PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE, **147** 331-336 (1974)

Unidentified Growth Factor(s) Present in Alfalfa Water-Solubles for Young Guinea Pigs[1] (38336)

P. A. LOFGREN, P. S. REYES, K. W. MCNUTT, G. M. BRIGGS, AND G. O. KOHLER

Department of Nutritional Sciences, University of California, and Western Regional Research Laboratory, USDA, Berkeley, California 94720

Diets consisting of synthetic or highly purified ingredients have been developed to satisfactorily raise many types of experimental animals. However, reports are still being published concerning unidentified growth factors present in a variety of natural ingredients needed by various animal organisms (1, 2). Reid and Mickelsen (3) substituted an isonitrogenous amount of alfalfa for 20% of the casein in a purified diet for guinea pigs and obtained growth equal to that with a stock diet. Lakhanpal *et al.* (4) published evidence for an unidentified growth factor(s) from alfalfa and other plant sources for young guinea pigs and reviewed the pertinent literature. Maximum growth response was obtained when 10% dehydrated alfalfa was added to the diet. The factor appeared to be distinct from known nutrients and was not present in alfalfa ash. Singh *et al.* (5) observed a growth stimulant effect in guinea pigs when 5% oven-dried alfalfa, water extracted oven-dried alfalfa or ethanol extracted oven-dried alfalfa was added to purified diets. These studies suggested that various animal species require a "plant factor" not yet identified.

Subsequent studies in this laboratory indicated that the "plant factor" had its greatest effect on young, rapidly growing guinea pigs and a graded response was obtained with up to 30% alfalfa in the diet.[2] The relationship of these effects to the "grass juice factor" (6) is not clear.

The present studies were initiated to further characterize this growth factor. Kohler and Bickoff have described a fractionation of fresh alfalfa[3] which offered several new fractions of alfalfa to test.

Materials and Methods. Animals and care. Weanling (3–5 day old) male and female guinea pigs[4] were used in all experiments. Following a minimum preliminary period of four days on the basal diet, the animals were randomly assigned to experimental diets. Average initial weight on treatments did not exceed 167 g. Each group consisted of five animals. The animals were weighed three times per week and fresh feed and tap water were provided daily. Feed was offered *ad libitum*. Each study was conducted for three weeks. The animals were housed in individual, stainless-steel cages with wire-mesh floors. The animal room was air conditioned with the temperature maintained at about 72° F.

Experimental diets. The composition of the basal diet (a modification of Diet 13, Reid and Briggs (7)) is shown in Table I. Supplements or test fractions (in freeze-dried form) were added to this diet at the expense of proportional amounts of cornstarch, cerelose, and sucrose. All alfalfa fractions for an experiment were prepared from the same initial lot of fresh alfalfa material.

Sufficient experimental diet was mixed for the entire three week experiment and stored in a refrigerator. A commercial pelleted diet[5] was used as a control stock diet.

[1] Supported in part by U.S. Public Health Service Grant GM-1188, and presented in part at the American Institute of Nutrition meeting in April, 1973 (Federation Proc. 32(3): 950, No. 4107, 1973).

[2] Briggs, G. M., McNutt, K., and Kohler, G. O. Eleventh Technical Alfalfa Conference Proceedings. ARS-USDA, ARS 74-60, p. 105, Jan. 1972.

[3] Kohler, G. O., and Bickoff, E. M. Proc. 3rd International Conference of Food Science and Technology, Institute of Food Technologists, 221 N. LaSalle St., Chicago, Ill. (1970).

[4] Supplied by Isaacs Lab Stock, Litchfield, IL.

[5] Purina Guinea Pig Chow supplied by Ralston Purina Co., St. Louis, MO.

Printed in U.S.A.

TABLE I. Composition of Basal Diet.

	g/kg
Vitamin-free Casein[a]	300
Cornstarch[b]	200
Glucose hydrate (cerelose)[b]	160
Sucrose[b]	173
Cellulose[c]	25
Corn Oil[b]	30
Mineral mix[d]	75
Water soluble vitamins in cerelose[e]	20
Fat soluble vitamins in cerelose[f]	10
Arginine HCl[a]	3
Ascorbic acid[a]	2
Choline chloride[a]	2

[a] Nutritional Biochemical Co., Cleveland, OH.

[b] Colonial Food Supply of California, Oakland, CA.

[c] Cellophane Spangles, Rayon Processing Co., Pawtucket, RI.

[d] The following amounts (g) were present per kilogram of mineral mix (or per kilogram diet); $CaHPO_4$, 388 (29.2); $CaCO_3$, 66 (5.02); NaCl, 64 (4.83); KCl, 86 (6.50); $MgSO_4$, 51 (3.83); Ferric citrate, 7.1 (0.533); $MnSO_4 \cdot H_2O$, 4.1 (0.31); Potassium acetate, 277 (20.84); MgO, 55 (4.16), $ZnCO_3$, 1.43 (0.108). Present in mg/kg: KIO_3, 170 (7.6); $CuSO_4$, 56 (1.7); $KCr(SO_4)_2 \cdot 12H_2O$, 32 (1.0); $Na_2MoO_4 \cdot 2H_2O$, 1.67 (0.2); $NiCl_2 \cdot 6H_2O$, 0.67 (0.05); Na_2SeO_3, 0.67 (0.10)

[e] The following amounts (mg) were present per kg of water-soluble vitamin mix (or per kg diet): thiamin · HCl, 800 (16); riboflavin, 800 (16); pyridoxine · HCl, 800 (16); calcium pantothenate, 2000 (40); niacin, 10,000 (200); biotin, 25 (0.5); folic acid, 500 (10); vitamin B-12, 2.5 (0.05).

[f] The following amounts (mg) were present per kg of fat-soluble vitamin mix (or per kg diet): vitamin A palmitate (250,000 IU/g) 5,400 (54); vitamin D_3 (400,000 IU/g), 4 (0.04); alpha-tocopherol (250 IU/g), 2000 (20); 2-methlyl-1, 4-naphthoquinone, 200 (2).

Fractionation of alfalfa[6]. See Fig. 1 for a schematic description of the alfalfa fractions prepared. Abbreviations were used to identify the various fractions.[7]

Hexane residue (HR) was prepared by extracting weighed quantities of dehydrated alfalfa meal twice with hexane (six and three times the amount of alfalfa) and centrifuging and washing the residue each time with an equal volume of hexane. The extracted alfalfa was dried in a vacuum oven at 60°. A portion of this HR was then washed with water (WWHR), with the water-extract saved also.

Wet fractionation of fresh alfalfa yielded several fractions.[8] If the green press juice was heated to 70° or higher, a coagulated protein concentrate, leaf protein concentrate (LPC), was produced which could be separated from the brown wheylike liquid, alfalfa solubles (AS).

Water-washed LPC (WWLPC) resulted from additional removal of AS from the LPC.

A portion of the WWLPC fraction was also extracted with either hexane or chloroform:methanol (2:1) as described for the preparation of HR.

The AS fraction was extracted three times with 70% methanol to yield methanol soluble (MSAS) and insoluble (MIAS) fractions. The MSAS fraction which was highly water soluble was further fractionated by dialysis filtration using hollow fiber units[9] with a molecular weight size cut-off of approximately 5000. This fraction was prepared by filtering a 40% solution of MSAS through #1 filter paper. The solution was then recirculated through the hollow fibers while being dialyzed against distilled water. This dialysis proceeded for at least four hours. The solution was then frozen and lyophilized. The yield was in the range of 20–30%.

Whenever possible, two levels of a fraction were fed, with the upper level being equivalent to 30% HR. This level of HR was known to support growth similar to 30% dehydrated alfalfa, or the stock diet.[10]

Statistics. Data for each experiment were analyzed by an analysis of variance with Duncan's Multiple Range Test used to isolate treatment differences (8).

Results and Discussion. Experiment 1. Significant results of the animal studies are shown in Table II, along with the treatment, level of fraction added to basal diet, and growth response.

All fractions tested exhibited some growth activity over that of the basal diet. Hexane-extracted alfalfa (HR) (30%) was equivalent to 30% dehydrated alfalfa meal and the stock diet.

[6] Alfalfa fractions were prepared in cooperation with the Western Regional Research Lab., USDA, Albany, CA.

[7] HR, Hexane Residue, WWHR, Water-Washed Hexane Residue, LPC, Leaf-Protein Concentrate, AS, Alfalfa Solubles, WWLPC, Water-Washed Leaf-Protein Concentrate, MSAS, Methanol Soluble Alfalfa Solubles, MIAS, Methanol Insoluble Alfalfa Solubles.

[8] Eleventh Tech. Alf. Conf. Proceedings ARS-USDA, ARS 74-60, p. 10, Jan. 1972.

[9] Supplied by Bio-Rad Co., Richmond, CA.

[10] Unpublished data.

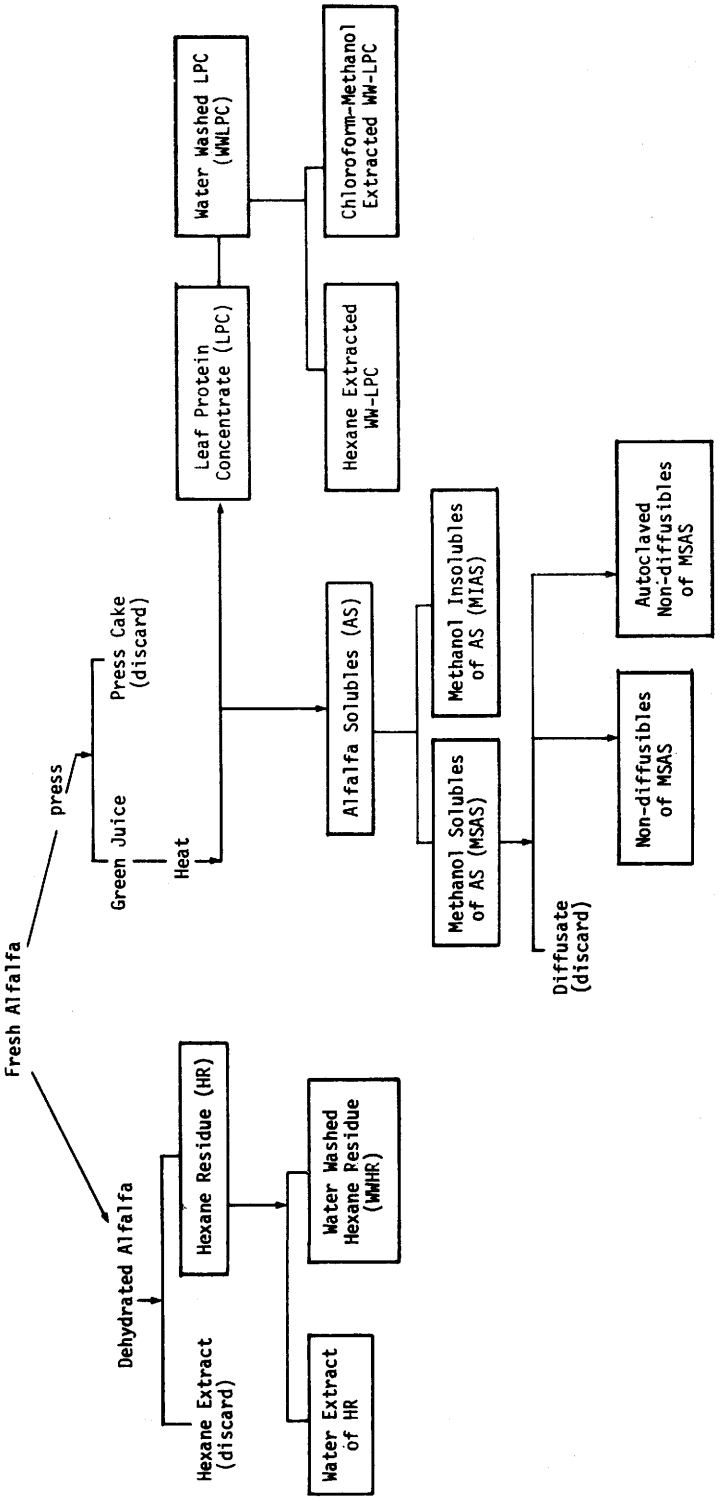

FIG. 1. Schematic relationship of alfalfa fractions (fractions in boxes were assayed for UGF).

TABLE II. Growth Response of Guinea Pigs Fed Various Alfalfa Fractions in Experiment 1 over a Three-Week Period (5 animals per group).

Group No.	Treatment	HR equiv. %	% Fraction in diet	Weight gain(g)
1	Stock diet			202^{d}
2	Basal			103^{a}
3	Basal + hexane residue (HR)	30	30.0	198^{d}
4	Basal + leaf-protein conc. (LPC)	30	15.0	167^{bcd}
5	Basal + hexane extracted WWLPC	30	9.0	156^{bcd}
6	Basal + chloroform: meth. extracted WWLPC	30	9.0	143^{abc}
7	Basal + alfalfa solubles (AS)	30	9.0	185^{cd}
8	Basal + methanol solubles of AS (MSAS)	30	6.9	81^{cd}
9	Basal + dehydrated alfalfa (30%)	30	30.0	197^{d}

^{a,b,c,d} Different superscripts in a column denote significant differences ($P < 0.05$).

Water-washing appeared to reduce activity in HR and LPC fractions. This supports the higher level of activity found in the AS component (group 7). In all cases, where two levels of a fraction were fed, growth activity was greater when a higher level was fed, although this was not always significantly different (Table II shows results for the higher levels). The trend to greater activity in the hexane-extracted WWLPC than the chloroform–methanol extracted WWLPC suggests that polar solvents may extract more of the factor (s). In addition, MSAS was found to be quite active at a level of 6.9% in the diet.

Experiment 2. This experiment attempted to repeat some of the results of Experiment 1, and demonstrate any effect due to level of addition of the methanol treated fractions (MIAS, MSAS). An ash fraction (from MIAS) was also tested. It should be noted water-washed HR (WWHR) was used in this experiment rather than HR.

At levels we used there was no graded response to the factor. As shown in Table III, this experiment did indicate significant growth activity in the MSAS material. The MIAS fraction exhibited reduced activity and its ash portion was not significantly active.

Feed intake and efficiency data for diets common to Experiments 1 and 2 are summarized in Table IV. Addition of water-soluble alfalfa fractions resulted in significantly ($P < 0.05$) improved feed efficiency compared to the basal diet fed alone.

Experiment 3. The MSAS fraction was partitioned further by dialysis filtration. In order to determine heat stability, one portion of the solution containing the nondiffusible fraction was autoclaved for 30 min at 120° (15 psi) prior to freezing and lyophilization.

The results of this experiment are shown in Table V and confirm the presence of growth factor (s) activity in the nondiffusible fraction of MSAS. Autoclaving did not reduce the activity of this fraction significantly. All treatment

TABLE III. Growth Response of Guinea Pigs Fed Various Alfalfa Fractions in Experiment 2 over a Three-Week Period.

Group No.	Treatment	No. of animals	HR equiv. %	% of fraction in diet	Weight gain (g)
1	Stock diet	5		—	193^{f}
2	Basal	4		—	90^{a}
3	Basal + water-washed hexane residue (WWHR)	5	30	30.0	151^{cde}
4	Basal + alfalfa solubles (AS)	5	30	9.0	187^{ef}
5	Basal + methanol solubles of AS (MSAS)	5	15	3.4	166^{def}
6	Basal + methanol solubles of AS (MSAS)	5	30	6.9	134^{bcd}
7	Basal + methanol solubles of AS (MSAS)	5	45	10.4	153^{cde}

^{a,b,c,d,e,f} Different superscripts in a column denote significant differences ($P < .05$).

TABLE IV. Feed Efficiency Data for Treatments Common to Experiments 1 and 2.

Treatment	No. of animals	HR equiv. (%)	F/G (g)
Stock diet	10	—	3.24[a]
Basal	9	—	4.14[b]
Hexane residue (HR) & (WWHR)	10	30	3.28[a]
Alfalfa solubles (AS)	10	30	2.80[a]
Methanol solubles of AS (MSAS)	10	15	2.91[a]
Methanol solubles of AS (MSAS)	10	30	2.92[a]
Methanol insolubles of AS (MIAS)	9	30	2.83[a]

[a,b] Different superscripts in a column denote significant differences ($P < 0.05$).

groups exhibited slower rates of growth (compared to Experiments 1 and 2), possibly due to an undetermined infection, since several animals did die during the course of the experiment.

These studies have concentrated a portion of the unidentified growth factor (s) activity which is present in 30% HR in a basal diet into a fraction (nondiffusible MSAS) which represents only 1% (or less) of the basal diet.

These studies suggest that the factor (s) is a large compound or forms a complex (> 5000 mol wt), which is water-soluble, partially methanol soluble and heat stable.

In contrast to the results of the investigation of Singh *et al.* (5) these experiments demonstrated growth activity in the AS and MSAS fractions. These experiments also used fresh alfalfa as a starting material rather than oven-dried alfalfa. The studies of both laboratories demonstrated little activity in the ash fraction. Singh *et al.* (5) demonstrated a possible factor in a residue fraction, while these studies suggest a factor in the water-soluble fraction. It is not known if these are the same factors. The present studies have been made in an attempt to further characterize this factor (s).

Hematocrit (9) and hemoglobin (10) determinations were made on all the experimental animals at the time of autopsy. In Experiment 1, the hematocrit and hemoglobin values were significantly ($P < 0.05$) increased in 14 of 16 treatment groups when alfalfa fractions were included in the basal diet. In Experiments 2 and 3, hematological values were more variable. Although not statistically significant, both hematocrit and hemoglobin values tended to be higher (in seven of ten treatment groups in Experiment 2 and in all treatment groups in Experiment 3) in the animals on the alfalfa fraction containing diets. With these limited data, it appears that a factor may exist in alfalfa which is responsible for changes in hematocrit and hemoglobin levels. However, further studies would be necessary to clarify the existence of a hematological factor (s) in alfalfa and its relationship to the growth factor. We do not believe it is directly related to folic acid, vitamin B_{12}, iron, copper, or any known nutrient, since the diet contains more than adequate amounts of these nutrients for the guinea pig. This is not to say that the action of these fractions may not indirectly affect the utilization of existing nutrients.

Summary. At least one unidentified factor is present in alfalfa which stimulates growth (and may alter hematological values) in young guinea

TABLE V. Growth Response of Guinea Pigs Fed Various Alfalfa Fractions in Experiment 3 over a Three-Week Period.

Group No.	Treatment	No. of animals	HR equiv. %	% of fraction in diet	Weight gain (g)
1	Stock diet	4	—	—	146[a]
2	Basal	4	—	—	76[b]
3	Basal + alfalfa solubles (AS)	5	30	9.0	142[a]
4	Basal + methanol solubles of AS (MSAS)	5	15	3.5	131[a]
5	Basal + nondiffusibles of MSAS	3	15	0.6[c]	143[a]
6	Basal + nondiffusibles of MSAS (autoclaved)	5	15	1.0	141[a]

[a,b] Different superscripts in columns denote significant differences ($P < 0.05$).

[c] Level added restricted by amount available.

pigs. These factor (s) are organic, water soluble, partially methanol soluble, heat stable, and of relatively large molecular size (> 5000 mol wt) or attached to large molecules. They are active at low levels (1% or less) in a purified basal diet. Further studies are in progress to characterize these factors.

The authors are grateful to Bennie Thomas, Diane Bray, and Jan Alberts for their assistance in animal care, and to K. Smith for assistance in making alfalfa fractions.

1. Blair, R., Scott, M. L., and Young, R. J., J. Nutr. **102,** 1529 (1972).

2. Neville, P. F., and Luckey, T. D., J. Nutr. **101,** 1217 (1971).

3. Reid, M. E., and Mickelsen, O., J. Nutr. **80,** 25 (1963).

4. Lakhanpal, R. K., Davis, J. R., Typpo, J. T., and Briggs, G. M., J. Nutr. **89,** 341 (1966).

5. Singh, K. D., Morris, E. R., Regan, W. O., and O'Dell, B. L., J. Nutr. **94,** 534 (1968).

6. Kohler, G. O., Elvehjem, C. A., and Hart, E. B., J. Nutr. **15,** 445 (1938).

7. Reid, M. E., and Briggs, G. M., J. Nutr. **51,** 341 (1953).

8. Steele, R. G., and Torrie, J. H. "Principles and Procedures of Statistics," McGraw-Hill Book Co., New York (1960).

9. Gradwohl, R. B., "Clinical Laboratory Methods and Diagnosis," 5th edition, Vol. 1, p. 594. C. V. Mosby & Co., St. Louis, MO (1956).

10. Cannan, R. K., Clin. Chem. **4,** 246 (1958).

Received July 24, 1973. P.S.E.B.M., 1974, Vol. 147.