Effect of Chloroquine and Certain Amines, Vitamins, and Arthritis-Influencing Agents on the Zinc-Deficient Chick[1] (35603)

E. M. REIMANN, M. L. SUNDE, AND W. G. HOEKSTRA

Department of Biochemistry and Department of Poultry Science, University of Wisconsin, Madison, Wisconsin 53706

Previous work in this laboratory (1) has shown that chicks fed zinc-deficient diets based on isolated soybean protein develop a leg abnormality characterized by a shortening and thickening of the long bones, an enlarged hock joint, and a resultant stiff and unsteady gait. The stiff gait and the reluctance of the chicks to move suggest that the joints, like those of rheumatoid arthritics, are painful. Furthermore, oral administration of the antiarthritic agents aspirin, indomethacin, cortisone, and phenylbutazone can largely prevent the development of this condition, with little influence on the other signs of zinc deficiency (2). This condition can also be alleviated by the addition of histamine (3) or histidine (3) to the diet. The mechanisms by which these agents exert their beneficial effects have not yet been established. Two experiments were therefore conducted to test the effects of the following types of agents on the leg abnormality of zinc-deficient chicks: (a) vasoactive amines, (b) compounds which might influence histamine metabolism, (c) supplemental amounts of vitamins which are needed for normal hock development, and (d) certain other agents which are known to influence experimental or human arthritis. The last group included an antiviral agent (statolon), an immunosuppressive agent (*N*-methyl-2,2′-dichloro diethylamine hydrochloride, *i.e.*, mechlorethamine), and an inhibitor of lysosomal enzymes (gold sodium thiosulfate). It was reasoned that either an effectiveness or an ineffectiveness of the various agents in alleviating the leg abnormalities would give information on the possible mechanisms whereby histamine and anti-inflammatory agents act to alleviate this condition.

Methods. Two experiments were conducted with day-old New Hampshire × single comb white leghorn chicks without segregation according to sex. The chicks were housed in a stainless steel battery at 37 to 40° after random allotment to treatment groups. The number of chicks in each treatment group is indicated in Table I. Feed and water were provided *ad libitum* in aluminum troughs except for the diet-restricted group of Expt. 2. Feed was mixed every 2 weeks and refrigerated until fed. The basal isolated soybean protein diet described previously was used (1). In Expt. 1, 10 other diets were fed. Each diet consisted of the basal diet plus one of the following additions: 0.2% chloroquine diphosphate,[2] 1.0% *p*-aminobenzoic acid,[3] 0.2% niacin,[4] 0.1% pyridoxine·HCl,[4] 0.2% serotonin creatinine sulfate complex,[5] 0.2% L-norepinephrine·HCl,[5] 0.2% L-epinephrine,[5] 0.2% histamine·2HCl,[6] 8 ppm zinc (as zinc oxide), or 80 ppm zinc. Nine additional treatments consisted of injecting chicks fed the basal diet with the following agents: 4 or 40 mg of gold sodium thiosulfate[7]/kg of

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[2] Aralen diphosphate, Sterling-Winthrop Research Institute, Rensselaer, N.Y.

[3] General Biochemicals, Inc., Laboratory Park, Chagrin Falls, Ohio.

[4] Merck and Co., Inc., Rahway, N.J.

[5] Sigma Chemical Co., St. Louis, Mo.

[6] Nutritional Biochemicals, Corp., Cleveland, Ohio.

[7] Prepared from gold chloride and sodium thiosulfate as described in the Merck Index, ed. 7, 1960. Merck and Co., Inc., Rahway, N.J., page 495.

TABLE I. Effect of Zinc and Certain Amines, Vitamins, and Arthritis-Influencing Agents on Body Weight, Mortality, and Leg Scores of Chicks Fed Soy Protein Diets.

Treatment [dietary addition (% of diet)]	Supplemental zinc (ppm) Expt.: 1	2	No. of chicks 1	2	Mortality (%) 1	2	Body wt (g) 1	2	Leg score 1	2
None	0[a]		26		12		89 A[b]		3.9 AB[c]	
	8		16		13		176 C		3.4 BC	
	80		10		30		200 E		1.7 D	
		5		10		10		134 DE		3.8 FG
None (restricted fed)		80		10		20		152 E		1.0 A
None		80		10		10		230 F		1.3 A
Histamine • 2HCl, 0.2%	0		10		0		80 AB		2.7 BCD	
		5		10		0		116 CD		1.7 AB
Serotonin,[d] 0.2%	0		10		0		84 A		3.9 AB	
L-Epinephrine, 0.05%		5		10		20		103 BC		3.6 EFG
0.10%		5		10		20		80 AB		3.6 EFG
0.20%	0		10		80		57 AB		2.5 BCD	
L-Norepinephrine • HCl, 0.05%		5		10		0		114 CD		3.5 EFG
0.10%		5		10		20		105 BC		3.3 DEFG
0.20%	0		10		70		51 B		2.0 CD	
p-Aminobenzoic acid, 0.2%		5		10		40		131 CDE		3.0 CDEF
0.5%		5		10		40		115 CD		3.0 CDEF
1.0%	0		10		60		68 AB		3.0 BCD	
Chloroquine diphosphate, 0.05%		5		10		0		103 BC		2.5 CD
0.10%		5		10		10		68 A		2.3 BC
0.20%	0		10		100		—		—	
Niacin, 0.2%	0		10		0		76 AB		3.8 AB	
Pyridoxine • HCl, 0.1%	0		10		30		93 A		4.1 AB	

TABLE I (continued)

Treatment (injection)	Supplemental zinc (ppm) Expt.: 1	2	No. of chicks 1	2	Mortality (%) 1	2	Body wt (g) 1	2	Leg score 1	2
Saline, 0.1 ml/chick, sc[e,h]	8		8		13		157 CD		3.6 ABC	
0.1 mg/chick, sc[g]		5		4		0		131 CDE		4.3 G
5.0 ml/kg, ip[f]	0		8		25		95 A		4.2 AB	
10.0 ml/kg, ip[i]		5		4		0		134 CED		4.0 FG
Heparin, 200 IU/kg, sc[h]	0		8		13		92 A		4.9 A	
2000 IU/kg, sc[h]	0		8		13		87 A		3.6 ABC	
200 IU/kg, sc[h]	8		8		25		153 D		2.5 BCD	
2000 IU/kg, sc[h]	8		8		0		175 CD		4.4 AB	
Gold thiosulfate, 4 mg/kg, im[f]	0		8		13		86 A		3.7 ABC	
40 mg/kg, im[f]	0		8		13		82 AB		3.6 ABC	
Mechlorethamine • HCl, 1 mg/kg, sc[f]	0		8		0		81 AB		3.1 BC	
5 mg/kg, sc[f]	0		8		100		—		—	
1 mg/kg, sc[i]		5		6		17		99 BC		3.0 CDEF
2 mg/kg, sc[g]		5		10		0		124 CD		3.9 FG
Statolon, 50 mg/kg, ip[f]	0		8		25		82 AB		3.0 BCD	
100 mg/kg, ip[f]	0		8		13		79 AB		3.0 BCD	
25 mg/kg, ip[i]		5		10		10		110 CD		3.1 CDEF
100 mg/kg, ip[i]		5		10		30		98 BC		2.9 CDE

[a] Unsupplemented diet contained 8 ppm zinc by analysis.
[b] Values within a column followed by the same capital letter are not significantly different ($p > .05$).
[c] 1 = normal; 5 = very abnormal.
[d] Creatinine sulfate complex.
[e] Route of injection: subcutaneous (sc); intramuscular (im); or intraperitoneal (ip).
[f] Injected twice weekly.
[g] Injected every 3 days.
[h] Injected 3 times weekly.
[i] Injected daily.

body weight (im),[8] 2 × weekly; 1 or 5 mg of mechlorethamine·HCl[9]/kg (sc), 2 × weekly; 50 or 100 mg of statolon[10]/kg (ip), 2 × weekly; 5 ml of saline/kg (ip), 2 × weekly; and 200 or 2000 IU of heparin[11]/kg (sc), 3 × weekly. Three other treatments consisted of injecting chicks fed the basal diet plus 8 ppm zinc with the following: 200 or 2000 IU of heparin/kg (sc), 3 × weekly or 0.1 ml of saline/chick, 3 × weekly (sc). The injections of gold sodium thiosulfate, mechlorethamine, and heparin were made in a volume of 0.1 ml of saline. Statolon was injected as a 1% solution in saline.

Because the doses of some agents tested in Expt. 1 caused high mortality rates and the doses of others may have been too low to be effective, treatment with certain agents was repeated in a second experiment. In the second experiment, lower levels of chloroquine, *p*-aminobenzoic acid, epinephrine, and norepinephrine, and more frequent injections of statolon and mechlorethamine were utilized. In the second experiment the basal diet contained 5 ppm supplemental zinc because of the severe zinc deficiency resulting in extremely poor growth in Expt. 1. In addition, 10 other diets were used. Each consisted of the basal diet plus one of each of the following additions: 0.05 or 0.10% chloroquine diphosphate,[5] 0.05 or 0.10% epinephrine, 0.05 or 0.10% norepinephrine·HCl, 0.2 or 0.5% *p*-amino benzoic acid, 0.2% histamine·2HCl, or 80 ppm zinc. Six additional treatments consisted of injecting chicks fed the basal diet as follows: 10 ml of saline/kg (ip) daily; 25 or 100 mg of statolon/kg (ip) daily; 1 mg of mechlorethamine·HCl/kg (sc) daily; 2 mg of mechlorethamine·HCl/kg (sc) every 3 days; and 0.1 ml of saline/chick (sc) every 3 days.

At 4 weeks of age, leg scores and body weights of the chicks were determined as described previously (2). A leg score of 1 is normal and 5 is severely abnormal. Duncan's test with Kramer's modification for unequal replication was used to determine the statistical significance of the treatment differences at the 5% probability level (4).

Results and Discussion. The results of these experiments are shown in Table I. The feeding of 80 ppm zinc, 0.2% histamine dihydrochloride, 0.05 or 0.1% chloroguine diphosphate significantly ($p<.05$) reduced leg scores. Daily injection of 100 mg of statolon/kg of body weight also significantly ($p<.05$) decreased leg scores; lower doses of statolon tended to produce similar effects, but they were nonsignificant ($p>.05$). Daily injection of 1 mg of mechlorethamine hydrochloride/kg of body weight significantly ($p<.05$) decreased leg scores when compared to saline-injected chicks, but not when compared to the uninjected control group. The other doses of mechlorethamine and the other agents tested had no significant effect on leg scores. The feeding of 0.2% norepinephrine also appeared to decrease leg scores, but only three chicks of greatly reduced body weight survived the treatment; and, therefore, no conclusions can be drawn from this observation.

Body weights were significantly ($p<.05$) different for all three levels of dietary zinc in Expt. 1 and were significantly ($p<.05$) increased by dietary zinc in Expt. 2. Body weights tended to be reduced as a result of many of the treatments imposed, but the decrease was significant ($p<.05$) only when the chicks were fed 0.1 or 0.2% norepinephrine, 0.05 or 0.1% epinephrine, 0.05 or 0.1% chloroquine diphosphate.

The significant ($p<.05$) increase in body weight and decrease in leg score resulting from supplemental zinc agrees with previous reports (1–3). Likewise, the beneficial effect of histamine on leg score confirms previous reports (2, 3). The reduced effectiveness of histamine in Expt. 1 may have been due to the severe zinc deficiency since previous studies utilized basal diets of higher zinc content.

Because the effect of histamine in preventing the development of leg abnormalities in zinc-deficient chicks may be related to its effects on circulation, other vasoactive amines were tested to see if they might also have this property. Furthermore, urinary norepi-

[8] Route of administration: intramuscular (im); subcutaneous (sc); or intraperitoneal (ip).

[9] Aldrich Chemical Co., Milwaukee, Wis.

[10] Eli Lilly and Co., Indianapolis, Inc.

[11] Organon, Inc., West Orange, N.J.

nephrine levels have been reported to decrease in rheumatoid arthritis (5) and histamine has been shown to cause the release of catecholamines (6, 7). Although norepinephrine was apparently effective in alleviating the leg disorder in Expt. 1, only three chicks survived the treatment and no effect was observed in Expt. 2 or with epinephrine in either experiment. The injection of serotonin has been demonstrated to cause a decrease in blood pressure in chickens presumably via histamine release (8). Serotonin did not significantly influence leg scores in either experiment. The ineffectiveness of serotonin, epinephrine, and norepinephrine indicates that the action of histamine is specific and cannot be carried out by other vasoactive amines at least when administered by diet.

The beneficial effect of histamine on the hock abnormality suggests that zinc deficiency might result in a derangement of histamine metabolism. However, in other studies no significant effect of zinc deficiency on total tissues histamine levels was found (9). A derangement may exist, however, in the turnover of histamine or in the size of a quantitatively small, but physiologically important, pool of histamine. Therefore agents influencing the metabolism of histamine might affect the development of the leg abnormality. Heparin was studied because of its association with histamine and zinc in mast cells (10), because of its histaminase-releasing capacity (11) and histamine-binding capacity (10) and because a direct relationship between serum heparin and the severity of arthritis has been suggested (12). Heparin had no significant influence, either beneficial or adverse, on the development of leg abnormalities in the chicks.

Chloroquine, a common antiarthritic agent, has been demonstrated to inhibit histamine methylation *in vivo* and *in vitro* (13). In Expt. 1, the level of chloroquine used resulted in 100% mortality within 3 weeks. In Expt. 2, each level of chloroquine significantly ($p<.05$) alleviated the hock defect as observed previously (2) for several other antiarthritic agents (aspirin, cortisone, indomethacin, and phenylbutazone). Since the latter compounds have not been demonstrated to inhibit histamine methylation, the significance of this property of chloroquine with regard to zinc deficiency is not clear.

A decreased histamine formation in rats resulting from pyridoxine deficiency has been described (14). Khairallah and Moore (15) confirmed this effect and reported that supplemental pyridoxine increased histidine decarboxylase levels above normal. If an increase in histidine decarboxylase activity occurred in zinc-deficient chicks supplemented with pyridoxine in the present experiments, it was not sufficient to influence hock development of zinc-deficient chicks.

Two other vitamins, folic acid (16) and niacin (17), have been reported as essential for normal hock development in the chick. These observations suggested the possibility that zinc deficiency could increase the requirement for these vitamins. Furthermore, niacin in large amounts is known to produce vasodilation similar to that produced by histamine (18). The feeding of *p*-aminobenzoic acid, a precursor of folic acid, or niacin did not significantly improve the legs of zinc-deficient chicks, suggesting that the hock abnormality is not a result of a functional deficiency of these vitamins in zinc deficiency. The ineffectiveness of niacin also suggests that the vasodilatory action of histamine may not be important in alleviating the hock defect.

Release of lysosomal enzymes from infiltrating leukocytes has been implicated in the etiology of joint diseases (19). Increased number of polymorphonuclear leukocytes in the blood of zinc-deficient pigs (20) and rats (21) have been reported. Gold salts have been shown to inhibit lysosomal enzymes and are well known for their antiarthritic properties. Nielsen *et al.* (2) found no effect of oral administration of gold salts to zinc-deficient chicks. However, gold salts are not effective when administered orally to humans. Therefore, gold sodium thiosulfate was injected intramuscularly at two levels to determine if it would alleviate the hock disorder. In contrast to all the other antiarthritic agents tested to date, gold thiosulfate was without effect. Even the lower dosage was severalfold higher than that used for humans; thus the ineffec-

tiveness was probably not due to inadequate dosage. However, gold salts or chloroquine must be administered to arthritic patients for several weeks before their effectiveness is manifested. The development of leg abnormalities in zinc-deficient chicks may occur too rapidly for gold salts to be effective and for chloroquine to be completely effective.

Because many anti-inflammatory agents also possess immunosuppressive activity, and because rheumatoid arthritis may be an autoimmune disease (22), the effect of injecting mechlorethamine (nitrogen mustard) was studied. Mechlorethamine has been used to suppress immunologic reactions and to treat arthritis and avian leukosis. In the second experiment, daily injection of mechlorethamine improved leg scores slightly, but significantly ($p<.05$) when compared to saline-injected chicks but not when compared to uninjected chicks. Since only five chicks of substantially decreased weight remained at the end of the experiment, the effect is not convincing. No significant effect was observed in either experiment when mechlorethamine was administered every 3 or 3.5 days.

Statolon, an antiviral agent, alleviates adjuvant-induced arthritis in rats (23). In these experiments, only the higher dose of statolon (100 mg/kg) administered daily was effective in alleviating the hock disorder, and then the effect was slight. Because growth was depressed substantially by this treatment and because improved leg scores are sometimes associated with depressed growth, it appears that statolon had little biologically significant effect.

Summary. The leg or hock abnormality of zinc-deficient chicks fed isolated soybean protein-based diets has previously been shown to be largely prevented by any of the following supplements: zinc, histidine, histamine, or any of several antiarthritic agents (aspirin, cortisone, phenylbutazone or indomethacin). Only zinc corrected all signs of deficiency while the other agents alleviated only the leg deformity. To better understand the mechanisms involved, the present experiments tested the effects on the leg abnormality of zinc-deficient chicks of certain vasoactive agents, compounds affecting histamine metabolism, certain vitamins required for normal hock development, and certain additional drugs which are known to influence experimental or human arthritis. Zinc, histamine, and chloroquine, an antiarthritic agent, were shown to alleviate the hock abnormality. The condition was not influenced by the dietary addition of the other vasoactive amines, serotonin, norepinephrine, and epinephrine, or by two vitamins required for normal hock development, niacin and *p*-aminobenzoic acid. Likewise, dietary addition of pyridoxine or injection of heparin, agents which could influence histamine metabolism, was without effect. The injection of gold sodium thiosulfate, in contrast to all other antiarthritic agents tested to date, was without effect. The injection of mechlorethamine, an immunosuppressive agent, or statolon, an antiviral agent, had only slight and questionable beneficial effects.

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