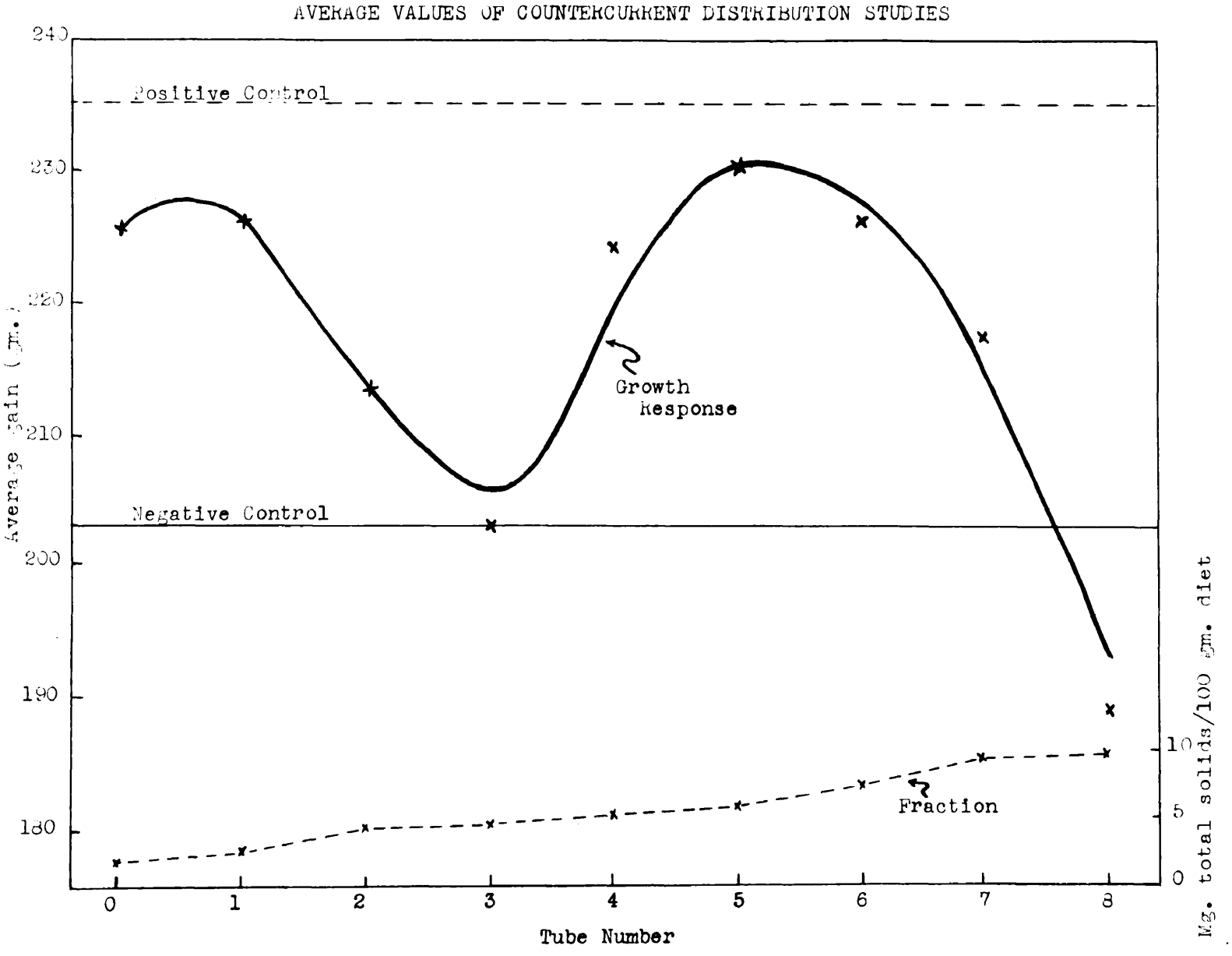
obtained in this experiment as in the previous trial with the exception that the two peaks of activity were shifted to the next consecutive tube, i.e., tubes 1, and 5, 6, and 7. This shift of the growth activity to the tube on the right may be due to (1) A slight change in the acidity of the solution, or (2) A change in the total solids content of the fraction. Although this fraction was prepared in exactly the same manner as the previous one, it is quite possible that changes in temperature of the solutions or other physical or chemical phenomena not under control may have altered the preparation. It has been noted, e.g., that the calculated percentage total solids of a dialysate varies as much as 100 percent between different preparations. This fact, alone, which is probably the effect of temperature, might account for certain changes in the entire procedure.

Inasmuch as the results obtained in the countercurrent distribution studies (experiments 18 and 19) were quite similar, the growth gains for each group were averaged and plotted in Figure 1. This procedure was considered logical even though two different types of diets were used because of the similarity of the results obtained in each experiment. The percentage total solids for each fraction was also averaged and plotted in Figure 1.

The two peaks of activity, although small, can be explained on the basis of at least two possibilities. First, that two forms of the same growth factor, each with slightly different solubility properties, were present, and second, that the growth responses were due to two different factors, one of which existed in two forms with somewhat different solubility properties. Either of these propositions would account for the growth curve obtained in these studies.

A comparison of the average percentage total solids in tube 5 (experiments 18 and 19) with that of the crude liver supplement shows an approximate 350-fold concentration of the growth factor.

Figure 1


AVERAGE VALUES OF COUNTERCURRENT DISTRIBUTION STUDIES
240
230
220
210
200
190
180
Positive Control
Negative Control
Growth Response
Fraction
0
1
2
3
4
5
6
7
8
Tube Number
10
5
0
Mg. total solids/100 gm. diet

DISCUSSION

A. Evidence for Two Unidentified Growth Factors

The results of the experiments described in this report show the need of the chick for two functionally distinct, unidentified growth factors. Liver fraction "L," liver fraction Biopar "C," and dried brewers' yeast were shown to contain one of these unidentified factors, and dried whey was demonstrated to be a source of the other factor. Moreover, the data strongly suggest that whey contains small quantities of the liver factor.

Considerable differences were noted in the growth responses of chicks to supplements of liver or dried brewers' yeast. The results of these studies demonstrate the effect of maternal depletion on the response of progeny to unidentified growth factors. Progeny of hens maintained on wire floors and fed a ration containing no animal protein supplements for a 9-month period showed a marked growth response to the unidentified factor in liver as well as to the factor in dried whey. These progeny exhibited an even greater response to combinations of dried whey and a liver fraction. However, when chicks from dams housed on litter and fed a complete breeder ration for a 12-month period were used, a difference in the growth response of the progeny was noted. These chicks did not show an appreciable response to supplements of Biopar "C," dried brewers' yeast, butyl molasses solubles, or combinations of Biopar "C" and either of the other crude supplements. The addition of dried whey, however, resulted in a marked increase in the rate of growth, and when whey was added in combination with the other crude supplements, an even greater response was noted.

The studies reported here have consistently shown a 2 percent supplement of liver to supply an adequate quantity of the "liver factor," whereas, a 3 percent level of dried whey was apparently not as effective as twice that amount. However, a combination of 2 percent liver and 3 percent whey stimulated growth equivalent to 6 percent dried whey. These results clearly show that two different unidentified growth factors are involved, one of which is present in liver and the other in dried whey. The data also strongly suggests that whey contains a quantity of the "liver factor." Combinations of liver, whey, and yeast did not improve the growth rate, hence, only two growth factors appear to be concerned in these studies.

Differences in nutritional requirements are known to exist, even among closely related species. However, the requirements of the turkey poult for certain recognized nutrients appear to bear a fairly direct relationship to the requirements of the chick. In the case of these two species, the requirements are usually greater for the poult. The differences are approximately in proportion to the increased rate of growth of the poult in comparison to that of the chick. Therefore, poults were used in the studies reported here because of their increased nutritive requirement and the fact that they might thus be a more sensitive assay animal than the chick.

Turkey poults from dams fed a complete breeder ration and housed on litter were shown to exhibit a marked growth response to Biopar "C" and fractions prepared from this material. The factor in liver which promoted growth of poults was apparently not identical with a factor present in the

same material which was effective against a swollen hock condition similar to that described by Scott (1950). Fractions prepared from Biopar "C" promoted rapid growth of the poult but did not alleviate the hock disorder, whereas, the poults which received the original Biopar fraction grew rapidly and did not show any incidence of the swollen hock condition. This factor(s), which is necessary for normal leg development in the poult, also appears to be present in fish meal, dried brewers' yeast, and butyl molasses solubles, as well as in liver (Combs, 1951b). It is apparent, therefore, that both the chick and the poult require an unidentified factor which is present in liver for rapid growth.

B. Fractionation of a Factor in Liver

In the fractionation studies reported in this paper, turkey poults responded to prepared fractions of Biopar "C" in much the same manner as chicks. The data obtained in experiment 16 suggest that the poults had responded to two different forms of the unidentified growth factor, each with different solubility properties. It is apparent that one form was preferentially soluble in phenol and was not removed through repeated extrations with water saturated with phenol, while the other form was removed by this procedure. Subsequent treatment of the resulting water layer with phenol saturated with water failed to extract the second form from this solution. Since each of the active fractions exhibited a growth response equivalent to the control group (4 percent Biopar "C"), it appears that the poults responded to different forms of the same biological growth factor rather than to different factors.

An approximate 75-fold concentration of the unidentified growth factor (fraction 8) was accomplished by means of dialysis, precipitation of impurities with 80 percent ethanol, separation into the phenol layer of a phenol-water, two-phase solubility system, followed by solution within the phenol-butanol phase of a two-phase solubility system composed of 25 percent phenol in butanol and water.

Chick growth responses obtained from liver fraction (8) subjected to an eight-plate countercurrent distribution indicate two peaks of activity. One of these was located in tubes 0 and 1, and the other in tubes 4, 5, and 6. The growth curve as shown in Figure 1 (page 61) can be explained on the basis of two possibilities:

(1). The chicks responded to the growth-stimulation of two forms of

the same growth factor; the forms differing from each other primarily in their solubility properties in the two-phase system used. The growth response of poults to a liver fraction prepared by separation into a two-phase system of phenol and water could be explained according to this theory. In this study, it appeared evident that the poults had responded to two forms of the same biological growth factor. One of the active substances remained in the phenol layer and was not removed by water, while the other was removed by the water, but not by subsequent extractions with phenol. It is possible, therefore, that in the countercurrent distribution process one form of the factor, being preferentially soluble in water, was concentrated in tubes 4, 5, and 6, whereas, the other form remained in tubes 0 and 1.

(2). The growth curve shown in Figure 1 might also have been the result of a stimulation from two functionally distinct growth factors (factors A and B), one of which was present in two forms with different solubility properties (factors A and A'). If this theory is correct, the growth stimulation observed by feeding the contents of tubes 0 and 1 was the result of the combination of factor A' and factor B, whereas, the growth activity demonstrated by feeding the contents of tubes 4, 5, and 6 was the effect of combinations of factor A and B. Conversely, the lack of response to tubes 2 and 3 could then be explained on the basis of a deficiency of factors A and A', and the poor growth obtained from feeding the contents of tubes 7 and 8 to a lack of factor B.

It is not possible to determine conclusively from these data which of the hypotheses noted above is correct. Studies involving combinations of tubes and super-imposed countercurrent distribution procedures are

necessary as further steps toward the final isolation and identification of this unidentified growth factor.

However, it is interesting to note that either of the proposed theories coincide with curves that can be plotted on the basis of assumed partition coefficients (K) in lieu of the actual values for K. These theoretical curves are quite similar to those that can be expected in a countercurrent distribution of a solution containing either two forms of a factor, or two factors, one of which is present in two forms with different solubility properties.

These observations are in agreement with the results obtained by Combs et al.(1950) who have reported the presence of two unidentified growth-promoting factors, not identical with vitamin B_{12}, in a refined liver paste dialysate. Countercurrent and super-imposed countercurrent distribution procedures were used by Combs and co-workers who also presented evidence of a mutual supplementary effect obtained by feeding combinations of prepared fractions containing the unknown growth factor.

C. Comparison with the Results Obtained by Other Workers

The growth response of chicks to unidentified factors has been observed in other laboratories. Additions of dried whey, liver, dried brewers' yeast, and fish products to the diet has stimulated chick growth responses which cannot be explained entirely on the basis of known required nutrients.

Schumacher et al. (1940) reported the presence of an unidentified chick growth factor (Factor S) in dried brewers' yeast. Later, Hill (1948) reported the presence of an unidentified factor in dried whey. Carlson et al. (1949) have presented data suggesting the existence of unidentified growth factors in fish meal, and dried brewers' yeast. These research workers used a synthetic diet similar to the one employed in the present study. Hill and Briggs (1950), using a similar diet, obtained comparable results from the addition of dried brewers' yeast or liver substances. Savage et al. (1950), also using a synthetic diet, found that a liver fraction promoted growth of chicks. These workers obtained data which indicated that soybean oil meal is a fair source of this unrecognized growth factor.

Combs et al. (1950), using fractions obtained from a refined liver paste dialysate by countercurrent distribution, reported results by means of chick growth and microbiological assays to indicate the existence of four substances which promote rapid growth of chicks. Microbiological assays indicate that two of these factors are different forms of vitamin B_{12}, and that the other two substances are not identical with vitamin B_{12} or any of the known vitamins. Evidence of a mutual supplementary effect was also obtained by feeding combinations of fractions containing the

unknown growth factors. An unidentified chick growth factor present in green leaves of alfalfa and cereal grasses was reported by Kohler and Graham (1950). Whole liver and dried brewers' yeast appeared to be good sources of this factor while dried whey, distillers' solubles, and fermentation solubles were not. These workers also presented evidence which indicated that condensed fish press water contained an additional growth factor which was not present in the "grass juice factor" concentrate or fish meal. However, no definite chick growth responses to this concentrate were obtained in this laboratory.

Arscott and Combs (1950), using a soybean oil meal chick starter, obtained growth responses which plateaued suboptimally when either fish meal or butyl molasses solubles were added singly at different levels. However, a combination of these substances elicited a significantly greater growth response than when either was added alone. Dried brewers' yeast and dried whey also promoted increased growth, but combinations of either of these with butyl molasses solubles did not cause a further increase in the rate of growth. These results suggest the activity of two growth factors, one of which is contained in dried brewers' yeast and dried whey, and the other in fish meal. These observations are in agreement with the results presented in this report since data are presented which show that the chick requires two different unknown factors for rapid growth.

Sunde et al. (1950b) obtained increased growth responses through the addition of corn, dextrin, or a combination of yeast and whey to a purified diet containing alpha-protein. These workers used sucrose as the carbohydrate source in their basal diet. Liver powder, fish meal,

or fish solubles did not improve the growth or livability of the chicks. Similar observations reported by Hill and Briggs (1950) indicated the presence of an unidentified growth factor in corn meal and soybean oil meal. However, these workers observed that whole liver substances and dried brewers' yeast stimulated chick growth as well as corn meal, ground corn cobs, and certain other carbohydrate fractions.

An ethanol-soluble, heat and alkali-stable component which promoted chick growth was extracted from dextrin by Dietrich et al. (1951). Sucrose was used as the carbohydrate source in these studies. Growth activity was also obtained from the addition of yeast, whole liver powder, bran, casein, and dried whey. The growth stimulation by the various carbohydrate fractions is not believed to be identical to either factor described in the present report since similar results have been observed on diets containing corn or corn starch.

Cravens et al. (1951), also using an alpha-protein, synthetic diet, have presented evidence for two different unidentified chick growth factors. One of these was shown to be present in torula yeast while wheat bran was a source of the other. Animal products tested were poor sources of either factor. These workers have observed a thiamin deficiency in some of their trials. Definite precautionary measures were followed throughout the course of the studies conducted in this laboratory; therefore, thiamin deficiencies were not encountered.

Huff, et al. (1950) isolated and identified a factor which was required for growth of Lactobacillus bulgaricus 09. The microbiologically active material was isolated from one of its natural sources, whey, and identified with orotic acid. Later, Emerson and Folkers (1951) reported

synthetic lyxoflavin to have growth-promoting or vitamin-like activity in rats. In the same year, Fraenkel (1951) found that the mealworm, Tenebrio molitor requires, in addition to eight known B-vitamins, a factor present in yeast or liver extract, which has been called B_t. This factor appears to have certain properties which are similar to those described for the liver factor reported in the present paper. These substances (orotic acid, lyxoflavin, and vitamin B_t) elicited only very slight and inconsistent growth responses when assayed with chicks in this laboratory; however, since a limited number of trials were employed, it appears that further studies are necessary before conclusive results can be obtained.

The growth responses obtained from the addition of various supplements to the diets used in the present studies are not considered to be identical with those obtained from the feeding of antibiotics. However, it is quite possible that antibiotics may be effective in the establishment of certain "desirable-type" microorganisms which may stimulate intestinal synthesis of required nutrients. Groschke (1950) has reported that the feeding of antibiotics in the presence of high levels of five B-complex vitamins exerted an indirect sparing effect on the requirement for an unidentified factor in dried whey. These observations are supported by the results obtained in this laboratory by Jones and Combs (1951) in which antibiotic supplements were found to reduce the requirement for unidentified growth factors in practical chick rations. Romoser, et al. (1951) also presented evidence which suggests that bacterial synthesis of an unidentified growth factor(s) is responsible in part for the improved chick growth as a result of antibiotic feeding.

Studies on the growth responses of poults to crude animal protein materials are not as numerous as those conducted with chicks. However, the poult, as well as the chick, appears to require an unidentified factor(s) for rapid growth. These reports have been considered in Review of Literature, Section B. Studies with Poults. It may be well, however, to reconsider the findings of Scott (1951) who reported that poults require an unidentified factor, or factors, present in grass and alfalfa juice, as well as dried brewers' yeast, dried distillers' solubles, and dried skim milk. This factor(s) was concluded to be distinct from those of the animal protein factor complex since liver and other animal products were shown to be poor sources of the factor. However, since grass and alfalfa juices were considered excellent sources of this growth factor for poults, it may well be that the factor is identical with the "grass juice factor" reported by Kohler et al. (1936). Samples of the "grass juice factor" were not assayed with the poult in this laboratory.

SUMMARY

1. Evidence has been presented which clearly shows that at least two functionally distinct, unidentified factors are required by the chick for rapid growth. One of these growth factors was shown to be present in liver fraction "L," liver fraction Biopar "C," and dried brewers' yeast. Dried whey was demonstrated to be a good source of the other factor which was apparently lacking in the liver fractions and dried brewers' yeast.

2. The results of the studies presented here show that the "liver factor" is transmitted from the dam through the egg and thence to the chick. Progeny of hens housed on wire floors and fed a ration devoid of animal protein supplements for a 9-month period showed a marked growth response to the liver substance; whereas, chicks from dams housed on litter and fed a complete breeder ration for a similar period did not show an appreciable response to the liver fraction. However, the progeny of both groups of dams did exhibit a marked growth response to whey, and when whey was added in combination with either liver or dried brewers' yeast, an even greater growth response was observed.

3. Poults from dams fed a complete breeder ration and maintained on litter exhibited a marked growth response to a crude liver substance and to fractions prepared from this material. These results clearly demonstrate that the poult, as well as the chick, requires an unidentified factor present in liver for rapid growth. Evidence has also been obtained which suggests that two forms of the same unidentified growth factor exist. One of these forms is primarily soluble in the phenol layer, while the

other form is preferentially soluble in the water layer of a phenol-water, two-phase solubility system. Observations based on the incidence of a peculiar "swollen hock condition" indicate that the "liver factor" is not identical with the factor(s) in the crude liver substance which appears to be required for the prevention of this syndrome.

4. Fractionation procedures have been developed which show clearly some of the properties of a growth factor found in certain crude liver preparations. The unidentified growth factor, as determined by chick and/or poult assay, is dialyzable and is insoluble in a 75 percent saturated ammonium sulfate solution at pH 3.0. The factor is soluble in water, phenol, and 80 percent ethanol, but insoluble in ether. Separation of the factor from a phenol solution of the crude liver preparation was accomplished through a continuous extraction with 95 percent ethanol. The factor is stable to light, to a temperature of 100°C (wet) over extended periods, to mild alkaline hydrolysis at pH 11.5, to drying in vacuo over calcium chloride or concentrated sulfuric acid, and it is also stable within the acid range of pH 2.0 through 4.7.

5. A 350- to 400-fold concentration of the unidentified chick and poult growth factor has been accomplished by means of dialysis, precipitation of impurities with 80 percent ethanol, separation into the phenol layer of a phenol-water, two-phase solubility system, followed by separation into the phenol-butanol phase of a two-phase system composed of 25 percent phenol in n-butanol and water, and finally by subjection to further fractionation by means of countercurrent distribution using the above-mentioned phenol-butanol-water system. This concentrate stimulated growth of chicks when only 4.8 to 5.6 mg. were supplied per 100 gm. of diet.

6. The growth curve obtained through the application of counter-current distribution procedures suggests the existence of two forms of the same growth factor with different solubility properties, or two functionally distinct factors, one of which is present in two forms with somewhat different solubility properties. Each of these possibilities are subject to further study.

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