in place of R in compound I. The *O,O*-diethyl compound was found to give complete control of powdery mildew and late blight at concentrations of about 10 and 200 parts per million (ppm), respectively; the *O,O*-di-*n*-propyl ester was somewhat more effective against these diseases. The *S,S*-diethyl compound, at a concentration of 150 ppm, almost completely controlled late blight but had no effect on powdery mildew.

Fungicidal activity was tested in the laboratory by spraying test plants to run-off with the formulated test chemicals. Plants were allowed to dry for 3 to 4 hours and were then inoculated with viable spores of the fungus either by spraying with a spore suspension or by direct contact with leaves of infected plants. Disease was allowed to develop in the proper environment for 5 to 7 days before readings on activity were taken.

The overall result of our investigation was the emergence of two potential fungicides, IA (R is OC_2H_5; m.p., 83° to 84°C) and IB (R is OC_3H_7; m.p., 53° to 54°C). Both were white crystalline substances of low toxicity to mammals. Compound IB, however, was about three times as toxic to mammals as was IA, which showed an LD_{50} (lethal dose, 50 percent effective) of 5600 mg/kg when administered orally to male rats.

Field experiments showed that the diethyl ester IA consistently controlled a variety of diseases satisfactorily (Table 1). Concentrations of the spray given in Table 1 were for protective spray schedules in which applications were made every 7 to 14 days, depending on the type of disease to be controlled. At these concentrations the diethyl ester IA is safe on both fruit and foliage.

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22 September 1966

Diagnosis of Gaucher's Disease and Niemann-Pick Disease with Small Samples of Venous Blood

Abstract. *Enzymes which catalyze the hydrolysis of glucocerebroside and sphingomyelin have been demonstrated in preparations of washed human white blood cells. The level of activity of these respective enzymes is markedly decreased in leukocyte preparations obtained from patients with Gaucher's and Niemann-Pick diseases. Assay of these enzymes may be useful in the differential diagnosis of the sphingolipidoses.*

The metabolic defect in Gaucher's disease has been shown to be a deficiency of the enzyme which catalyzes the cleavage of glucose from glucocerebroside, the substance which accumulates in various tissues in patients with this disease (*1*). In like manner, the metabolic lesion in Niemann-Pick disease is a deficiency of the enzyme which catalyzes the hydrolysis of phosphorylcholine from sphingomyelin which accumulates in Niemann-Pick disease (*2*). These enzymes are present in a number of tissues in the body (*3*), and there appears to be a generalized attenuation of these enzymes in several organs in patients with these diseases (*2, 4*). Since human white blood cells contain a number of hydrolytic enzymes (*5*), it was considered of interest to develop methods for determining the quantity of glucocerebroside-cleaving enzyme and sphingomyelin-cleaving enzyme in human leukocytes. We now describe procedures for determining the activity of these sphingolipid hydrolases in human leukocytes. Our data show that the activity of these enzymes in leukocyte preparations from patients with Gaucher's disease and Niemann-Pick disease is very much lower than in preparations from normal human beings and patients with other diseases.

Glucocerebroside labeled with ^{14}C in the D-glucose portion of the molecule was prepared as described (*6*). Sphingomyelin was labeled with ^{14}C in the methyl carbon atoms of the choline portion of the molecule (*3*). Leukocytes were separated from erythrocytes by differential sedimentation in the following fashion. Ten milliliters of venous blood were added to a test tube containing 2 ml of a solution containing per 100 ml: 5 g of dextran, 0.7 g of sodium chloride, and 50 mg of heparin. The contents were mixed, and the red blood cells were allowed to settle (45 minutes at room temperature). The plasma containing the suspended leukocytes was removed with a capillary pipette, and the suspension was centrifuged for 10 minutes at 600*g*. The supernatant was discarded and the leukocyte pellet was suspended and washed twice with 0.85 percent sodium chloride solution. The white blood cells were suspended in a fresh portion of this isotonic sodium chloride, and the cells were counted with a Coulter counter. The suspensions were adjusted with saline so that they contained from 20,000 to 60,000 leukocytes per microliter. The incubation mixtures for determination of glucocerebroside-cleaving enzyme activity contained 50 to 100-μl portions of the leukocyte suspension, 15 μmole of potassium phosphate buffer (*p*H 6.0), 300 μg of sodium cholate, 200 μg of Cutscum (isooctylphenoxypolyoxyethanol, Fisher Chemical Co.), and 125

Table 1. Glucocerebroside-cleaving enzyme in human white blood cells. One unit of enzymatic activity is defined as the amount of enzyme required to catalyze the hydrolysis of 1 mμmole of glucocerebroside per hour under the conditions of incubation described.

Age (yr)	Sex	Units of enzymatic activity: Per mg of protein	Per 10^9 leukocytes
		Normal individuals	
40	F	3.4	480
20	F	3.7	440
32	M	2.7	530
48	M	4.1	550
39	M	3.8	480
37	M	4.2	420
31	M	3.4	530
17	M	3.4	610
21	M	6.2	640
31	F	4.6	690
48	F	3.1	590
		Familial hyperlipoproteinemia Type II	
17	M	3.8	490
32	M	2.8	450
		Tangiers disease	
12	F	3.3	490
		Refsuns syndrome	
27	F	3.0	450
28	M	3.0	480
		Niemann-Pick disease	
18	M	3.4	400
3	M	4.4	460
		Lipidosis of unknown etiology	
8	F	3.4	410
8	M	3.1	380
	Mean ± SE	3.64 ± .18	498 ± 18
		Gaucher's disease	
40	F	.47	120
48	M	.77	162
47	F	.78	119
10	M	.46	68
14	M	.57	68
	Mean ± SE	.61 ± .09	107 ± 18
	P	<.001	<.001

mμmole of glucose-1-^{14}C–cerebroside (440,000 count min^{-1} mole^{-1}) in a final volume of 0.15 ml. After incubation for 1 hour at 37°C in air, the amount of glucose-1-^{14}C cleaved was determined by liquid scintillation spectrometry (*3*).

In order to determine the activity of sphingomyelin-cleaving enzyme, the washed white blood cells obtained from 5 ml of venous blood were suspended in 0.3 ml of a solution of sodium cholate (5 mg/ml). The suspended cells were chilled in a bath at −10°C and subjected to sonic oscillation for 15 seconds (Heat Systems Co. sonifier, Melville, N.Y.). The suspensions were allowed to stand for 1 hour at 0°C and then centrifuged at 37,000*g* for 20 minutes. Under these conditions, over 92 percent of the sphingomyelin-cleaving enzyme was recovered in the clear supernatant in preparations from patients with Niemann-Pick disease and from normal individuals. The incubation mixtures contained from 10 to 50-μl portions of the leukocyte enzyme preparation, 25 μmole of potassium acetate buffer (*p*H 5.0), 70 mμmole of sphingomyelin-^{14}C (327,000 count min^{-1} μmole^{-1}), 50 μg of sodium cholate, and water in a final volume of 0.18 ml. The mixtures were incubated for 1 hour at 37°C in air, and the amount of sphingomyelin hydrolyzed was determined (*2*).

Table 2. Sphingomyelin-cleaving enzyme in human white blood cell preparations. The procedures for the preparation of the enzyme and conditions of incubation are described in the text. One unit of enzymatic activity is defined as the amount of enzyme required to catalyze the hydrolysis of 1 mμmole of sphingomyelin per hour using the conditions of incubation described in the text.

Age (yr)	Sex	Units of enzymatic activity per mg of protein
	Normal	
20	M	5.2
1½	M	4.1
32	M	4.6
42	F	4.5
7	F	3.2
28	F	5.6
22	F	3.3
	Paroxysmal nocturnal hemoglobinuria	
18	M	4.5
	Hypernephroma	
47	M	4.7
	Lymphosarcoma	
28	M	6.0
	Fabry's disease	
23	M	4.8
	Gaucher's disease	
10	M	3.4
30	F	4.3
	Lipidosis of unknown etiology	
8	M	3.3
1	M	5.0
		Mean ± SE 4.4 ± 0.22
	Niemann-Pick disease	
3	M	0.25
26	F	0.84
8	M	0.00
		Mean ± SE 0.36 ± 0.25

The activity of glucocerebroside-cleaving enzyme could be readily demonstrated in preparations of whole white blood cells. The amount of glucocerebroside cleaved was proportional to the amount of protein in the washed leukocyte suspensions, from 0 to 960 μg of protein (Fig. 1) and to the number of leukocytes in the incubation mixture from 0 to 8 × 10^6 white cells (Fig. 2). The glucocerebroside-cleaving enzyme activity in leukocyte suspensions prepared from normal human subjects and from patients with various disorders was compared with the activity of this enzyme in similar preparations obtained from five patients with Gaucher's disease (Table 1). There is a marked attenuation of glucocerebroside-cleaving enzyme activity in the leukocyte suspensions obtained from patients with Gaucher's disease. This enzymatic defect appears to be quite specific for patients with Gaucher's disease since the level of activity appears to be within the normal range in leukocyte preparations from patients with Niemann-Pick disease. An occasional discrepancy was observed in the level of activity when the values were expressed in terms of protein or number of leukocytes. This may be due to variations within leukocyte populations since the glucocerebroside-cleaving enzyme activity seems to be mainly associated with granulocytes and is quite low in lymphocytes (*7*).

Although the presence of sphingomyelin-cleaving enzyme activity could be detected in intact leukocytes, considerable difficulty was encountered in trying to obtain a relation between the amount of activity and the number of cells or the amount of protein in the leukocyte suspensions. This limitation was overcome by disrupting the cells with sonic oscillation of washed leukocytes while they were suspended in a dilute solution of sodium cholate. Under these conditions, the amount of sphingomyelin hydrolyzed was proportional to the amount of protein in the supernatant after centrifugation at 37,000*g*, over

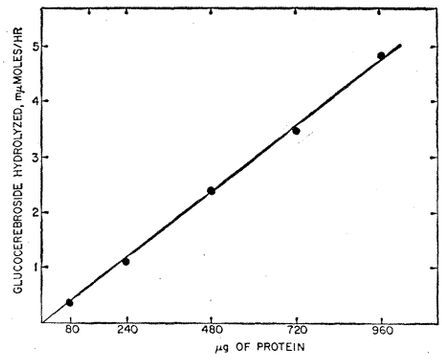

Fig. 1. Proportionality between the amount of glucocerebroside-cleaving enzyme activity and leukocyte protein in the incubation mixtures.

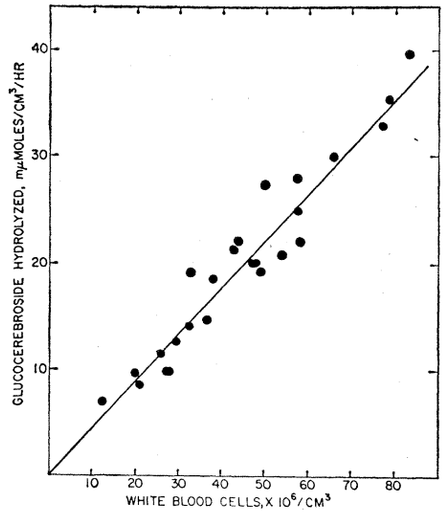

Fig. 2. Proportionality between the amount of glucocerebroside-cleaving enzyme activity and the number of leukocytes in the incubation mixtures.

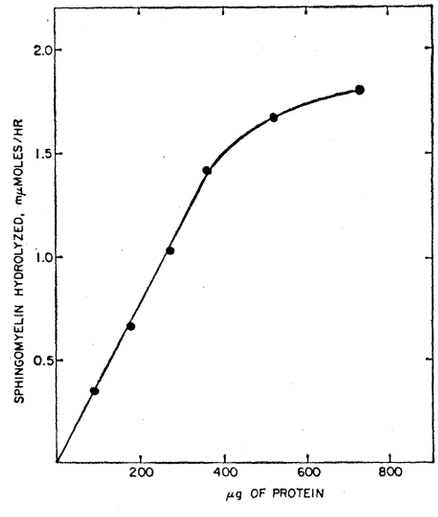

Fig. 3. Proportionality between the amount of protein added and sphingomyelin-^{14}C hydrolyzed. The procedure for the preparation of the sphingomyelin-cleaving enzyme from human white blood cells and the conditions of incubation are described in the text.

a range from 0 to 400 μg of protein (Fig. 3). There was a profound diminution of activity of the sphingomyelin-cleaving enzyme in preparations from patients with Niemann-Pick disease compared with those obtained from normal human beings and patients with various other disorders (Table 2). Although we studied only three patients with Niemann-Pick disease the mean value of sphingomyelin-cleaving enzyme in the control series was 4.4 units per milligram of protein, whereas it was 0.36 unit per milligram of protein for patients with Niemann-Pick disease. This difference in mean is highly significant (*8*). The specificity of this assay is indicated by the fact that enzyme activity in the leukocyte preparations obtained from patients with other sphingolipodystrophic conditions, such as Gaucher's disease and Fabry's disease, was normal. Thus, our techniques should be useful in the diagnosis of Gaucher's disease and Niemann-Pick disease. Whether these procedures can be used to detect heterozygous carriers of these diseases remains to be seen.

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9. A portion of this work was performed under Section 104(K) of Public Law 480, 83rd Congress, Agreement No. 42515. We thank Dr. D. S. Fredrickson of NHI for his interest and assistance in obtaining some of the blood samples.

31 October 1966

D-Gluconic Acid: Isolation from the Defensive Secretion of the Cockroach Eurycotis decipiens

Abstract. *The major water-soluble constituent of the defensive secretion of* Eurycotis decipiens *was identified as gluconic acid, isolated in the form of calcium* D-*gluconate. The acid, in equilibrium with its lactones, is present in unusually high concentration.*

Adults of *Eurycotis decipiens* (Kirby) have a large defensive gland which opens through the intersegmental membrane between the sixth and seventh sternites. Except for its white color, the large secretion-filled reservoir is similar in appearance and position to the gland which is found in *Eurycotis floridana* (Walker) (*1*) and which produces and stores 2-hexenal (*2*) for defensive purposes.

The aqueous, acidic, milky fluid (0.58 ml) from 72 specimens of *E. decipiens* (*3*) was dissolved in methanol. Volatiles which included 2-hexenal (about 5 percent) (identified by infrared spectra and gas-liquid chromatography) were removed in a vacuum. After extraction with methylene chloride, the residue (39.6 mg) was dissolved in methanol (1 ml). The infrared spectrum of a disc obtained from a lyophilized aqueous potassium bromide solution of the methanol-soluble fraction was similar (essentially identical from 1900 to 500 cm^{-1}) to that derived from an equilibrated aqueous solution of δ-D-gluconolactone. A chromatogram of the methanol-soluble fraction was developed with a mixture of butanol, acetic acid, and water (4:1:5) and sprayed with silver nitrate in acetone and then alcoholic sodium hydroxide. By comparison with chromatograms of known materials the pattern of spots could be interpreted in order of increasing mobility as an *O*-gluconylgluconic acid (*4*), δ-gluconolactone and gluconic acid, methyl gluconate, and γ-gluconolactone. Methyl gluconate was an artifact of storage in methanol. Gas-liquid chromatography (*5*) of material subjected to trimethylsilylation indicated the presence of a gluconolactone but not of glucose.

The gluconic acid fraction was passed through a cation exchange resin (H^+ form). After the effluent was treated with calcium carbonate, calcium D-gluconate monohydrate ($[\alpha]_D^{28} = +5°$) was isolated by crystallization from water and identified by comparison of infrared spectra (Fig. 1). The D-gluconic acid, in equilibrium with its lactones, is a major constituent (6.8 to 8.2 percent, weight to volume) of the secretion. After *Eurycotis decipiens* ejects its secretion, the volatiles, 2-hexenal and water, evaporate and leave a mixture of gluconic acid and lactones as a residue. The secretion effectively repelled the fire ant (*6*), probably because of the presence of 2-hexenal.

The compound 2-hexenal has previously been identified in the defensive secretions of Hemiptera, cockroaches, and an ant (*7*).

Aldonic acids have not been found previously in plants or animals in appreciable amounts. L-Arabonic acid has been isolated from gum arabic (*8*) and from *Austrocedrus chilensis* (D. Don), Florin and Boutelje (*9*). Gluconic acid (about 0.18 percent by weight) occurs in pasteurized honey (*10*) where it originates in a honey

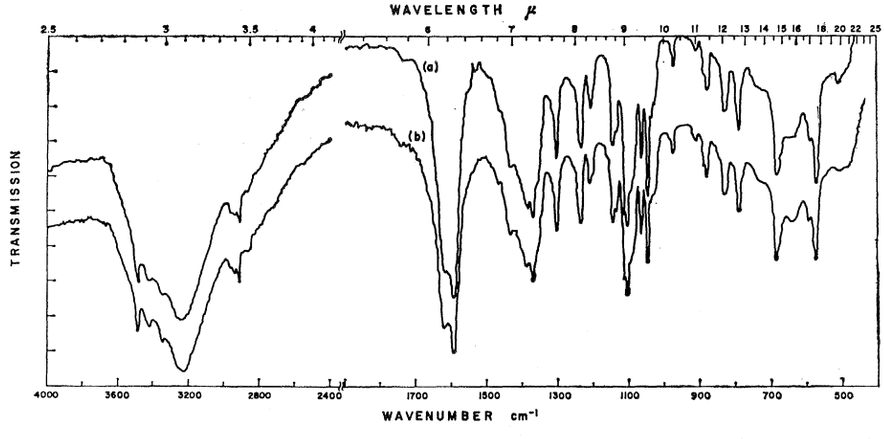

Fig. 1. Infrared spectra of calcium D-gluconate monohydrate (KBr disc). Curve *a* is the spectrum of the salt obtained from the methanol-soluble fraction of *E. decipiens* secretion. Curve *b* is that of an analytically pure sample from the recrystallization of commercial material.