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Long Term Toxicologic Assessment of Nickel in Rats and Dogs*

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Nickel sulfate hexahydrate was added to the diet of rats and dogs for 2 years in amounts yielding dietary concentrations of 0, 100, 1000, and 2500 ppm as nickel and 0, 250, 500, and 1000 ppm for 3-generation reproduction studies in rats. In the two year study in rats and dogs, growth was significantly depressed in rats on 1000 and 2500 ppm diets, and in dogs on the 2500 ppm diet. Hemograms in rats were within normal range, and in dogs on 2500 ppm diet slightly lower hematocrit and hemoglobin values were observed. Urinary findings were normal except for marked polyuria in 2 dogs on 2500 ppm diet. Tissue storage of nickel in various organs indicated no important storage sites. Organ-to-body weight data for rats indicated increased heart and decreased liver ratios for females on 1000 and 2500 ppm diets; in dogs on 2500 ppm diet increased kidney and liver ratios. In multigeneration study in rats, a higher incidence of stillborn was observed only in the first generation at all dietary levels of nickel and decreased body weights of weanings on 1000 ppm diet in all generations. Histopathologic studies on rats in the two year study and of F/3b weanlings revealed no lesions. Dogs on 2500 ppm diet showed lung lesions, and in two, granulocytic hyperplasia of the bone marrow was observed.

Nickel (Ni) has been referred to as a relatively non-toxic ubiquitous trace metal, it is found in the tissues of man, in many organs and tissues of animals, in a variety of plants, soil, and in sea foods. Of the 29 trace elements found in the tissues of man, nickel ranks third in the universe and solar atmosphere, eleventh in the earth's crust, fifteenth in sea water, and fifteenth in the body of man, in this respect ranking with the essential elements iron, cobalt, copper and zinc. Its physiologic role, if any, has not been established. As far as is known, nickel does not play any role in human, animal and plant nutrition, in disease, or is required in known enzyme systems.¹⁻³

Acute and subacute toxicity of various nickel compounds in various species of experimental animals by different routes of administration have been documented in the literature. However, there is conflicting evidence on their systemic toxicity by mouth.^{4,5} In humans, systemic poisoning from orally ingested nickel salts is almost unknown from occupational and non-occupational exposure.⁴⁻⁶ In studies on the effects of feeding nickel carbonate, nickel soaps, and nickel catalyst to rats and monkeys, Phatak and Patwardhan⁷ found no toxic effects after continuous feeding of nickel

for 4 to 6 months at diet levels of 250, 500, and 1000 ppm nickel. In rats, reproductive performance was not significantly affected after 3 to 4 months of continuous feeding of nickel-containing diets. Further studies by Phatak and Patwardhan⁸ on the retention and excretion of nickel at 4 month intervals for 16 months in rats on nickel catalyst diet containing 250 ppm nickel revealed no progressive accumulation of nickel in tissues assayed. Bone showed the highest concentration, about 8 mg per cent, kidney, heart, and spleen about 2 mg per cent, liver, intestine, testes, blood, and skin averaged less than 1 mg per cent. Of the total nickel ingested, about 95 per cent was excreted in the feces and about 1 per cent in the urine. Other toxicologic studies have been summarized by Schroeder⁹. The present study, started in 1960, describes the results of two year feeding of nickel as nickel sulfate hexahydrate to rats and dogs, and a three generation reproduction study in rats.

Material and Methods

Nickel sulfate hexahydrate fines (NiSO₄·6H₂O) containing 22.3 per cent nickel by analysis (theoretically 22.32 per cent) served as the test material in this study.

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Two-year feeding study in rats: Four groups of 25, 28-day old weanling albino rats (Wistar derived^(a)) of each sex, culled to a narrow starting weight (56-65g), individually caged, were placed on diets consisting of 0, 100, 1000, and 2500 ppm Ni. Littermate distribution between diets was used within each sex. Finely ground meal^(b) served as the basic diet and to this was added with thorough mixing nickel sulfate hexahydrate fines in amounts calculated to yield the above dietary levels of nickel. Body weight was recorded weekly and food consumption was measured over 3-day periods at the end of 1, 3, 6, 12, and 24 months.

Hematologic studies (hematocrit, hemoglobin, and total and differential leukocyte counts) were made on 5 rats of each sex and dietary level at 3-month intervals. Due to poor survival, smaller numbers were used at 24-months for control male and female rats and for male rats on 2500 ppm diet. Semiquantitative tests for urinary reducing substances and protein were made on pooled urine from 5 rats of each sex and dietary level at 3-month intervals through 21 months.

At the end of 23 months, one female rat from each diet was sacrificed. Their gastrointestinal tracts were washed free of contents and small sections of liver and kidney were removed for histopathologic study. The remaining carcasses were homogenized and aliquots were taken for nickel analysis. At sacrifice of two-year survivors, samples of liver, kidney, bone (femur), and fat were taken from each rat. Each tissue was pooled as to sex and diet for subsequent analysis for nickel content.

Nickel analyses on tissues (wet basis) were performed by La Wall and Harrison Research Laboratories using the spectrophotometric method of Alexander *et al.*¹⁰ as modified by the Research Laboratories of the Rohm and Haas Company and identified as their Test Method 200 2-2, second revision, 1956.

At autopsy of 2-year survivors, gross examinations were carried out and absolute and relative organ-to-body weight ratios for heart, spleen, kidneys, liver, and testes were analysed statistically. Tissues preserved in formalin for histopathologic study were in addition to the above: lung, urinary bladder, stomach, small and large intestine, skeletal muscle, brain, skin, bone marrow, pituitary, thyroid, adrenal, pancreas, and gonad.

Body weight changes and organ-to-body weight ratios were evaluated by analysis of variance and Duncan multiple range and multiple F tests.¹¹

Two-year feeding study in dogs: Groups of three male and three female purebred beagle dogs of about 6-months of age, individually housed, were maintained for two years on diets providing 0, 100, 1000, and 2500 ppm Ni.

Prior to being placed on the above diets, the dogs were immunized against distemper, infectious hepatitis and leptospirosis, and treated as needed for intestinal parasites. Finely ground dog kibbled meal^(b) served as the basic diet to which was added with thorough mixing, nickel sulfate hexahydrate in amounts calculated to yield the above diet levels of nickel. Weighed amounts of feed, 400 g/day through the first 22 weeks and 450 g/day thereafter, moistened and thoroughly mixed with an equal weight of water, were offered to the dogs once a day. Feed consumed was recorded daily and body weights were recorded weekly.

Hematologic studies (hematocrit, hemoglobin, and total and differential leukocyte counts) were made prior to placing the dogs on the respective diets, at 2, 4, and 13 weeks, and at 3-month intervals thereafter. Semi-quantitative tests for urinary reducing substances and protein concentration were made before placing dogs on respective diets, at one-month, and at 3-month intervals thereafter.

Urine and feces for nickel analysis: At approximately 12 months, one week collections of excreta were made on one dog of each sex on each of the respective diets for analyses of Ni content. During the 23rd and 24th months, one week collections were made on two dogs of each sex and diet level, and three successive weekly collections were made from two other dogs of each sex and diet level. The sixth dog of each sex on each of the Ni-containing diets for 24 months was returned to the control diet. Two successive one-week collections of excreta were made following passage of charcoal-marked feces.

At autopsy, specimens of bone, liver, kidney, lung, skeletal muscle, and fat were taken from each dog for Ni analyses. Nickel analyses for excreta and tissues were conducted by the La Wall and Harrison Research Laboratories, as for rats. Feces were oven-dried and ground and tissue Ni was determined on wet basis.

At sacrifice, organ-to-body weight ratios were determined for heart, spleen, kidneys, liver, and testes and data evaluated by Duncan test¹¹. Tissues preserved in formalin for histopathologic study, in addition to the above, included: lung, stomach, large and small intestine, urinary bladder, skeletal muscle, brain, skin, bone marrow, pituitary, thyroid, adrenal, pancreas, and gonad.

Reproduction studies in rats: A three-generation study was undertaken in albino rats (Wistar derived^(a)). For this study, 28-day-old littermate rats, within but not between sexes were separated into 4 groups of 30 rats of each sex, to constitute the parent F/0 generation.

(a) Albino Farms, Red Bank, New Jersey.

(b) Radlston Purina, St. Louis, Missouri.

Mean body weight and weight range, in so far as possible were similar for all groups. One group was placed on each of the following dietary concentration of nickel: 0, 250, 500, and 1000 ppm.

Finely ground laboratory chow^(b) served as the basic diet. Stock aqueous solutions of nickel sulfate hexahydrate were prepared in appropriate concentrations so that the addition of 100 ml for each 6 kg of diet resulted in the desired nickel content, respectively. Additions of nickel solutions were thoroughly blended into the diet by mixing in a rotary-type blender. Diets were prepared fresh each week. Rats were individually caged and had free access to water and diet. Weekly body weight records were obtained, except during mating through weaning of litters.

After 11 weeks on the above dietary regimen, 20 females from each diet were transferred to individual breeding cages and each was mated with a male of the same dietary level of nickel for the F/1a generation. Male rats within each group were rotated to a different female on each of three successive 7-day periods. On the 20th mating day all males were removed. Records were maintained of mating, number of pregnancies, litters cast (alive and dead), pups in litter at 1, 5, and 21 days (weaning), and total weight of the litter at weaning. Litters containing more than 10 offsprings were randomly reduced to 10 on day 5. All surviving F/1a siblings were sacrificed and autopsied at weaning. Approximately 10 days after weaning of F/1a litters, F/0 parent generation rats were remated for F/1b litters. Procedure and observations recorded were the same as those described for F/1a litters. Following weaning of F/1b litters, surviving F/0 rats were sacrificed and autopsied.

For the F/2 generation 30 male and 30 female F/1b

offsprings from each diet level were continued on their respective parents' diet for 11 weeks at which time 20 of each sex within each group were mated and the same procedure followed as with the F/0 generation through production and weaning of F/2a and F/2b litters. At weaning of F/2b rats, F/1b parents were sacrificed and autopsied. For the F/3 generation, the same procedure as with the previous generations was followed through the production of F/3a and F/3b litters. All matings in each generation were made with rats from different litters. The following indices were calculated for each generation: Fertility (F.I.)=(pregnancies/matings) × 100; Gestation (G. I.)=(litters cast/pregnancies) × 100; Viability (V.I.)=(live pups at day 5/live pups born) × 100; and Lactation (L.I.)=(weaned/live pups-discards on day 5) × 100.

Histopathologic studies were performed on 10 male and 10 female F/3b weanlings from each diet level. Tissues included were the same as those described in the two year rat study.

Results and Discussion

Two-year feeding study in rats: Data on body weight at representative periods and on number of survivors at two-years are summarized in Table 1. Two-year survival was poor, particularly among control rats of both sexes and males on 2500 ppm, but there is no indication of an effect due to nickel. Nickel had a depressant effect on body weight in both sexes on 2500 ppm and sporadically for rats on 1000 ppm diet. Food consumption indicated no consistent trends, but it appeared that the lesser weight gains, particularly on the 2500 ppm diet, may be in part a result of lower food consumption.

Hematologic values for hemoglobin, hematocrit and differential leukocyte counts, obtained at 3-month

TABLE 1. BODY WEIGHT AND MORTALITY DATA ON RATS** RECEIVING NICKEL SULFATE IN THEIR DIET FOR TWO YEARS

Sex	Diet concn (ppm)	Average body wt (g±S.D.)								
		Start	1 wk	3 wk	6 wk	13 wk	26 wk	52 wk	78 wk	104 wk
Female	0	57	87±14	147±25	205±25	267±31	309±39	369±72(5)	393±115(9)	496±84(21)
	100	57	87±15	144±24	205±16	268±25	307±30	356±59	376±68(7)	409±72(18)
	1000	57	82±16	146±13	191±17*	250±32	277±37*	304±30(4)*	324±54(7)*	325±62(18)*
	2500	57	78±13*	131±13*	180±17*	232±17(1)*	261±20(3)*	269±29(4)*	266±35(6)*	303±36(18)*
Male	0	60	97±13	185±19	285±37	406±45(1)	467±66(2)	551±49(6)	562±58(11)	483(23)
	100	61	98±17	186±27	298±25(1)	425±38	506±51	551±53(2)	537±101(5)	511±81(17)
	1000	61	94±15	176±15	266±30	392±43(3)	459±52	494±69(9)*	519±69(11)	446±61(18)
	2500	60	88±15*	154±11*	241±16*	331±30(2)*	382±38(5)*	389±43(9)*	365±45(9)*	367(23)

*Value differs significantly from control, P=0.05. Figures within the parentheses indicate cumulative mortality.

**At start 25 rats of each sex/group.

TABLE 2. TISSUE CONCENTRATIONS OF NICKEL IN RATS RECEIVING NICKEL SULFATE IN THEIR DIET FOR TWO YEARS

Sex	Diet concn (ppm)	Tissue concentration of Ni (ppm) on wet wt basis			
		Bone	Liver	Kidney	Fat
Female	0	0.53	0.094	0.14	0.51
	2500	0.82	0.64	3.4	1.0
Male	0	<0.096	0.055	<0.14	<0.055
	2500	0.64	0.68	4.9	1.4

intervals, for rats of all dietary levels of nickel did not depart significantly from those of the controls. Results of tests for urinary reducing substance at three month intervals were negative. Results of semiquantitative tests for urinary protein at the same time intervals were quite variable and inconsistent, with no clear trends.

Results of nickel analyses on individual body tissues (bone, liver, kidney, fat), summarized in Table 2, indicate no important storage sites of nickel. Kidneys showed the highest content (about 4 ppm), fat averaged 1 ppm, bone and liver, about 0.7 ppm. Results on the whole carcasses, on wet basis, on diet levels of 0, 100, 1000, and 2500 ppm for 23 months, are 0.38, 0.33, 1.5, and 3.0 ppm, respectively. The total nickel content of one rat weighing 251 g, on the 2500 ppm diet, was 0.75 mg.

Organ-to-body weight ratios obtained at sacrifice of two-year survivors are summarized in Table 3. A tendency toward increased heart-to-body weight ratios and decreased liver-to-body weight ratios appears in female rats on 1000 and 2500 ppm diets.

Gross pathologic findings on rats sacrificed at term were negative. Histologic findings were essentially negative. The distribution of the lesions found was not indicative of any characteristic effect of nickel in the diet.

Two-year feeding study in dogs: All dogs survived

the two year experimental period. During the first 3 days, all six dogs on 2500 ppm Ni diet vomited, usually within an hour. On the fourth day they were returned to the control diet. All but one dog readjusted within 3 days. The one dog readjusted after parenteral feeding and intravenous fluids. At the start of the second week 5 of the dogs were placed on 1500 ppm Ni and the sixth dog was included at the start of the sixth week. This level of Ni apparently was well tolerated, as no emesis, salivation or gastro-intestinal irritation was observed. At two-week intervals the diet level of Ni was raised to 1700, 2100 and 2500 ppm, respectively, with no further evidence of emesis, salivation, or gastrointestinal irritation.

Analyses of body weight data at representative intervals suggest no effect for dogs on 100 and 1000 ppm diets. At 13 weeks dogs on 0, 100, and 1000 ppm diets gained 20 per cent in body weight as compared to 10 per cent for dogs on 2500 ppm. At 52 weeks dogs on the two lower levels of Ni and the controls had gained 39 per cent as compared to 20 per cent for dogs on 2500 ppm. Similar comparison at 65, 78, 91, and 104 weeks showed a gain of about 45 per cent in body weight for dogs on 0, 100, and 1000 ppm as compared to less than 20 per cent for dogs on 2500 ppm.

Cumulative food consumption data for all dogs, except for dogs on 2500 ppm during the terminal weeks when they were caged for collection of excreta, are quite comparable and do not explain the difference in body weight.

Hematology: Hematologic values obtained at three month intervals were quite variable but within normal range. However, the greatest variability appeared in the hematocrit and hemoglobin values with a tendency toward lower values in dogs on 2500 ppm Ni, suggestive of a simple hypochromic anemia.

Urinary tests for reducing substances and protein gave comparable values for all groups of dogs. Urine volume data during the 23rd and 24th month revealed high urine volumes for dogs on 2500 ppm diet. Two weeks of

TABLE 3. ORGAN-TO-BODY WEIGHT RATIO DATA ON RATS RECEIVING NICKEL SULFATE IN THEIR DIET FOR TWO YEARS

Sex	Diet concn (ppm)	Rats No.	Organ-to-body wt ratios (g/kg \pm SD)				
			Heart	Spleen	Kidney	Liver	Testes
Female	0	4	2.5 \pm 0.3	2.5 \pm 1.3	6.8 \pm 1.1	36.6 \pm 5.2	
	100	7	2.8 \pm 0.3	2.3 \pm 0.5	7.9 \pm 1.1	34.5 \pm 7.4	
	1000	6	3.8 \pm 0.7 ^a	2.0 \pm 0.4	8.6 \pm 1.5	29.0 \pm 4.1 ^a	
	2500	7	3.2 \pm 0.4 ^a	1.9 \pm 0.2	7.8 \pm 0.8	29.1 \pm 2.4 ^a	
Male	0	2	2.8	1.9	8.2	38.6	7.9
	100	8	3.3 \pm 0.5	2.0 \pm 0.3	7.6 \pm 0.9	25.9 \pm 3.6	6.7 \pm 1.6
	1000	7	3.3 \pm 0.9	2.3 \pm 0.6	10.3 \pm 5.8	30.4 \pm 8.1	7.2 \pm 1.1
	2500	2	3.3	1.5	7.1	31.0	9.5

^aValue differs significantly from control, P = 0.05

TABLE 4. ORGAN-TO-BODY WEIGHT RATIO DATA OBTAINED ON DOGS RECEIVING NICKEL SULFATE IN THEIR DIET FOR TWO YEARS

Diet concn (ppm)	Dogs No.	Organ-to-body wt ratios (g/kg \pm SD)				
		Heart	Spleen	Kidney	Liver	Testes
0	6	8.0 \pm 1.3	7.4 \pm 2.2	5.7 \pm 0.9	28.1 \pm 2.8 ^a	2.4 \pm 0.5
100	6	8.0 \pm 0.9	6.8 \pm 1.8	5.9 \pm 0.8	29.5 \pm 3.3	2.4 \pm 0.3
1000	6	7.1 \pm 0.6	6.8 \pm 2.3	6.1 \pm 0.6	29.2 \pm 3.6	2.4 \pm 0.4
2500	6	8.4 \pm 1.1	6.9 \pm 1.8	7.8 \pm 0.8 ^b	33.1 \pm 3.1 ^b	3.1 \pm 0.5

^aAverage of 5.

^bValue differs significantly from control, $P = 0.05$.

additional observations on all dogs on 2500 were made. Urine volumes for two of the dogs (one of each sex) were unusually high. On the eighth day the antidiuretic effect of pitressin tannate (5 and 10 units) by IM injection was tested. In the two dogs with polyuria no effect was observed. Whether these findings bear any relationship to the dietary intake of Ni does not appear to be answerable by this small number of observations.

Data on the excretion of Ni in urine and feces obtained at 12 months and during the 23rd and 24th months showed variable amounts of Ni in the feces, inconsistent with the amounts in the diets and ingested. No explanation is offered on the inconsistency and variability of the results obtained. However, only approximately 1 to 3 per cent of the ingested Ni was excreted in the urine and tissue levels (below) were small, indicating little body retention of Ni.

Tissue storage: Tissue analyses on bone, liver, kidney, lung, skeletal muscle, and fat obtained at sacrifice indicated limited retention of Ni, highest values found were for kidney (4-7 ppm in dogs receiving 2500 ppm), and in one dog 1.6 ppm was found after withdrawal of Ni diet for two weeks.

Organ-to-body weight data are summarized in Table

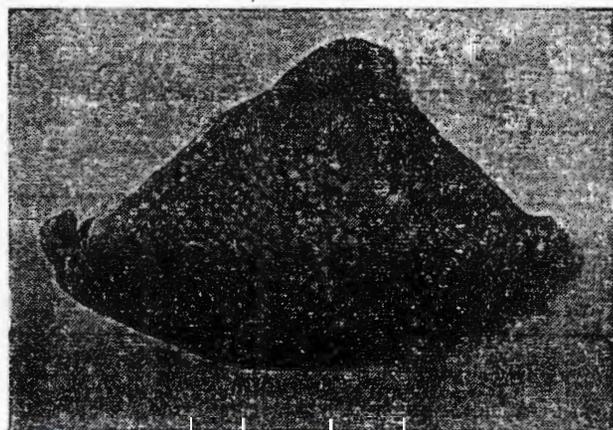


Fig. 1. Formalin-fixed lung from dog on 2500 ppm nickel, showing multiple, subpleural, peripheral cholesterol granulomas.

4. Statistical by significant higher kidney and liver ratios appeared only in dogs on 2500 ppm for two years.

Histopathologic findings showed no characteristic lesions in dogs receiving 100 and 1000 ppm. All dogs on 2500 ppm showed histologic changes in their lungs. Grossly 5 of 6 dogs displayed multiple subpleural peripheral cholesterol granulomas, (Fig. 1; photomicrographs reproduced elsewhere, Hennigar¹²). The only dog not showing this had been returned to control diet for two weeks prior to sacrifice. Other lung pathology included bronchiolectasis (4 dogs), emphysema (3 dogs), and focal cholesterol pneumonia (4 dogs). The only other change observed consisted of granulocytic hyperplasia of the bone marrow in two dogs on 2500 ppm.

Reproduction studies: Body weights for parent generation rats on 250 and 500 ppm diets, before mating and at weaning of respective litters, were not adversely affected, but rats on 1000 ppm exhibited slightly lower body weights. The average decrease in body weight did not exceed 8 per cent for females and 13 per cent for males.

Data on reproduction through three generations are summarized in Table 5. On fertility, gestation, viability and lactation indices, no adverse effects were noted at any of the dietary levels of nickel. Data on the number of pups born dead showed higher incidence of stillborn in the first generation at all levels of Ni, not observed to any extent in subsequent generations. The number of siblings (alive and dead) cast per litter averaged 10.3, 10.6, 9.8, and 9.0 for 0, 250, 500, and 1000 ppm diets, respectively. The number of siblings weaned per litter were progressively fewer with increasing dietary level of nickel, averaging 8.1, 7.2, 6.8, and 6.4 for 0, 250, 500, and 1000 ppm diets, respectively. On average weaning body weight, a clear-cut adverse effect is only apparent in weanlings of females on 1000 ppm diet, averaging 73 per cent of control. However, offsprings maintained on 1000 ppm diet from weaning, to mating of succeeding generations recovered considerably from this deficit, averaging 92 per cent of controls.

TABLE 5. SUMMARY OF REPRODUCTION DATA FOR RATS ON VARIOUS DIETARY LEVELS OF NICKEL^a THROUGH THREE GENERATIONS

Generation	Diet concn (ppm)	Number of rats			Total number of pups					Weanlings mean body wt. (g)	Indices (%) ^b			
		Mated ^c	Pregnant ^d	Whelped ^e	Born alive	Born dead	Alive ^f day 5	Discarded ^g	Weaned		F.I.	G.I.	V.I.	L.I.
F ₀	0	20(1)	15(1)	14	113	5	113(10)	2	89	37.5	79	93	100	87
	250	20(1)	11	11(1)	72	17	69	5	60	40.1	58	100	96	94
	500	19	14	14(1)	96	13	95	5	72	34.4	74	100	99	80
	1000	20	12	12(1)	93	16	91	5	83	27.8	60	100	98	97
		0	17	14	14	143	3	139	0(8)	137	33.8	82	100	97
F _{1b}	250	19	16	16	164	6	150	0(12)	134	31.6	84	100	91	89
	500	19	14	14(3)	109	27	106	0(9)	98	28.1	74	100	97	93
	1000	20(1)	15	15	93	31	77	0(3)	67	25.1	79	100	83	87
		0	20	15	15	142	0	138	13	115	35.9	75	100	97
F _{1a}	250	20	18	18(1)	188	2	181	30	119	31.1	90	100	96	79
	500	20	17	17	171	0	141	10	129	34.6	85	100	82	98
	1000	20	16(2)	14	127	6	120	8	96	25.9	80	88	94	86
		0	20	14	14(1)	149	1	146	30	116	37.9	70	100	98
F _{2b}	250	20	17	17(1)	181	16	177	39	130	35.7	85	100	98	94
	500	20	16(1)	15	166	0	160	22	131	36.9	80	94	96	95
	1000	18	11	11(1)	109	3	103	10	90	26.9	61	100	94	97
		0	20	18(1)	17	180	5	175	20	125	36.7	90	94	97
F _{2a}	250	20	19	19	207	4	201	21	121	34.5	95	100	97	67
	500	20	19(1)	18(1)	184	5	172	20	102	35.6	95	95	93	67
	1000	20	20(1)	19	168	7	162(10)	6	107	28.0	100	95	96	73
		0	18	18	18	216	0	206	38	158	41.9	100	100	95
F _{3b}	250	20	18	18	204	2	198	32	161	41.4	90	100	97	97
	500	18	16	16(1)	142	6	138	22	102	41.2	89	100	97	88
	1000	17	17	17	139	7	138	13	114	29.7	100	100	99	91
		0	18	18	18	216	0	206	38	158	41.9	100	100	95

^aNickel sulfate hexahydrate (22.3% Ni).

^bIndices: F.I., G.I., V.I., and L.I. refer to Fertility, Gestation, Viability, and Lactation Index, respectively, defined in text.

^cNumbers within parentheses refers to non-pregnant dams found dead during mating period, not included in calculation of F.I.

^dWithin parentheses, dams found dead and autopsied to confirm pregnancies.

^eNumbers within parentheses refers to litters born dead

^fNumbers within parentheses refers to number of siblings sacrificed after day 5 and before weaning due to death of dam, not used in calculation of L.I.

^gTo reduce litter size to 10 on day 5; and within parentheses are totals in excess of 10/litter not discarded as planned, due to oversight, and carried to weaning.

Gross observations on siblings cast, at all dietary levels of nickel through three generations showed no teratogenic effects. Histopathologic findings on F/3b weanlings, 10 of each sex on each dietary level, were entirely negative.

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Production of Fungal Rennet Substitute for Cheese Making

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An aqueous extract obtained from the mouldy bran of *Rhizopus oligosporus* grown at 30°C for 72 hr possessed high milk clotting and relatively low proteolytic activity. The preparation could be stored actively under refrigeration. The organism produced less milk clotting enzyme on liquid medium. The enzyme was influenced by Ca in its activity. The concentrate of the enzyme extract was brown in colour, bland in taste with a pleasant vegetable aroma. The enzyme was free from pathogens and aflatoxin. Cheddar cheese trials involving 50-litres of milk per batch with the enzyme preparation, showed that curd working properties at cutting, cooking, cheddaring and pressing were similar to those with rennet. Fat loss in whey was slightly higher than that of control. The yields of ripened cheese (30 days) with rennet and fungal enzyme were about the same. Both types developed flavour and aroma without marked differences in body and texture during early ripening (30 days).

Calf-rennet is by far the most common milk clotting enzyme in cheese manufacture. Shortage of rennet for commercial cheese production has led to a world wide search for suitable rennet substitutes. Several proteolytic enzymes of animal, plant and microbial origin have been tried for the purpose. Some of the available bacterial enzymes have their own characteristic differences and limitations for use as rennet substitutes¹⁻⁴.

Among the several milk clotting enzymes from fungi "Surecurd" from *Endothia parasitica*⁵ and Meito microbial rennet from *Mucor pusillus* Lindt. have been commercially produced for use in cheese manufacture. Many types of cheese have been produced with the above milk clotting enzymes and the qualities of some of these cheese have been even superior to that made with rennet^{6,7}. However, development of bitterness and body defects in cheese made with microbial proteases have also been reported. Besides the above commercial preparations enzymes of certain strains of *Rhizopus chinensis* and *Rhizopus oligosporus* have been used as rennet substitutes for cheese manufacture^{8,9}.

The cheese industry is finding it difficult to get its requirements of rennet though no precise data are available regarding its actual needs. Rennet substitute from microbial sources are not commercially available in the country. There is considerable potential for the development of cheese industry, if suitable rennet substitutes can be made available.

This paper discusses the production of a rennet substitute from *Rhizopus oligosporus* (CFTRI 1104) for use in cheese manufacture.

Materials and Methods

Organisms: Fungal cultures of *Aspergillus*, *Penicillium*, *Rhizopus* and a few other important genera known to produce proteolytic enzymes were selected from culture collections maintained at the CFTRI, Mysore, for use in these trials.

Media: Four types of media were used for screening the organisms. (i) The liquid medium consisting of 10 per cent each of corn-starch and wheat bran prepared on the formulae of Arima¹⁰. The inoculated

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