## THE DIFFUSION FACTOR IN LEPROUS SKIN

by

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#### I. INTRODUCTION

In 1928 Durán-Reynals (1) discovered, in extracts of normal organs of laboratory animals, a factor that increases the permeability of tissues with which it comes in contact and the velocity of diffusion of germs and vital stains in connective tissue. This factor has since been called "diffusion factor," "R factor," or "Reynals' factor." Further research showed that testicular extract has the greatest diffusion capacity (2, 3, 4, 5, 6, 7) but that the factor is also present to a much lesser degree in extracts of kidney, skin, brain, and liver. Later it was discovered in some snake venoms, in cancerous cells, and in spermatozoa; also that it is a characteristic of certain bacteria, especially of certain strains of staphylococci, streptococci, pneumococci, and the bacillus of gas gangrene. A number of additional papers have been published on the subject (8, 9, 10) and biochemical studies have identified the R factor with a hydrolytic enzyme (mucinase).

In 1934 Thomas and Durán-Reynals (11) published a paper on the influence of the R factor on the tuberculin reaction in tuberculous guinea pigs, showing that the reaction area becomes larger but less intense than in the controls. To see the effect of testicular extract, an area of skin is prepared with R factor and a few hours later the germ, antigen, or stain is injected. Germs are inoculated intravenously and localized where the testicular extract has been injected. Negroni (12) in 1943 made intradermal inoculations of R factor in man and found that antigens (Frei's, tricophytin, oidiomycin, tuberculin) inoculated in the same place one to five hours later give a considerably larger (352 per cent) area of reaction in sensitive subjects. This author believes (13) that the diffusion factor facilitates the entrance of antigens into sensitized cells.

### II. OBJECT OF PRESENT RESEARCH

The present investigation was undertaken in an attempt to answer the following questions:

1) What is the relation between the R factor (testicular) and the diffusion factor of normal human skin?

2) What difference, if any, is there between the diffusion factor in normal and leprous skin? That is, does leprous infection alter the behaviour of normal skin as a diffusion agent? What difference, if any, is there between tuberculoid and lepromatous skin with respect to the diffusion factor? Results have shown that tuberculoid skin has a diffusion action approximately equal to that of normal skin, while that of lepromatous skin is considerably inferior and similar to that of a saline control. This difference was attributed by Mom (14) to *Mycobacterium leprae* which might inhibit or destroy the R factor normally present in skin. When the skin reacts, according to the Jadassohn-Lewandowsky law, with a tuberculoid response, the Reynals' factor apparently is not affected and can act normally.

3) What relation is there between the diffusion activity of the skin and its content of *M. leprae*?

#### III. METHODS AND TECHNIQUES

The Durán-Reynals technique was used (2).

Testicular extract. Bull testicles were used. The vaginal sheath was removed, the tissue ground with sand in a mortar and suspended evenly in Ringer's solution, the homogenous fluid centrifuged at 4,000 r.p.m. for 30 minutes, and the supernatant fluid stored in the icebox. All operations were made aseptically.

Normal skin extract. Adipose tissue was removed and the extract prepared in the same manner as the testicular extract.

Tuberculoid leprosy skin extract. Skin was obtained from patients bacteriologically-negative and with positive lepromin test. Lepromatous leprosy skin extract. Three types of extracts were prepared: 1) with few bacilli; 2) with a moderate quantity of bacilli; 3) with abundant bacilli. All these patients had a negative Mitsuda test.

Extract of skin from tuberculoid leprosy in reaction. Extracts were made with skin from two patients in a state of reaction of one and two months duration respectively, with few acid-resistant bacilli, and positive lepromin test.

These extracts also were used after filtration through a 3 G 4 filter. No difference was observed in the behaviour of filtered and non-filtered extracts.

Lepromin. Two types were used: 1) integral or standard, prepared according to the Mitsuda-Hayashi and Muir techniques; 2) bacillary (suspension of pure ground bacilli) prepared by the Fernández-Olmos Castro technique (15). This latter type also was prepared with unground or slightly ground bacilli, and also as a suspension of pure bacilli in Ringer's fluid without phenol or glycerol.

All these extracts were injected intradermally (0.1-0.2 cc.) into the depilated skin of the flanks of white male rabbits of 1,500 Gm. weight.

At first a 1:2 suspension of India ink in saline was used as an indicator of diffusion of the extracts; later a 1 per cent solution of trypan blue was substituted as this made reading easier. The dye was injected at the same time as the extracts in amounts of 0.1 cc. Isotonic solution of hemoglobin prepared from sheep red cells also was used for the same purpose; it gave good readings up to 2 hours after injection, but for longer periods it was less satisfactory.

The area of diffusion of each extract was measured 30 minutes, and 1, 2, 5, 8, and 24 hours after injection.

In all the experiments control injections of 0.1 cc. trypan blue plus 0.1 cc. saline were made.

Skin extracts were used in dilutions of 1:2, 1:20, and 1:50. Durán-Reynals (10) has already observed that testicular extract activates the diffusion of India ink even in a dilution of 1:100,000.

#### IV. RESULTS

A) Diffusion factor of testicular extract. The results obtained in 3 rabbits are summarized in table 1. Giving a value of 100 to the control diffusion, that of testicular extract was 497. Therefore in 24 hours the dye diffused five times more in the skin that had received testicular extract than in the control.

 
 TABLE 1. Diffusion of a 1 per cent solution of trypan blue, under the influence of testicular extract.

	Diffusion of trypan blue		
Time after inoculation	Testicular extract	Saline	
30 minutes	28x20	16x15	
1 hour	30x24	25x21	
2 "	35x34	30x23	
5 "	41x36	33x23	
8 "	70x38	35x24	
24 "	80x55	36x24	
24 hour diffusion area*	8455.76	697.44	

\* The areas are considered as elipses; the first figure corresponds to the larger diameter and the second to the smaller (in mm.). The surface was calculated at the 24th hour according to D.d.  $\pi/4$  in sq. mm. The figures are the average for 3 rabbits. This method of calculation of area is used in all subsequent tables.

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B) Diffusion factor in normal skin. The results obtained in 3 rabbits are summarized in table 2. Again giving a value of 100 to the control diffusion, that of normal skin was 248.

 
 TABLE 2. Diffusion of a 1 per cent solution of trypan blue under the influence of normal skin extract.

	Diffusion of trypan blue		
Time after inoculation	Extract of normal skin	Saline	
30 minutes	39x18	16x15	
1 hour	44x25	25x21	
2 "	48x28	30x23	
5"	54x29	33x23	
8"	60x34	35x24	
24 "	<b>6</b> 3x35	36x24	
24 hour diffusion area	1731.81	697.44	

A comparison of tables 1 and 2 shows that up to the fifth hour diffusion was greater with normal skin extract than with testicular extract; between the fifth and eighth hours the diffusion rate increased with testicular extract and remained the same with skin extract. Including all the experiments, diffusion with testicular extract was five times that with saline and two times that with extract of normal skin.



Comparative diffusion effect of testicular factor (R), and normal skin extract (N) with respect to saline (t), 24 hours after inoculation into the skin of a white rabbit.

C) Diffusion factor in leprous skin. The skin from cases of both principal forms of leprosy was studied to find if M. leprae destroyed or inhibited the diffusion factor.

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a) Skin with tuberculoid leprosy. The skin extract was prepared and tested as in the case of normal skin but 5 white rabbits were used. In table 3 the maximum and minimum values observed are given. Diffusion increased in rate between the fifth and eighth hours. In the early stages of the experiment it was not possible to say which animal would give the greatest diffusion area at 24 hours.

TABLE 3. Diffusion of a 1 per cent solution of trypan blue under the influence of

extract of tuberculoid leprosy skin. Diffusion of trypan blue Time after Tuberculoid skin extract inoculation Max. Saline Min. 22x20 30 minutes 17x15 14x10 28x24 1 hour 20x19 14x11 2 " 28x25 21x20 14x13 " 32x30 5 25x2017x15 " 8 65x30 52x30 20x16 " 24 80x35 60x3423x20 24 hour diffusion area 2199.12 1602.22 361.29

b) Skin with lepromatous leprosy. Preliminary experiments with diseased skin taken from patients in different stages of lepromatous leprosy showed differences in behaviour of the extracts. It was therefore decided to make three types of extracts: 1) with skin from cases with discrete lesions and poor in *M. leprae* (L1); 2) with skin containing a moderate amount of *M. leprae* (L2); 3) with skin containing large quantities of *M. leprae* (L3). These extracts were prepared and tested as in the previous cases. Results are summarized in table 4, using the average figures for 5 rabbits.

	Diffusion of trypan blue			
Time after	Extracts of lepromatous skin			
inoculation	L1	L2	L3	Saline
30 minutes	17x15	20x19	21x17	17x16
1 hour	22x20	23x22	22x21	23x20
2 "	29x25	25x25	24x21	28x25
5"	34x33	29x28	27x25	28x27
8 "	45x38	38x32	30x28	32x28
24 "	50x40	43x34	32x28	34x29
24 hour diffusion area	1570.80	1148.25	703.72	774.40

 
 TABLE 4. Diffusion of a 1 per cent solution of trypan blue under the influence of extract of lepromatous leprosy skin.

In table 5 the results obtained with the extract of skins of normal persons and those of patients with tuberculoid and lepromatous forms of leprosy are compared. It will be seen that diffusion of trypan blue under the influence of extracts of normal and tuberculoid skin is approximately three times that of saline but with extract of lepromatous skin it is slightly less than that of the control.

 
 TABLE 5. Diffusion area of a 1 per cent trypan blue solution under the influence of extracts of normal, tuberculoid, and lepromatous skin.

Diffusion area of trypan blue 24 hours after inoculation			
Normal	Tuberculoid	Lepromatous	Saline
1995.70	1924.23	678.72	774.40



Comparative diffusion effect of extracts of tuberculoid (T), normal (N), and lepromatous (L) skin with respect to saline (t), 24 hours after inoculation into the skin of white rabbits. The stippled area represents the difference between the relative maximum and minimum results obtained.

c) Tuberculoid leprosy in reaction. The foregoing experiments seem to indicate a certain relation between the diffusion activity of the skin and its content of M. leprae; it is therefore of interest to compare the results obtained with skin from tuberculoid leprosy patients who had no bacilli in the skin, as was proved by careful microscopic examination of serial sections, and those with skin from patients with the same form of leprosy during a period of tuberculoid leprosy reaction, when M. leprae is found in the skin. It has not yet been possible to examine the skin of the same patient with tuberculoid leprosy before and during a leprosy reaction, with a negative and then a positive bacillary examination.\* The skin of two patients in the midst of reactions of one and two months' duration respectively was used. A moderate number

<sup>\*</sup> Experiments with a provoked leprosy reaction obtained by the subcutaneous injection of lepromin (Fernández' method) are being carried out.

of bacilli were found in the skin. The results are summarized in table 6. Extracts of tuberculoid skin in reaction have a diffusion activity intermediate between that of L3 lepromatous and tuberculoid leprosy.

 

 TABLE 6. Diffusion area of a 1 per cent trypan blue solution under the influence of extracts of normal, L3 lepromatous, tuberculoid, and tuberculoid in reaction skin. Average values of 4 rabbits 24 hours after inoculation.

Normal	L3	Tuberculoid reaction	Tuberculoid	Saline
1374.45	219.91	549.78	1308.48	392.70

D) Comparison of diffusion factor in apparently normal and diseased skin of patients with tuberculoid and lepromatous leprosy. Extracts were made with apparently healthy skin from patients with tuberculoid and lepromatous leprosy. The diffusion factor in the skin of tuberculoid patients is the same as in the skin of normal subjects, both in the diseased and the apparently healthy parts. In this respect the skin of these patients has a uniform behaviour, as also occurs with respect to the lepromin reaction. The skin from areas macroand microscopically normal in patients with lepromatous leprosy showed some alteration in diffusion activities, but results so far obtained cannot be considered conclusive. Should this be confirmed it would indicate that leprous infection also affects in some way the clinically normal skin areas. With respect to the diffusion activity all the skin of lepromatous patients may have a common behaviour. Nevertheless, it should be kept in mind that Fernández (16) has seen a sarcoid reaction to lepromin in some cases of lepromatous leprosy. Further experiments to clear up these questions are being carried out.

E) Diffusion factor of lepromin. In a previous paper Mom (14) studied the diffusion activity of lepromin. Bacillary lepromin (Fernández-Olmos Castro) was seen to act similarly to the extracts of skin with abundant bacilli; standard lepromin (Mitsuda-Hayashi or Muir) had a diffusion activity similar to that of lepromatous skin with few bacilli or rather to that of the skin of tuberculoid leprosy in reaction. It was concluded that the bacilli probably inhibited the diffusion of the dye.

Later experiments with other batches of lepromin gave irregular results that could not be compared with those already published. This lack of agreement might be due to one or more causes: 1) Standard lepromin contains whole bacilli, while bacillary lepromin is a suspension of pure bacilli submitted to prolonged grinding, and therefore caution should be used in comparing diffusion activity; 2) large quantities of sodium chloride which are used in the preparation of bacillary lepromin (Fernández-Olmos Castro) are not completely eliminated before suspending the bacillary powder in glycerol and may interfere with the activity of the diffusion enzyme; 3) glycerol and phenol may also interfere with diffusion activity. For these reasons a bacillary suspension was prepared by the Fernández-Olmos Castro technique, deprived of salt by

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repeated washing, and divided into three parts: (a) unground, (b) partially ground, and (c) completely ground. Diffusion of trypan blue with (a) was similar to that seen when extracts of skin with abundant bacilli were used; (b) and (c) gave irregular results that could not be fruitfully analyzed.

F) Apparent antagonism between extracts of tuberculoid and lepromatous skin. Using trypan blue as an indicator, the following experiments were designed to determine whether extract of lepromatous skin would diminish or inhibit the diffusion activity of extract of tuberculoid skin. On one flank of a rabbit tuberculoid skin extract was injected in four places, and 30 minutes, 1, and 2 hours later lepromatous skin extracts with abundant bacilli (L3) were injected into the same areas. On the other side of the same animal the order of injection was reversed, the lepromatous skin extract being injected first.

In the areas reinjected with a 30 minute interval, in both series, a certain balance was established between the diffusion activity of tuberculoid skin and the antidiffusion activity of lepromatous skin. The diffusion observed was intermediate between that obtained with the extracts used separately.

G) Antigenic activity of the diffusion factor. The diffusion areas observed in rabbits used for successive experiments were smaller than those seen when they were used for the first time. This may be due to the development of antibodies for the extract, which reduce its activity. As the antigenic properties of the diffusion factor are not unanimously accepted, further experiments are being made in order to explain this observation.

### V. DISCUSSION

This series of experiments seem to prove that leprous infection modifies the diffusion factor R found in normal skin. Modifications are due to the presence of *M. leprae*, and are more pronounced the greater the number of bacilli in the skin. In apparently normal skin of lepromatous patients, which shows no bacilli, the diffusion factor suffers some alteration though not as marked as in the areas in which bacilli are present. It cannot be admitted, therefore, that the presence of bacilli is the only factor causing inhibition of the diffusion activity in the skin of leprosy patients.

In the tuberculoid and the lepromatous forms of the disease the skin reacts uniformly with respect to this factor. In the former the normal diffusion rate is present, in the latter it is inhibited. This reactional uniformity has already been proved in the case of the lepromin test, both with standard and bacillary lepromin.

Uniformity of reaction is nevertheless more apparent than real. Fernández (16) has shown that in lepromatous patients lepromin can give a tuberculoid reaction of sarcoid type, therefore the "lepromatous" manner of response is not an unmodifiable quality in this type of leprosy. Furthermore with clinical and bacteriological improvement a previously negative lepromin MOM, BASOMBRIO)

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- 1 = Testicular extract 2 = Normal skin extract 3 = Saline



L = Lepromatous extract 1 = Few bacilli 2 = Moderate bacilli 3 = Abundant bacilli



S = Saline1-2-3-4 = Lepromatous skin extract, abundant bacilli

Diffusion areas 24 hours after injection

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test can become positive. This may be due either to sensitization, which implies a different form of reaction, or to the removal of inhibition of the Mitsuda phenomenon produced by a massive infection. These considerations open new avenues for research into the pathogenesis of leprosy, and make it probable that there is no hard and fast barrier between the clinical forms of leprosy that would make impossible the passage of one to the other.

A further argument in this same direction is found in the behaviour of normal and diseased skin of tuberculoid leprosy in reaction, which becomes, in respect to its diffusion activity, similar to the lepromatous skin, while it behaves like normal skin when not in a reaction period. Inhibition of the diffusion factor produced by M. leprae may be a transitory and not a permanent condition.

Certain experiments of Joyner and Sabin (17, 18) made with a vital dye, pontamine sky blue, upon allergic tuberculous guinea pigs show that diffusion is about one half of that seen in the controls. They maintain that "... if we may assume that the rate of diffusion of dye is approximately an indicator of the diffusion of the protein, an animal in which the tuberculous infection has induced a delay in the rate of diffusion in the tissues is able to react to a smaller amount of tuberculo-protein, because a larger proportion of material is retained in contact with cells instead of being rapidly diffused throughout the body." Diffusion activity conditions concentration of a substance in the locus of injection. In a moribund, anergic, tuberculous guinea pig the dye diffuses more rapidly than in the normal animal.

If we admit that the lepromatous form is anergic and the tuberculoid hypergic it will be seen that this interpretation of what happens in the tuberculous guinea pig cannot be applied in the case of leprosy, where the capacity to react coincides with a normal diffusion activity in hypergic cases and where there is a lowered diffusion factor in anergic cases. The conditions are too dissimilar to allow a legitimate comparison.

On the other hand Negroni (12) has shown that in the initial phases of the specific reactions in bacterial allergy the R factor increases by 352 per cent the intensity of the reaction. (This is contrary to Thomas and Durán-Reynals experiments.) It may be argued that the lack of the R factor in lepromatous leprosy is the cause of the negativity of the lepromin test. This argument is reinforced by the observation made by us and by others that in tuberculoid leprosy during a reaction period when bacilli appear in the lesions, the diffusion activity diminishes and the lepromin test decreases, even if it does not completely disappear. It may be possible to obtain a positive lepromin test in lepromatous leprosy with adequate use of the R factor. Work on these lines is now being performed.

#### VI. SUMMARY

- 1) The diffusion activity (R factor) of human skin is 50 per cent that of bovine testicular extract.
- The R factor is not modified in diseased and in apparently healthy skin of patients with tuberculoid leprosy.
- In cases of lepromatous leprosy the diffusion activity disappears completely from the skin.
- 4) The diffusion activity of leprous skin is inversely proportional to the amount of *M. leprae* it contains.
- 5) Extract of lepromatous skin appears to exercise an antagonistic effect on the diffusion action of extract of tuberculoid skin.

#### Addendum

In the above discussion an assumption is made that one of the factors responsible for the negativity of the lepromin reaction may be the lack or the inhibition of the R factor. One of us (A.M.M.) performed a series of experiments on ten lepromatous patients of from 9 to 30 years of age, preparing the site of the intradermal lepromin injection (bacillary antigen, Fernández-Olmos Castro or Dharmendra technique) with 0.1 cc. of a 1:20 solution of dry testicular powder in sterilized distilled water. Inoculations with 0.1 cc. of the bacillary antigen were made at variable intervals of 2 to 4 hours. The early reaction (tuberculin type at 48 hours) showed erythema of more than 10 mm. in 9 of the 10 cases, while the Mitsuda reaction was positive (progressive and persistent nodule above 5 mm., third week) only in two cases, with intensive early reaction (erythema of 40 mm.).

Further investigations are now being made on a larger scale following this hypothesis.

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