added *in vitro* to normal rat kidney submitochondrial phosphorylating complexes, however, suggests a primary effect. It is suggested that the mechanism of action of aminonucleoside may be bimodal, producing some of its metabolic effects either directly or indirectly through inhibition of reactions producing energy-rich ATP and some directly through inhibition of reactions by which the energy of ATP is released.

1. Bartlett, P., Ford, L., *Fed. Proc.*, 1960, v19, 38.
2. Fisher, E. R., Tsuji, F. I., Gruhn, J., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 448.
3. Fisher, E. R., Gruhn, J., *A.M.A. Arch. Path.*, 1958, v65, 545.
4. Weinbach, E. C., *Anal. Biochem.*, 1961, v2, 335.
5. Hiller, A., Plazin, J., Van Slyke, D. D., *J. Biol. Chem.*, 1948, v176, 1401.
6. Weinbach, E. C., Garbus, J. J., *ibid.*, 1959, v234, 412.
7. Fiske, C. H., Subbarow, Y., *ibid.*, 1925, v66, 375.
8. Hiller, A., Greif, R. L., Beckman, W. W., *ibid.*, 1948, v176, 1421.
9. Fisher, R. A., *Statistical Methods for Research Workers*, 1948, Oliver & Boyd, Ltd., Edinburgh.
10. Abood, L. G., Alexander, L., *J. Biol. Chem.*, 1957, v227, 717.
11. Potter, V. R., in *Manometric Techniques*, by Umbreit, W. W., Burris, R. H., & Stauffer, J. F., Burgess Publishing Co., Revised Edition 1957, p. 185-187.
12. Kielley W. W., Kielley, R. K., *J. Biol. Chem.*, 1953, v200, 213.
13. Hess, R., *Am. J. Path.*, 1960, v37, 583.
14. DiSabato, G., Fonnesu, A., *Biochim. et Biophys. Acta*, 1959, v35, 358.
15. Skou, J. C., *ibid.*, 1962, v58, 314.
16. Sjostrand, F. S., *Int. Rev. Cytol.*, 1956, v5, 515.
17. Rhodin, J., *ibid.*, 1958, v7, 506.
18. Novikoff, A. B., in *Biology of Pyelonephritis*, International Symposium by Quinn, E. L., and Kass, E. N., Little, Brown & Co., 1960, p. 113-144.
19. Persijn, J. P., Daems, W. T., DeMan, J. C. N., Meijer, A. E. F. N., *Histochemie*, 1961, v2, 372.

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Pancreatic Hypertrophy and Chick Growth Inhibition by Soybean Fractions Devoid of Trypsin Inhibitor.* (27962)

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Discovery of the presence of trypsin inhibitors in soybeans(1,2) and other legumes (3,4), and the ability of concentrates of these factors to inhibit growth in rats(5,6) and chicks(7), led to the assumption that the trypsin inhibitor was responsible for the inferior nutritive value of raw soybean meal. However, contradictory results were later obtained and the mechanism of growth inhibition by raw soybean remains obscure. Feeding a purified trypsin inhibitor was shown to have an innocuous effect on growth of chicks (8,9) as well as of rats(8,10). Furthermore, fractions of beans (*Phaseolus vulgaris*) that were devoid of anti-tryptic activity inhibited growth markedly(11). Trypsin inhibitor concentrates prepared from raw soybean meal depressed fat absorbability, caused pancreatic hypertrophy, although the levels used were only slightly growth depressing(12).

Results of a study designed to determine if trypsin inhibitor was the factor that depressed growth and caused pancreatic hypertrophy in the chick are presented here.

Experimental. Crossbred (New Hampshire × Delaware) chicks were used in the experiments and were maintained in electrically heated, thermostatically controlled battery brooders with raised wire screen floors located in a temperature controlled laboratory. Feed and water were supplied *ad libitum*.

A semi-purified type diet (Table I) was used, which contained all nutrients known to be required by the chick. The heated soybean meal was prepared from soybean brew

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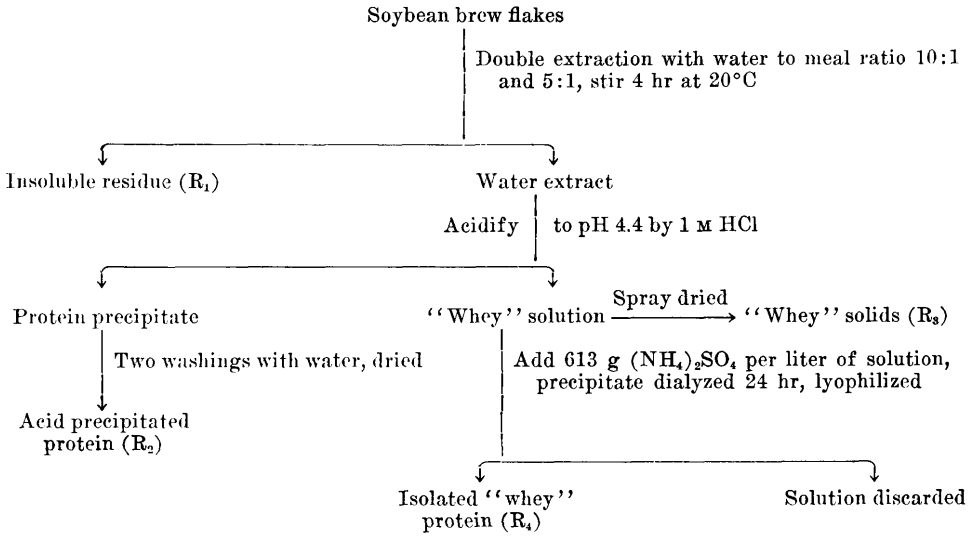

FIG. 1. Preparation of soybean meal fractions.

flakes† by heating in thin layers in an autoclave at atmospheric pressure for 30 minutes. Chicks were weighed in groups at end of the experiment, and their feed consumption was recorded. Three chicks per pen were randomly selected, weighed individually, and then sacrificed for purpose of pancreas measurements. Pancreas weights were expressed as mg per 100 g body weight.

Two crude soybean trypsin inhibitors were prepared from soybean brew flakes. The alcohol-insoluble (Ali) and the acetone insoluble (Ai) fractions were prepared according to Bowman(13), omitting the 2.5% trichloracetic acid 95°C treatment for Ai. Nitrogen contents of Ali and Ai were determined to be 9.0 and 1.5%, respectively.

Soybean meal fraction insoluble at pH 4.4 (SBM 4.4) was prepared by removing the fraction soluble at that pH (Fig. 1). The insoluble fraction was dried in an oven at 50°C and then ground. The resulting yield of SBM 4.4 was 75% and its nitrogen content, 10.0%

Whey protein fraction (R_4) was prepared by adding 613 g $(NH_4)_2SO_4$ per liter of "whey solution" obtained after preparing SBM 4.4. The precipitate was dialyzed in cold tap water and lyophilized. The resulting yield of R_4 was 0.5% and its nitrogen content 15.7%.

"Whey" solids (R_3) were prepared by spray drying the "whey" solution. Exact yield of the product could not be determined because of the loss that resulted by drying small quantities of "whey" in a fairly large spray dryer. Nitrogen content of the product (R_3) was 1.9%.

Water insoluble residue (R_1) was prepared by removing the fraction soluble in water. This fraction was extracted twice in cold water (20°C) for approximately 4 hours with water to meal ratios of 10:1 and 5:1. The residue was dried in an oven at 50°C and then ground. Resulting yield of R_1 was 35% and its nitrogen content, 7.13%. Water extract was brought to pH 4.4 by addition of 1 M HCl. The acid precipitated protein (R_2) was washed twice with water, dried in the oven at 50°C and then ground. The resulting yield of the product, R_2, was 40% with a nitrogen content of 13.9%. All fractions were added to basal diet at the expense of cerelose.

Trypsin-inhibiting activity was determined by the casein digestion method of Kunitz (14) as described by Laskowski(15) at 280 mμ with a Beckman Spectrophotometer, Model DU, using trypsin (1:250), vitamin

† Specially processed, low temperature desolventized soybean flakes obtained from Archer-Daniels-Midland Co., Cincinnati, Ohio.

free casein (Hammersten quality) and Sørenson 0.1 M phosphate buffer (pH 7.6) prepared from $Na_2HPO_4 \cdot 12\ H_2O$ and $NaH_2PO_4 \cdot 2H_2O$ according to Gomori(16). Antitryptic activity in O.D. units of various soybean fractions is given in Table II.

Raw residue (R_1) was treated with HCl to bring the pH to 1.6. It was then treated with pepsin (1:3000) at the rate of 175 mg/100 g R_1, then incubated for 24 hours at 37°C. After this period, the pH was brought to 7.6 using .05 M NaOH and the material was further treated with trypsin (1:300) at a rate of 2.5 g/100 g R_1, and incubated again for a period of 24 hours at 37°C. The material was then dialyzed in cold tap water with constant stirring and then lyophilized.

Results. *In vitro* anti-tryptic activity, as measured by the optical density measurements at 280 mμ (Table II), showed that besides raw soybean meal, only the fractions designated as R_3 and R_4 possessed a very high antitrypsin activity. Adding crude antitryptic fractions, prepared according to the procedure of Bowman(14), did not depress the growth rate of chicks, nor did they produce pancreatic hypertrophy or reduced feed efficiency (Table III). Ai and Ali were added in amounts equivalent to the amount of raw soybean meal in the diet of the chicks.

TABLE I. Composition of Basal Diet.

Ingredients	%
Soybean brew flakes	50.0
Cellulose	2.0
Glucose	33.4
Corn oil	10.0
Limestone	1.0
Dicalcium phosphate	2.0
Iodized sodium chloride	.5
Mineral-vitamin mixture*	.5
DL-methionine	.6
Protein, calculated	25.0

* Premix supplied the following per kg of diet: manganese ($MnSO_4$), 55 mg; zinc ($ZnSO_4 \cdot 7H_2O$), 44 mg; iron ($FeSO_4 \cdot 7H_2O$), 20 mg; copper ($CuSO_4$), 2 mg; cobalt ($CoCl_2 \cdot 6H_2O$), 1 mg; potassium (KH_2PO_4), 2.5 g; magnesium ($MgSO_4 \cdot 7H_2O$), 440 mg; vitamin B_{12}, 9 μg; riboflavin, 3.3 mg; ca-pantothenate, 9.3 mg; niacin, 26.4 mg; thiamin, 1.76 mg; folic acid, 0.55 mg; pyridoxine · HCl, 2.8 mg; choline, 1320 mg; vitamin A, 4400 I.U.; vitamin D_3, 440 I.C.U.; menadione, 2.2 mg; vitamin E, 5 I.U.; biotin, 0.09 mg; and oleandomycin, 22 mg.

TABLE II. Anti-tryptic Activity of Various Soybean Fractions.

	Optical density at 280 mμ, 20 min incubation at 37°C
Casein	.830
Heated soybean meal	.830
Raw soybean meal	.410
SBM 4.4 fraction	.780
Residue 1 (water insoluble residue)	.810
" 2 (acid ppt. protein)	.790
" 3 ("whey" solids)	.420
" 4 (isolated "whey" protein)	.200

Three fractions, SBM 4.4, R_3, and R_4 were each fed to 2 pens of 10 chicks (5 males, 5 females) from day of age to 2 weeks (Table IV). Only the fraction designated as SBM 4.4 (raw) depressed chick growth, reduced feed efficiency and caused pancreatic hypertrophy. The SBM 4.4 contained both the residue (R_1) and the acid precipitated protein (R_2) fractions.

When R_1 and R_2 fractions were fed (Table V), only the R_1 (raw) significantly depressed chick growth, accompanied by pancreatic hypertrophy and reduced feed utilization. This experiment was repeated; similar results were obtained (Table V, Exp. II).

Treatment of raw residue (R_1) with pepsin and trypsin did not overcome the growth depressing effect of the R_1 fraction (Table VI). In each case, improvement by heat treatment was obtained.

Discussion. Results of the study reported here show that soybean fractions devoid of anti-tryptic activity caused growth depression, poor feed efficiency, and pancreatic hypertrophy. Crude anti-trypsin inhibitor preparations and fractions possessing high anti-tryptic activity had little effect on growth or pancreatic hypertrophy. Recently, Birk and Gertler(17) reported that a soybean meal fraction SBM 4.2, having about 50% of the original raw soybean meal trypsin inhibitory activity and almost no hemagglutinating activity, was only slightly superior to raw meal when added to diets of chicks. Our chick growth results with a similar fraction confirm these findings but also show that growth was depressed even though the fraction was devoid of anti-tryptic activity.

TABLE III. Effect of Bowman's Anti-tryptic Factors on Chick Growth, Feed Efficiency and Pancreatic Hypertrophy.

Soybean meal treatment	Avg wt 10 days, g	Feed efficiency g feed/wt gain	Pancreas wt, mg/100 g body wt
Raw	61*	3.19*	807*
Heated	81	1.69	595
Heated + acetone insoluble	81	1.80	604
Heated + alcohol insoluble	82	1.78	602

* $P < .05$.

TABLE IV. Effect of Feeding Soybean Fractions on Growth, Feed Efficiency and Pancreatic Hypertrophy in Chicks.

Soybean fraction or treatment	Avg wt 2 wks, g	Feed efficiency g feed/wt gain	Pancreas wt mg/100 g body wt
Raw meal	120*	2.80*	781*
Heated meal	155	1.78	496
SBM 4.4, raw	122*	2.78*	797*
" " heated	152	1.62	525
Heated + R_3, raw	144	1.89	509
" " heated	149	1.71	512
Heated + R_4, raw	140	2.12	540
" " heated	144	1.98	534

* $P < .05$, compared to appropriate treatments.

TABLE V. Chick Growth, Feed Efficiency and Pancreatic Hypertrophy on Water Insoluble Residue (R_1) and Acid Precipitated Protein (R_2).

Treatment		Avg wt gain per chick – 7 days, g		Feed efficiency, g feed/wt gain		Pancreas wt mg/100 g body wt	
Soybeans	Fractions	Exp I*	Exp II*	Exp I	Exp II	Exp I	Exp II
Raw	—	30.8†	24.8†	2.45†	2.42†	854†	824†
Heated	—	53.5	57.8	1.86	1.75	511	485
Heated	20% R_1 raw	31.9†	24.2†	3.13†	3.02†	771†	787†
"	20% R_1 heated	53.7	56.7	1.86	1.82	507	501
—	30% R_2 raw	51.4	—	1.67	—	470	—
—	30% R_2 heated	56.0	—	1.53	—	450	—

* 3-day-old chicks fed experimental diets for 7 days; duplicate pens of 10 chicks (5 male, 5 female) used per treatment in each experiment.
† $P < .05$, compared to appropriate treatment.

TABLE VI. Effect of Enzymatic Digestion of Water Insoluble Residue (R_1) from Raw Soybean Meal on Chick Growth, Feed Efficiency and Pancreatic Hypertrophy.

Soybean treatment	Avg wt gain per chick* 9 days, g	Feed efficiency, feed/gain	Pancreas wt mg/100 g body wt
Raw meal	42.9†	2.35†	718†
Heated meal	63.4	1.63	425
Heated + 20% R_1, raw	47.3†	2.49†	698†
" " " heated	64.1	1.88	442
Heated + 15% R_1, pepsin, trypsin, digested, dialyzed and lyophilized, raw	44.0†	2.50†	615†
Heated + 15% R_1 as above, heated	52.0	2.13	445
Heated + 20% R_1, pepsin, trypsin digested, raw	46.5†	2.47†	680†
Heated + 20% R_1 as above, heated	66.3	1.79	450

* 3-day-old cross-bred chicks fed experimental diet for 9 days; duplicate pens of 10 chicks (5 male, 5 female) used for each treatment.
† $P < .05$, compared to appropriate treatment.

Predigestion of raw soybean meal with pepsin or papain has been shown to destroy most of its *in vitro* anti-tryptic activity(18). In studies reported here, feeding raw soybean meal fraction R_1, predigested with pepsin and trypsin, failed to improve chick growth significantly. These findings support the results of Brambila *et al.*(19), which showed that trypsin supplementation did not overcome the growth depressing properties of raw soybean meal. The fact that treatment of soybean fraction R_1 with pepsin and trypsin followed by dialysis failed to overcome the growth depression suggests that the fraction depressing growth may not be entirely of protein nature or origin, or that it is simply resistant to action of these enzymes.

Pancreatic hypertrophy in the chick was caused by raw soybean meal, SBM 4.4 and the insoluble residue (R_1) fractions and was accompanied by a growth depression. It is possible that the fraction in raw soybean meal which depresses growth has its primary effect on the pancreas. Subsequent loss of amino acids through increased pancreatic secretions as suggested by Lyman and Lepkovsky(20) may deprive the chick of essential nutrients. The relation of increased O_2 consumption(21) by chicks fed raw soybean meal to pancreatic hypertrophy is not clear.

Summary. Fractionation studies on raw soybean meal have shown that the major chick growth inhibiting factor resides in the water insoluble residue. This fraction was devoid of anti-trypsin activity. Other fractions which contained high anti-trypsin activity were found to have an innocuous effect on growth of chicks. In all cases, where growth depression occurred, the pancreas was markedly hypertrophied.

1. Ham, W. E., Sandstedt, R. M., *J. Biol. Chem.*, 1944, v154, 505.

2. Bowman, D. E., PROC. SOC. EXP. BIOL. AND MED., 1944, v57, 139.

3. Borchers, R., Ackerson, C. W., Kimmett, L., *Arch. Biochem.*, 1947, v13, 291.

4. Sohonie, K., Bhandarkar, A. P., *J. Sci. Ind. Res.*, 1954, v13B, 500.

5. Klose, A. A., Hill, B., Fevold, H. L., PROC. SOC. EXP. BIOL. AND MED., 1946, v62, 10.

6. Haines, P. C., Lyman, R. L., *J. Nutrition,* 1961, v74, 445.

7. Ham, W. E., Sandstedt, R. M., Mussehl, F. E., *J. Biol. Chem.*, 1945, v161, 635.

8. Borchers, R., Ackerson, C. W., Mussehl, F. E., *Arch. Biochem. Biophys.*, 1948, v19, 317.

9. Riley, D. J., Thesis, Washington State Univ., 1961.

10. Sohonie, K., Apte, U. V., Ambe, K. S., *J. Sci. Ind. Res.*, 1958, v17C, 42.

11. Honavar, P. M., Shih, Cheng-ven, Liener, I. E., *J. Nutrition,* 1962, v77, 109.

12. Garlich, J. D., Nesheim, M. C., *Fed. Proc.*, 1962, v21, 393.

13. Bowman, D. E., PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 547.

14. Kunitz, M., *J. Gen. Physiol.*, 1947, v30, 291.

15. Laskowski, M., *Methods in Enzymology,* Vol. 2, Eds., S. B. Colowick and N. O. Kaplan. Academic Press, Inc., New York, p33.

16. Gomori, G., *ibid.*, v1, p143.

17. Birk, Y., Gertler, A., *J. Nutrition,* 1961, v75, 379.

18. Birk, Y., Bondi, A., Gertler, A., *Proc. 4th Cong. Sci. Soc.* (Rehovoth, Israel), 1961.

19. Brambila, S., Nesheim, M. C., Hill, F. W., *J. Nutrition,* 1961, v75, 13.

20. Lyman, R. L., Lepkovsky, S., *ibid.*, 1957, v62, 269.

21. Saxena, H. C., Jensen, L. S., McGinnis, J., *Poultry Sci.*, 1962, v42, 1304.

Received September 18, 1962. P.S.E.B.M., 1963, v112.