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ANIMAL LEPROMIN WITH LEPROSY BACILLI FROM INFECTED RATS

PREPARATION AND COMPARATIVE STUDY WITH HUMAN LEPROMINS [1]

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In previous articles ([1-3]) I reported the multiplication of *Mycobacterium leprae* inoculated into rats fed a prooxidant diet. In animals of the last of three serial inoculations there were demonstrated great quantities of acid-fast bacilli. These germs, when seeded on Loewenstein-Jensen medium and incubated at different temperatures, gave no growths. For this reason it was concluded that they were the Hansen bacillus, the one which had been originally inoculated. It was stated at that time that when the number of inoculated animals and the abundance of bacilli in them would permit, a lepromin would be prepared from the testicles to be tested in man, this being another procedure which might afford evidence that the Hansen bacillus had been caused to multiply in other than the human body.

To this end the experiment here described was carried out, to study the behavior in the lepromin reaction of the bacillus found in the third passage from the human leproma in rats fed with the prooxidant diet. It was also the purpose to see if it might be possible to prepare an animal lepromin for routine immunological studies.

MATERIAL AND METHODS

The animal used was rat No. 110, from the group which had been given the second serial transfer (reinoculation), as shown in Fig. 1. This animal was sacrificed nine months after it has been inoculated intratesticularly with 0.1 cc. of a bacillus suspension from a first-transfer rat.

The rat weighed 325 grams. At autopsy yellowish points were seen on the surface of the liver. The spleen was large and dark. The testes were atrophic, and on section there exuded an albuminous fluid which coagulated spontaneously. The paravertebral glands were enlarged and of yellowish color. The fat showed nothing special.

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[1] Translation, with certain additions, of an article from *Semana Medica*, **113** (1958) 1119-1124.

In the bacteriologic examination of the inoculated testicle, the right one, an impression smear showed great numbers of homogeneous acid-fast bacilli, predominantly short and thin, similar to those seen in previous inoculations which I called "young" bacilli. There were also seen a great number of bacillus "nests": enormous accumulations of bacilli with the characteristics described. There were practically no granulated bacilli, or separate acid-fast granules.

With that testicle an integral lepromin was prepared in the following manner. The testicle was decapsulated, discarding the albuginea. The parenchyma was triturated in a mortar with the addition of 0.4 per cent phenol-saline, then heated in a water bath for one-half hour, the volume of liquid lost by evaporation being replaced. Then, because of the coagulation of the proteins by heat, the material was again triturated until there was obtained a fine suspension which did not need filtering. This was placed in a bottle with a perforatable rubber stopper and heated for one-half hour. The final volume was about 2 *cc.*

FIG. 1. Scheme showing the series of inoculations in the experiments previously reported. Rat No. 110, the source of the lepromin tested, was from the second-reinoculation group, sacrificed after nine months.

For the comparative study two lepromins prepared from human lepromas were used. One was an "integral" lepromin made by the classical Hayashi-Mitsuda method, in the usual 1:20 proportion. The other was a "bacillary" antigen of the Dharmendra type, the bacilli extracted from the tissue with chloroform and the suspension made 1:2000 by weight.

The three lepromins were injected intradermally in the region between the scapula and the vertebral column, in such a way as to produce adequately-separated wheals each approximately 1 cm. in diameter. Nine individuals, all adults, were tested: 2 had lepromatous and 3 had tuberculoid leprosy, and 4 were nonleprous controls with other skin diseases. The number of persons tested was limited because of the small amount of the rat-testis lepromin available.

RESULTS

The results of the tests are given in Table 1, and are illustrated by the selected photographs and photomicrographs in the plates. For obvious reasons not all of the pictures that were made can be reproduced, only examples of the various findings.

CLINICAL FINDINGS

Early reaction.—As regards the early or 48-hour reaction of Fernandez, the lepromatous patients gave negative results with all three lepromins. On the other hand, speaking generally, the tuberculoid cases and the nonleprous controls reacted similarly to the three lepromins. The discordance noted in the table, for the most part only with respect to the intensity of the reaction, may have been due partly to the heterologous nature of the experimental lepromin, which contained rat proteins, or to different concentrations of bacilli, or to other factors.

TABLE 1.—*Results of early and late reactions in 3 tuberculoid and 2 lepromatous leprosy patients and 4 nonleprous control patients, with the experimental rat-tissue lepromin and integral (Hayashi-Mitsuda) and bacillary (Dharmendra) human antigens.*

Case No.	Sex and age	Diagnosis	Early reaction[a] (Fernandez)			Late reaction[a] (Mitsuda)		
			R.L.	I.L.	B.L.	R.L.	I.L.	B.L.
1	M, 31	Tuberculoid l.	1+	2+	1+	3+	3+	3+
2	M, 34	Tuberculoid l.	—	1+	1+	1+	1+	1+
3	M, 24	Tuberculoid l.	2+	2+	3+	3+	3+	3+
4	M, 33	Lepromatous l.	—	—	—	—	—	—
5	M, 28	Lepromatous l.	—	—	—	—	—	—
6	M, 48	Prurigo	2+	2+	2+	2+	2+	2+
7	M, 21	Prurigo	—	—	—	1+	1+	1+
8	M, 26	Scrofuloderma	1+	2+	±	2+	2+	2+
9	M, 32	Epithelioma	±	1+	±	2+	2+	2+

[a] R.L. = rat leprosy lepromin; I.L. = integral (Hayashi-Mitsuda) lepromin; B.L. = bacillary (Dharmendra) antigen.

Late reaction.—As regards the late or 21-day reaction of Mitsuda, the results were extraordinarily uniform, and highly significant. In the lepromatous cases the results were completely negative, and it was very difficult even to locate the sites of the injections. The tuberculoid and the nonleprous patients all reacted positively, in degrees of intensity varying between 1+ and 3+. In each case the reactions to all of the antigens were of similar intensity.

The early reactions in two of the tuberculoid cases, Nos. 2 and 4, are shown in Figs. 2 and 5. Although in Case 1 these reactions were somewhat weaker than those in Case 2, the late reactions, shown in Fig. 3, were all strong, and equally so. Fig. 5 shows the weak late reactions, all 1+, in Case 2. The moderately strong early reactions of one of the prurigo cases (Case 6) are to be seen in Fig. 7, while the weak late reactions of the other prurigo case (Case 7, early reactions negative) are shown in Fig. 9. The 21-day appearances of the two lepromatous cases (Nos. 4 and 5), negative throughout, are shown in Figs. 10 and 11.

HISTOPATHOLOGY

The reaction lesions, or sites of injection, of several cases were removed 21 days after the injections for histopathologic examination.

The specimens were fixed in 10 per cent formalin and embedded in paraffin; sections were stained with hematoxylin and eosin.

In Case 1, one of the 3+ tuberculoid cases, the reactions to all three antigens were so severe that they produced central necrosis, with perforation of the surface in one instance (the reaction to the integral lepromin). These centers are represented in the sections (Figs. 12 and 13) by cavities around which are zones composed of epithelioid and lymphocyte cells, with some giant cells (Fig. 13).

The nodules of Cases 6 and 8, both 2+ reactors, one a case of prurigo and the other of scrofuloderma, all presented the typical structure of the Mitsuda reaction with no essential differences dependent upon the type of antigen. They all show intense infiltration of the corium, especially of the medial and deeper levels, usually but not always of follicular arrangement, with a preferential perivascular location. The infiltrate, composed of epithelioid cells, lymphocytes and some giant cells, tends to assume a typically tuberculoid structure (Figs. 15-19).

The sites of injection in Case 5, a reaction-negative lepromatous case of L_2 grade, had the appearance of normal skin. Sections show an absence of epithelioid cells or nodule formation; they show only a lepromatous infiltration of the corium with many bacilli.

CONCLUSIONS

From the foregoing results it is believed that three conclusions of importance can be drawn:

(1) The inability of the lepromatous patients to react against the rat-testis lepromin is evidence that the bacilli composing it are Hansen

DESCRIPTION OF PLATES

All of the photographs of this plate were taken vertically, but for reproduction they have been turned horizontally, to read from left to right: integral (Hayashi-Mitsuda) lepromin, rat-testis lepromin, and bacillary (Dharmendra) antigen.

FIG. 1. Early reaction, Case 1, tuberculoid leprosy. Readings, left to right: 1+, 2+, 1+. Noteworthy is the relatively marked prominence, here and to some extent in the other cases pictured, of the 48-hour reaction to the rat-testis lepromin.

FIG. 2. Late reaction, Case 1. Readings: all 3+.

FIG. 3. Early reaction, Case 3, tuberculoid leprosy. Readings, 2+, 2+ and 3+.

FIG. 4. Late reaction, Case 2, tuberculoid leprosy. Illustrating the results in a weakly-positive case, readings all 1+. The reaction to the rat-testis lepromin is no weaker (and it is somewhat more visible in the photograph) than those to the control antigens.

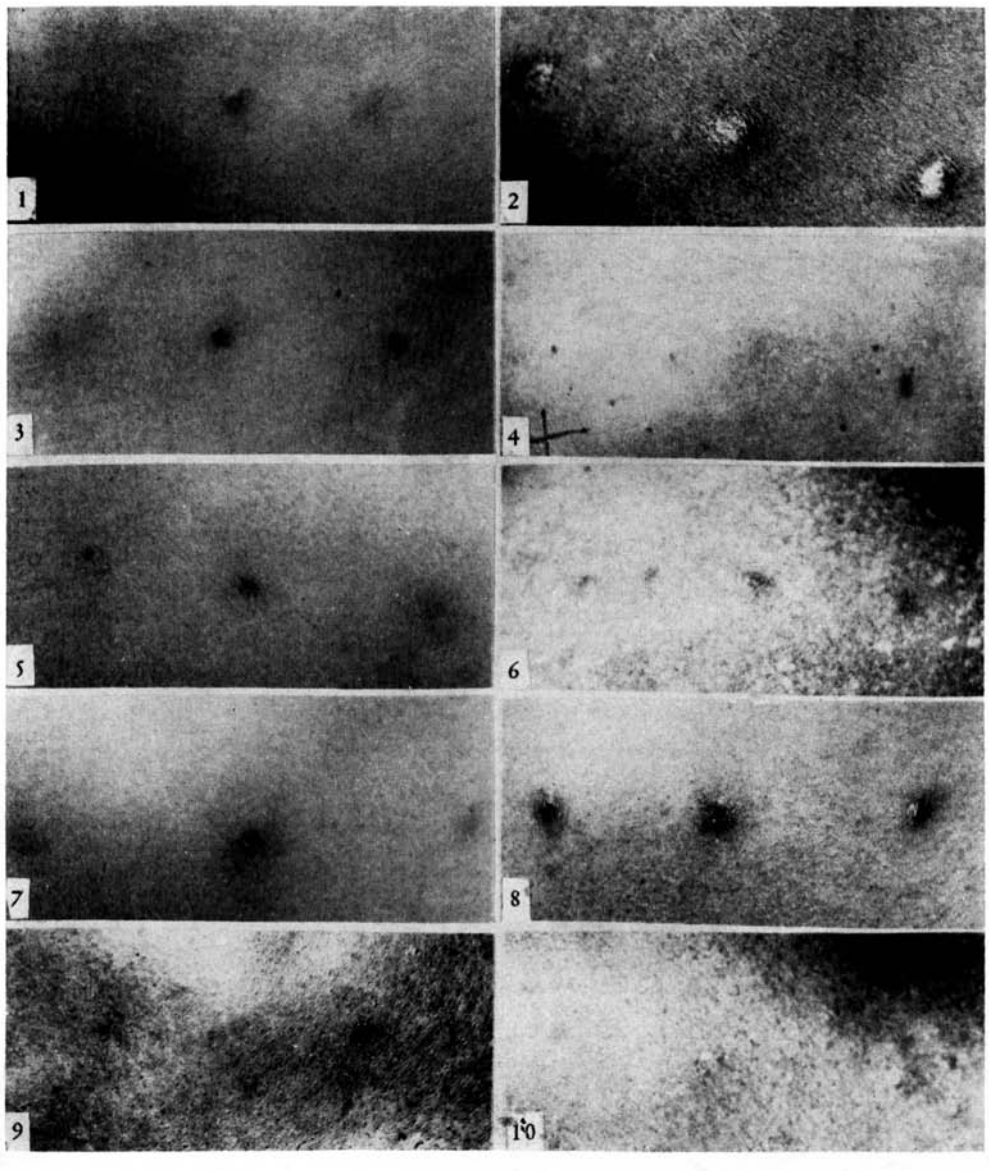
FIG. 5. Early reaction, Case 6, prurigo. Readings: all 2+. (The late reactions of this case were also all graded as 2+.)

FIG. 6. Late reaction, Case 7, prurigo. Unlike the late readings in the other prurigo case (above), these were all only 1+.

FIG. 7. Early reactions, Case 8, scrofuloderma. Readings 1+, 2+, ±. The relatively strong early reaction to the rat-testis lepromin in this patient with tuberculosis of the skin is noteworthy (but compare Fig. 8).

FIG. 8. Late reaction, Case 8. Readings: all 2+. The relatively strong early reaction to rat-testis lepromin (Fig. 7) was not followed by an especially strong late reaction.

FIGS. 9 AND 10. Late reactions in lepromatous leprosy, Cases 4 and 5. All readings negative, as were the early reactions.



bacilli, since only the lepromatous ones failed to react to this mycobacterium, whereas such cases react to suspensions of other acid-fast bacilli (e.g., the Stefansky bacillus, or *M. phlei*), with nodular reaction lesions of tuberculoid structure. In other words, the inability of the lepromatous cases to react is specific for the Hansen bacillus, and is not observed with any other acid-fast bacillus. This fact is used as maximal proof, so far, of the biological specificity of the Hansen bacillus in cultures and inoculation of acid-fast germs which are presumed to be *Mycobacterium leprae*.

2. The parallelism in the late, or Mitsuda, reactions to the three antigens employed in this experiment indicates, among other things, the great quantity of bacilli present in the rat-testis lepromin, which was capable of eliciting nodular reactions similar in intensity to those provoked by the control antigens used, which were rich in bacilli. Also this parallelism may be regarded as an indication that the three antigens contained the same germ, that is, the Hansen bacillus.

3. Thus, by the findings of the present report and the three previous ones cited, I believe that I have demonstrated that the Hansen bacillus is capable of growing and multiplying actively in a living organism other than the human one, when the experimental animal is properly prepared by, for example, a prooxidant diet. I wish also to point out that this is the first time that an integral (Hayashi-Mitsuda type) lepromin with Hansen bacilli from an animal source has been prepared.

It should be borne in mind that the multiplication of the Hansen bacillus in an animal organism, apart from its being an extrahuman source of these bacilli, may be a point of departure of studies of pathogenesis, chemotherapy and immunology of unquestionable value.

SUMMARY

There is described the preparation of a lepromin containing Hansen bacilli obtained by serial intratesticular inoculations of rats fed a prooxidant diet. The behavior of this lepromin has been found, on

DESCRIPTION OF PLATES

(The magnifications stated are as given by the author. There has been a reduction of about 31 per cent in reproduction.)

FIG. 11. Section of the reaction lesion to the integral lepromin in Case 1, a tuberculoid case with 3+ reactions, producing local abscesses which in this instance perforated (Cf Fig. 1). The cavity is lined with a necrobiotic zone, with distinct infiltration of epithelioid cells. Beneath it was a nodular structure. 60X.

FIG. 12. The reaction to the rat-testis lepromin in Case 1, a small, nonperforating deep abscess. Around the cavity there is a histoleucocytic reaction, and farther away, in the several levels of the corium, a follicular infiltration composed of epithelioid and giant cells and a lymphocytic halo. 50X. (The lesion produced by the bacillary antigen was very similar.)

FIG. 13. A high-power field of the rat-testis reaction lesion (Fig. 12), composed of epithelioid cells with a tendency to transform to giant cells, with lymphocytic infiltration. 350X.

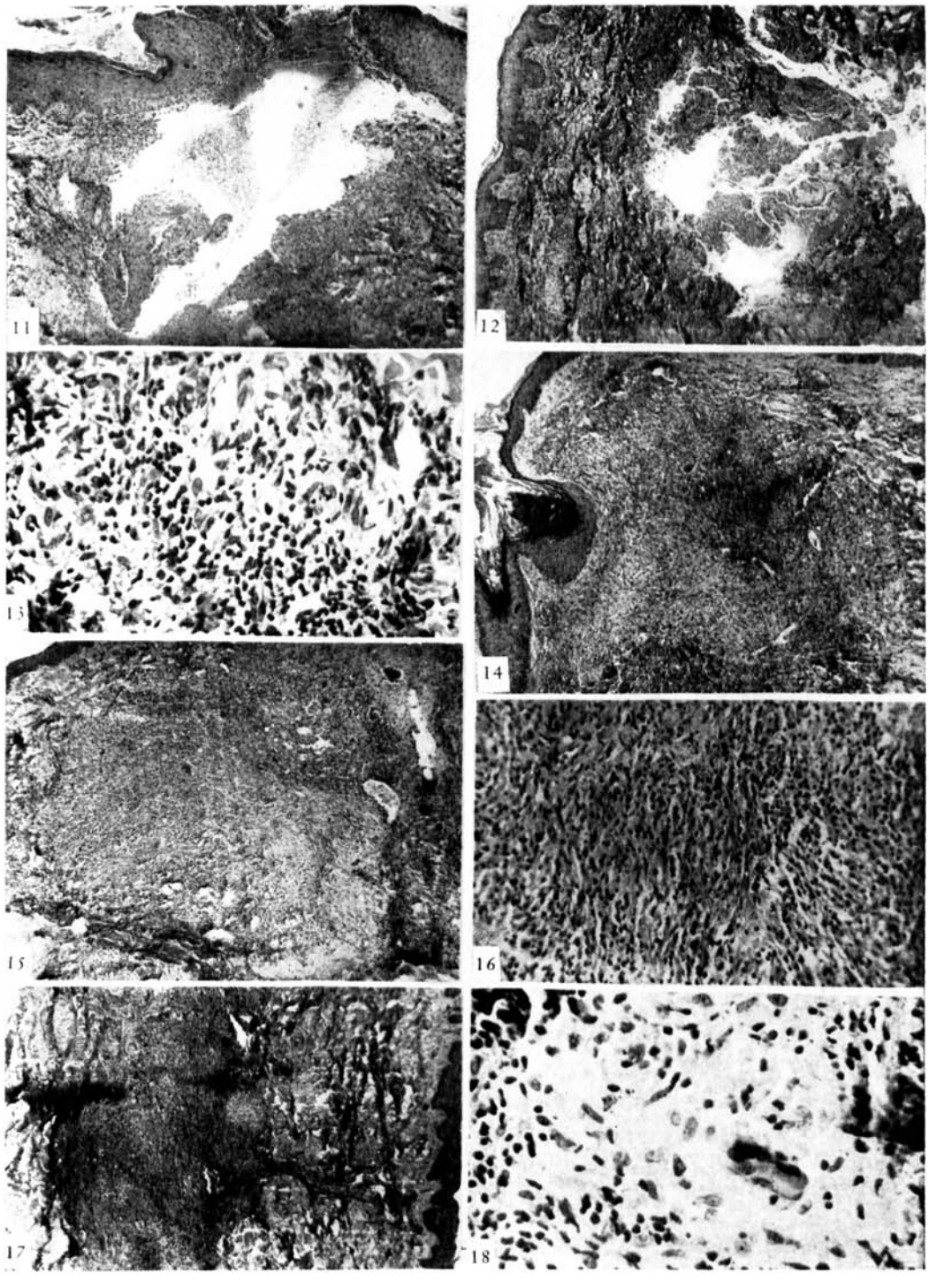
FIG. 14. Reaction to the integral lepromin in Case 6, a 2+ reactor with prurigo. Nodular structure with central necrobiosis, epithelioid cells, and distinct lymphocytic infiltration. 60X.

FIG. 15. Lepromin nodule in Case 6 produced by the rat-testis lepromin. Nodular structure with giant cells and distinct lymphocytic halo. 50X. (The section of the bacillary antigen lesion showed an infiltration of similar composition, but less distinctly localized and nodular.)

FIG. 16. The rat-testis lepromin lesion in higher magnification. Epithelioid structure with lymphocytic infiltration. 150X.

FIG. 17. Rat-testis lepromin nodule in Case 8, a 2+ reactor with scrofuloderma. In the medial and deep levels of the corium there is a follicular infiltration of epithelioid cells with a lymphocytic halo. In one of the follicular elements there is central necrosis. 50X. (The lesion produced in this case by the integral lepromin was very similar to that in Fig. 14. The one caused by the bacillary antigen was less localized.)

FIG. 18. A high-magnification field in a large epithelioid area of Fig. 17, rat-lepromin lesion. Around a giant cell are epithelioid and, peripherally, lymphoid cells forming a follicular structure. 400X.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testing nonreactive lepromatous cases and reactive tuberculoid cases and patients with other skin diseases, to be similar to that of integral (Hayashi-Mitsuda) lepromin and a Dharmendra type antigen. This is held to be further proof that the bacilli found in previously-reported rat lesions are Hansen bacilli.

RESUMEN

Descríbese la preparación de una lepromina que contiene bacilos de Hansen obtenidos por la inoculación intratesticular seriada de ratas a las que se había suministrado una alimentación prooxidante. Al comprobar casos lepromatosos irreactivos y casos tuberculoideos reactivos y enfermos con otras dermatosis, se ha observado que el comportamiento de esta lepromina es semejante al de la lepromina íntegra (Hayashi-Mitsuda) y de un antígeno de tipo Dharmendra. Se considera esto como nueva prueba de que los bacilos descubiertos en lesiones previamente descritas en ratas son bacilos de Hansen.

Acknowledgment.—The generous spirit of collaboration and helpfulness of Prof. Luis E. Pierini has made the present investigation possible. An expression of my gratitude is extended to him.

REFERENCES

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2. Bergel, M. Inoculación del *Mycobacterium leprae* a ratas alimentadas con dietas prooxidantes. II. Resultados bacteriológicos hasta los diez meses de la primera reinoculación. Semana Médica **111** (1957) 1148-1151.
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ADDENDUM

Preparation of rat-testis lepromin

To prepare the rat-testis employed in the study here reported it is advisable to proceed in the following manner.

Inoculate 0.1 cc. of a fresh suspension of Hansen's bacilli from a human leproma into each testicle of three or four rats previously fed from the 21st to the 40th day of life on a prooxidant diet, and maintain them on the same diet afterward. Sacrifice them 8 to 10 months after inoculation, triturate the testicles in saline, and inoculate 0.1 cc. of this suspension into each testicle of a group of 20 to 30 rats, under the same conditions as the previous ones, fed on the same type of diet. Then, at the end of another 8 to 10 months, these animals are also sacrificed. Their testicles are decapsulated and an integral lepromin is prepared from the parenchyma, as if they were lepromas. The passage from rat to rat, as indicated, increases the final richness in bacilli

The extraordinarily slow growth of the Hansen bacillus under the condition described has as yet made the preparation of animal lepromin by this procedure costly and impracticable. However, it is of indisputable scientific and academic value.

The prooxidant diet used in this study has the following composition (1):

Casein	23.8 gm.
Brewer's yeast, dry powder	8.9 gm.
Mineral salt mixture	3.0 gm.
Starch (*almidón*)	48.9 gm.
Linseed oil	15.5 gm.

The mineral-salt mixture is that of Hubbel and associates:[2] $CaCO_2$, 543; $MgCO_3$, 25; $MgSO_4$, 16; NaCl, 69; KCl, 112; KH_2PO_4, 212; $FePO_4$, 4 H_2O, 20.5; KI, 0.08; $MnSO_4$, 0.35; NaF, 1.0; Al^2 $(SO_4)_3$, K_2SO_4, 0.7; $CuSO_4$, 0.90. The linseed oil, minimal iodine index 170, is added daily to the dry mixture, in order to facilitate its autooxidation.

[2] HUBBEL, R. B., MENDEL, L. B. and WAKEMAN, A. J. New salt mixture for use in experimental diets. J. Nutrition **14** (1937) 273-285.