

ALLERGENIC EXTRACTS—A COMPARISON OF THEIR QUALITY AND RELIABILITY

DESCRIPTION OF TECHNIQUE FOR PREPARATION OF STABLE ALLERGENS AND PROPOSAL FOR BIOLOGIC ASSAY

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ALLERGENS and their application in the diagnosis and treatment of a variety of diseases have played a major role in the development of the specialty of clinical allergy. Yet, in recent years the skin test has lost much of its stature as an aid in allergy studies. Many physicians leave such studies entirely to technical assistants, who often are inexperienced in their application and interpretation. It is little wonder, therefore, that much of the medical profession is cynical about the value of skin testing, about specific allergy management based on such tests, and even about the specialty of allergy itself.

The value of skin testing as an aid to clinical allergy must depend on certain basic points:

1. Reliability of the allergen.
2. Method of application and interpretation of the reaction.
3. Incorporation of the results in over-all evaluation of the patient.

The second and third points were critically evaluated and offered by Tuft¹ fifteen years ago.

The present discussion will therefore be concerned only with quality and reliability of the allergens. Since most of the standard textbooks on allergy contain discussions of common methods of obtaining, handling, preparing, and standardizing extracts, there is no need for a detailed review of each allergen in common use. Certain basic general principles, however, will be outlined.

RAW MATERIAL

The source of raw materials, their handling, and their preservation represent the paramount preliminary steps in providing the final reliable allergens. Certain essential criteria must be fulfilled:

1. *The source of raw material should offer sufficient supply to permit the use of a reliable method for the concentration of an adequate quantity of the final allergenic product.* House dust, which is readily available in large quantities, permits elaborate methods for preparation of a highly concentrated and highly allergenic product.² On the other hand, many epidermals, which can be gathered only in limited quantity and which yield small amounts of active allergens, are often so inadequately concentrated as to provide testing materials with questionable allergenicity.

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2. *The raw material should be obtained and handled in a manner that will prevent the deterioration of the intrinsic allergenic factor.* In the case of many fruits and vegetables and some cereals, pigmentary oxidation may occur within a few hours.³ Epidermals, such as feathers and hog hair, are seriously affected in the course of removal with scalding hot water. Meats, fish, and insect material require rapid processing before autolytic changes occur.

3. *The raw material should be uncontaminated by other allergic substances.* This may necessitate acquaintance with the source of the raw material and with the manner and care of its handling before the extract is made. During storage many epidermals may be contaminated with house dust. On the other hand, house dust from an unknown source may be contaminated with the epidermals of household pets.

4. *Pollens, if obtained in original dry form, should be inspected grossly and microscopically to see that the following requirements have been met:*

(a) The pollen under the microscope is the same as that indicated on the label. (Errors in labeling do occur.)

(b) There are no other contaminating pollens present.

(c) The pollen is normal in appearance and does not show deterioration from age or moisture.

(d) There is no appreciable amount of moisture or debris. This is especially important when weight/volume is the method used for preparation of the pollen extract.

5. *Gathering of insects in quantity may require many hands and a general knowledge of entomology.*⁴ Some insects, especially those which swarm, can be readily caught in large numbers. A few insects, such as cockroach, mosquito, and other dipterae, can be raised in quantity in the laboratory.

Preservation of insects by quick freezing from the live stage will prove most successful. By this method, mildewing and putrefaction are avoided. Defatting, lyophilizing, and fine grinding in a Wiley mill will yield a product which may be stored indefinitely under desiccation until needed in the preparation of aqueous extracts. Some insects, such as moth, caddis fly, mosquito, and a few others, can be simply air dried, defatted, and then kept under desiccation without the more elaborate procedure used for the larger-bodied insects.

PREPARATION OF ALLERGENS

The ideal method for the preparation of allergenic extracts will fulfill certain rigid postulates:

1. The method will yield a final extract which is actively allergenic.

2. The method will yield a final extract which is free of nonspecific irritants.

3. The method will yield a final extract which is stable for many months or years.

4. The method will not be too complex or costly.

Unfortunately, no present method fulfills ideally such rigid postulates for the preparation of all allergens. The ideal method must adapt itself to the nature of the raw material and to the allergen being prepared.

Too frequently the compromise sacrifices reliability for the sake of simplicity and economy. Such shortsightedness yields an unreliable allergen which offers only costly misinformation to the physician and his patient.

Proposed Method of Preparing Most Allergens in Quantity.—In our laboratory there has been in use for several decades a method which has provided reliable and stabile allergens free from nonspecific irritants. This method, once established, is not cumbersome and not too costly in light of the quality of the final allergen.

Briefly, this method consists of adding four parts acetone to a clear, crude, buffered aqueous extract. The resultant flocculant precipitate is allowed to settle to the bottom over a period of hours, and the supernatant liquid is poured off. The precipitate, with a small amount of residual liquid, is poured into a filter funnel and, after filtration, is washed with isopropyl alcohol (99 per cent pure) and finally with ethyl ether. This final precipitate is placed in a desiccator for several days, at the end of which time it is thoroughly dried and readily pulverized. In the case of slow-drying allergens, lyophilization has proved more satisfactory.

A most important aspect of this procedure is the carrying-out of all steps under subfreezing temperature (about 28° to 30° F.). The final desiccated extract is finely ground in a Wiley mill, using an 80-mesh screen. This fine powder is then stored indefinitely under desiccation.

Before it is accepted for storage (and for future routine use), the dry powdered material is assayed by scratch testing on known sensitive patients. Only if it reacts as strongly as the allergen currently in use and, at the same time, does not cause any nonspecific irritation in nonsensitive persons, is it added to the common pool. Since only a few grams of such an allergen, freshly prepared and proved strongly reactive by skin testing, are added to their kind, there is maintained a reasonably constant quality which does not need repeated standardization chemically or immunologically.

For later clinical use, the dry powder is redissolved in a 50 per cent buffered glycerinated extracting fluid and passed through a sterilizing Seitz filter. The strength of dilution varies from 1:25 to 1:50 by weight/volume, depending upon the solubility of the powdered material. (This has been established through experience.) The allergen in solution is then ready for further dilution in testing and treatment of the patient.

This method of preparing powdered concentrate has been applied to epidermals, dusts, foods, and many miscellaneous allergens that require concentration. Although zinc sulfate, ammonium sulfate (saturated and half-saturated strength), mercuric chloride, and other precipitating salts were used experimentally in the past, especially for fractionation of various possible allergenic components, they required the employment of cumbersome equipment and lengthy methods that acetone precipitation has now eliminated.

This method of precipitating out the active allergen with a water-miscible solvent, sketchily outlined, omits some inevitable pitfalls and disappointments. Certain epidermals, such as those of the rabbit and the cat, yield a niggardly amount of precipitates in proportion to the effort and the raw material used. A number of precipitates (especially orris root) dry very slowly unless lyophilized; others are slow to redissolve. We have a "dossier" on each allergen prepared in our laboratory, which spares us the repetition of frustrating and disappointing experiences. With this method, epidermals and miscellaneous inhalants have provided excellent allergens that are uniformly reliable and have remained stable indefinitely in the dry form. Most of the foods have also been prepared by acetone precipitation and have provided actively allergenic material that is ready for use at any season of the year, thus eliminating the necessity of a hurried visitation to the market place for such items as fresh fruits or vegetables.

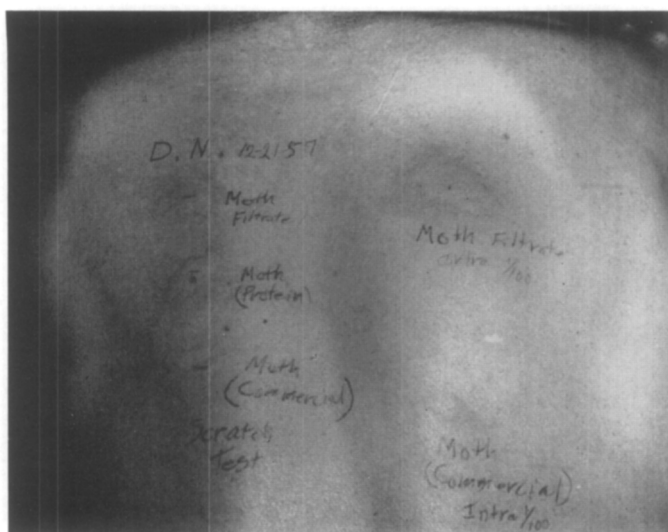


Fig. 1.—Moth extracts. Patient D. N., 44-year-old tire shop owner, had chronic asthma and extensive eczema since early youth. Skin testing showed moderate reactions to epidermals and house dust with strongest reactions to mold and insect extracts.

Comparison using moth filtrate (1:10 w/v) prepared from carefully handled material and "moth protein" prepared by acetone precipitation showed strong reaction by scratch testing. Intradermal test with the moth filtrate 1:100 gave a large reaction. Powdered moth from commercial source, in same strength, gave no reaction to the scratch test and an equivocal reaction on intradermal testing.

Pollens and Insects.—Pollens in the dry state and insects after lyophilization are defatted and then extracted for forty-eight hours at room temperature in 50 per cent buffered glycerin. In the case of pollens, a 1:50 by weight volume has been accepted as a standard. In the case of insects, the powdered material contains more inert material and a dilution of 1:10 by weight volume is the standard used. With pollens, little or no evident loss of allergenicity occurs throughout the entire season.⁵ Some insect extracts have retained high protein-nitrogen content and still have shown strong skin reactions after more than a year.⁴

Both orchard grass pollens and several insects (moth, honeybee, and mosquito) were highly concentrated by acetone precipitation. These, as we expected, yielded a high protein-nitrogen content and showed very strong skin reactions even in great dilution. At this time, however, there seems to be no practical advantage in using this method of concentration, since both pollens and insects can be stored indefinitely in a dry, defatted, powdered state. Non-specific reactions are more frequent when simple extracts are used, but these are represented chiefly by delayed "tuberculin type" reactions and are not clinically important. These nonspecific reactions are more frequent for insect extracts than for the pollens.

COMPARATIVE STUDY OF QUALITY OF ALLERGENS

For purposes of comparison of the quality of allergens currently in use, testing material was obtained from seven sources representing five commercial

Fig. 2.

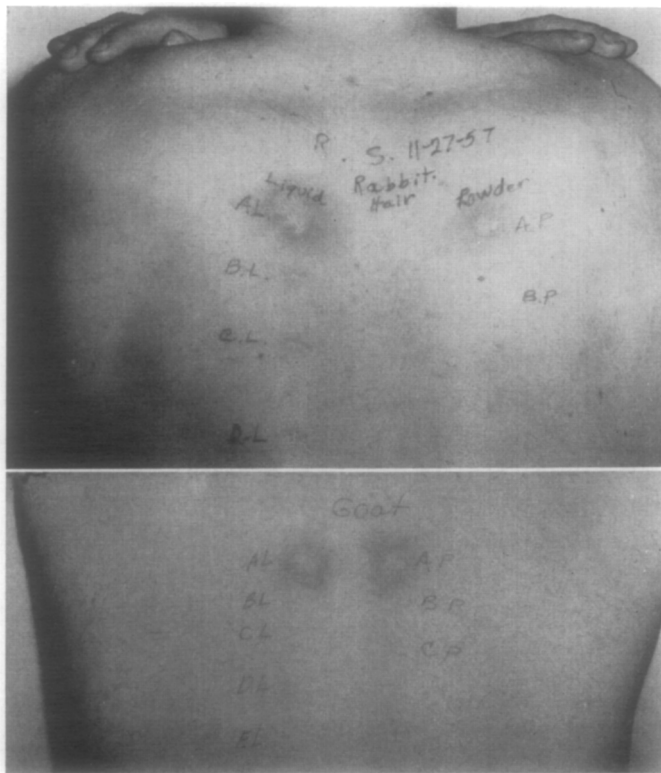


Fig. 3.

Fig. 2.—Rabbit epidermal. Patient R. S., 17-year-old son of a farmer. Perennial allergic rhinitis was present for several years with severe symptoms during the pollen season. The patient reacted strongly to pollens and epidermals.

Comparison, in this case, showed positive skin reactions only with "A.L." and "A.P." This contrast of reaction on several known sensitive patients was repeatedly demonstrated.

Fig. 3.—Goat epidermal. Patient E. M., 29-year-old rancher had asthmatic attacks and chronic perennial allergic rhinitis dating back to childhood. He strongly suspected epidermals and hay, and while in military service in Japan he was free of allergic symptoms. Most of the epidermals tested by the scratch method showed strongly positive reactions.

Comparison, using powder and liquid, showed that material "A.L." and "A.P." reacted strongly. The material from other sources failed to show any reactions.

laboratories and two allergy clinics. The commercial laboratories were widely distributed throughout the United States and would no doubt comprise the source of allergens currently used for skin testing by most physicians who do not prepare their own extracts.

For obvious reasons, the sources of supply of the various allergens are designated in the illustrations only by the letters "A" through "F." After each of these letters there is a designated letter, "P," for powdered material, and "L," for liquid material. "A" is revealed as the allergen from our laboratory, prepared by acetone precipitation. The liquid for allergen "A" is prepared from the dried powder by dissolving in 50 per cent buffered glycerin in a concentration of 1:50 by weight volume. No detailed information was available as to the method used in preparation of materials obtained from other sources. The allergens in powdered form were obtained from only two other laboratories. In the case of several epidermals, this material had the appearance, on close inspection, of finely ground hair. One commercial laboratory complied with our request for special preparation of an acetone precipitate of cat and dog epidermals. Although the quantity sent to us was too small for chemical analysis, it may be noted in the subsequent illustrations that the skin reactions were strong.

Patients who gave histories of hay fever or asthma and who even suspected specific epidermals were chosen. In the case of sensitivity to foods, the patient was certain of their offending quality. None of the patients represented in the illustrations (Figs. 1 to 10) had in recent years received specific desensitization or were currently on antihistaminics or other symptomatic medication. The illustrations reveal the unreliability of many of the allergens applied by the scratch method.

DISCUSSION

Although investigative studies in allergy and applied immunology are carrying current thought onto somewhat remote paths, it is still important to re-examine the basic clinical tools used daily in the study and care of the allergic patient. Figs. 1 to 10 demonstrate consistent findings of the unreliability of the allergens studied, which represent many of those allergens widely used. The epidermals chosen are used frequently in routine skin testing and are important in diagnosis and specific desensitization.

All the allergens were obtained (shortly before starting these studies) from commercial sources or from allergy clinics where they were in current use. Most of the vials showed some symbols of standardization, either as to nitrogen content or weight/volume dilution. Some labels also indicated the type of diluent used, the date of preparation, and the date of expiration. None of the labels indicated that positive skin reactions had been obtained with the contained allergen. We made no attempt to gain detailed information concerning the method of preparing these allergens. The illustrations, however, give evidence of consistent findings that only allergen samples "A" and several labeled "C" and "F," prepared by acetone precipitation, gave consistently strong, positive skin reactions.

Our method of preparing a concentrated allergen in stabile powder form by acetone precipitation is not a recent development. It has been the standard method for preparation of epidermals, foods, and most miscellaneous inhalants (except pollens and insects) in our laboratory for more than twenty years. It has been employed even longer by a few other allergists.

The preparation of purified house dust extract, using such a water-miscible organic liquid as that described by Boatner and associates,^{2, 6} includes

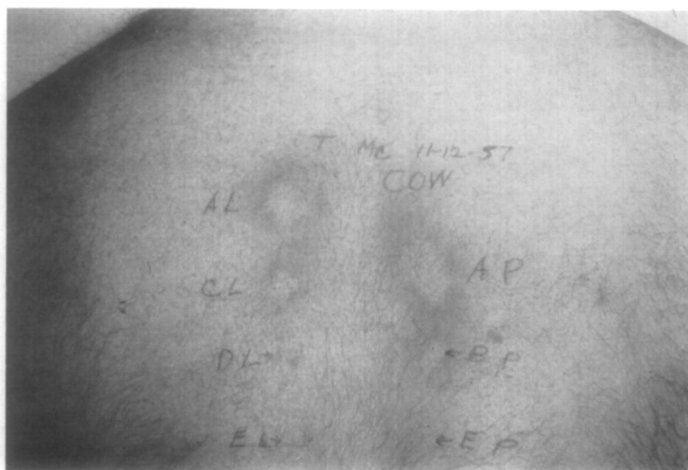


Fig. 4.—Cow epidermal. Patient T. McC., 56-year-old physician who raised registered beef cattle developed asthma each time he groomed cattle for stock show. Strongest reactions were to cow epidermal, and there were moderate reactions to hay, house dust, and several other epidermals.

Comparison shows strong positive scratches to samples "A.L." and "A.P." with moderate reactions to "C.L." and "B.P.," and no reaction to those from other sources.

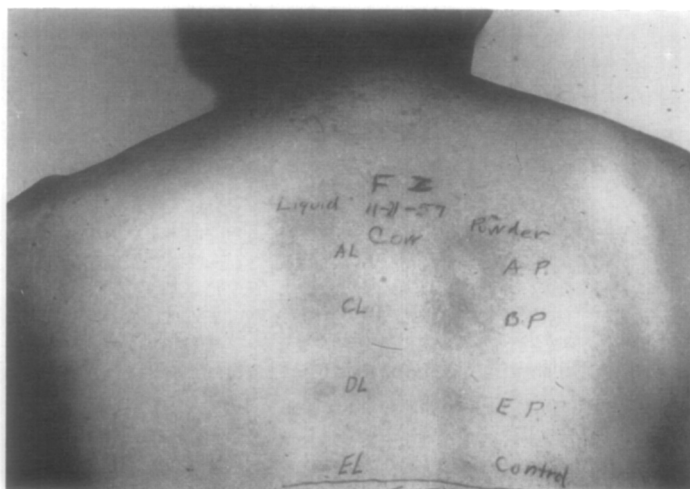


Fig. 5.—Cow epidermal. Patient F. Z., 47-year-old farmer. Asthmatic attacks are continual but most intense after contact with farm animals and hay. Strong positive skin reaction by scratch testing with epidermals and pollens.

Comparison shows sample "A.P." and liquid "A.L." producing strong whealing reactions. The powder "B.P." and liquid "C.L." from commercial sources reacted moderately. The remaining samples showed no reactions.

the preliminary step of fractional precipitation first with a "low concentration" of an organic liquid using two parts dioxine to three parts crude aqueous extract, thus removing the inert fraction. The active allergen is precipitated out with the "intermediate concentration." Further purification was done with ammonium sulfate. The high allergenic quality of house dust prepared by the method of Boatner and colleagues and sold as Endo house

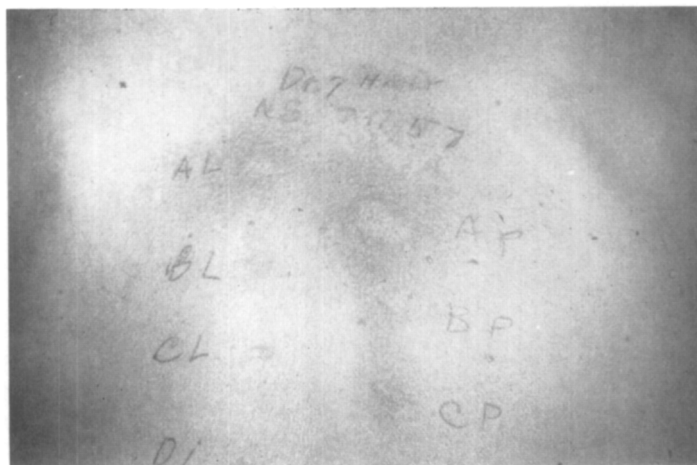


Fig. 6.—Dog epidermal. Patient N. S., 35-year-old paper maker. Perennial allergic rhinitis and mild asthma date back four years. He had no household pets at this time but showed, on testing, strong reactions to epidermals and house dust as well as to grain cereals.

Comparison, using materials "A.L." and "A.P.," showed consistently strong scratch reactions, while the others failed repeatedly to show any significantly positive reaction.

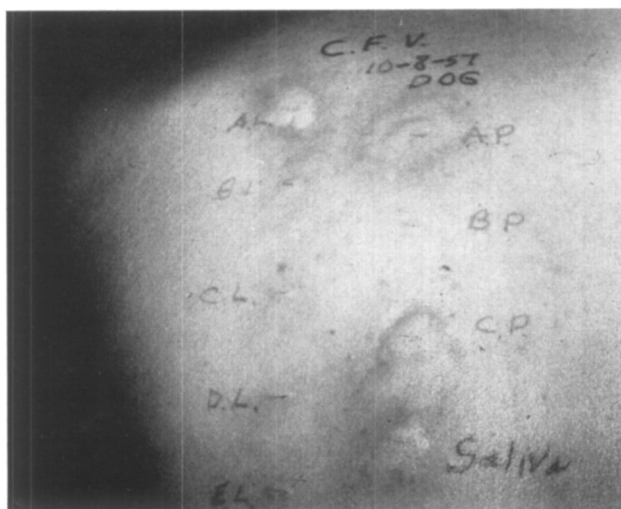


Fig. 7.—Dog epidermal. Patient C. F. V., 20-year-old construction worker. Recurrent eczema and minimal hay fever date back to childhood. Testing produced strong skin reactions to epidermals, pollens, and insect extracts.

Comparison showed reactions only with the liquid extract "A.L." and with the powdered allergens "A.P." and "C.P." and lyophilized saliva. The sample "C.P." was especially prepared by a commercial laboratory using the method of acetone precipitation (author's request). The other samples of dog epidermal failed to react.

dust has been well established, as evidenced by its wide use among many allergists. Their method of fractional precipitation is adaptable to house dust, since the crude material is available in limitless quantity and yields a relatively large amount of intrinsic allergenic material. Such epidermals as those

Fig. 8.

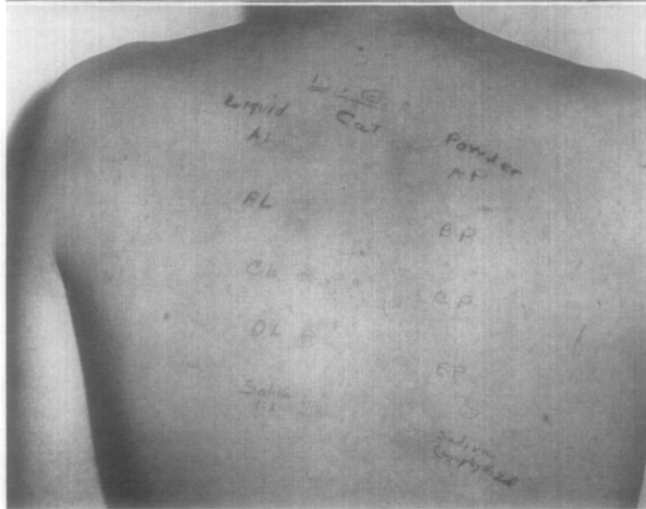
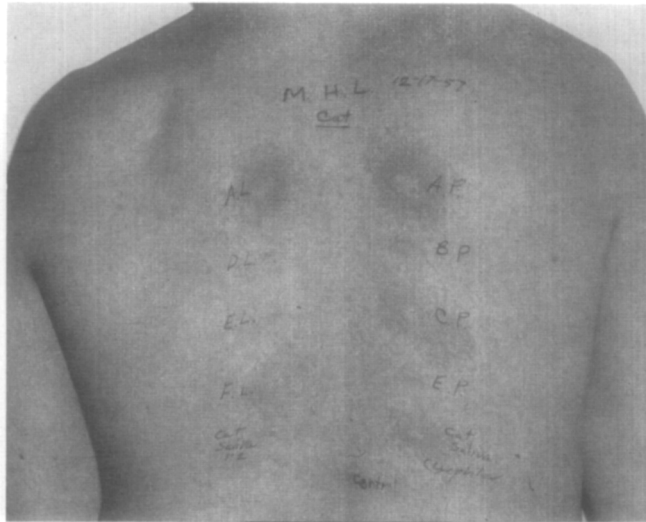


Fig. 9.

Fig. 8.—Cat epidermal. Patient M. H. L., 34-year-old mechanic. Allergic rhinitis and mild asthma dated back to childhood. Strongest skin reactions were obtained with epidermals and house dust.

Comparison showed that cat epidermal reaction was strongest to the prepared allergen "A.L." and "F.L." The powdered allergen "A.P." and "C.P." and lyophilized cat saliva also showed strong reactions. ("F.L." and "C.P." were from the same commercial source.)

Fig. 9.—Cat epidermal. Patient L. G., 29-year-old grocery clerk, developed asthma one year previous to being tested. He reacted strongly to cat epidermal and moderately to other inhalant allergens.

Comparison of concentrated cat epidermal showed the powder form "A.P." and "C.P." reacted strongly. Also, the powder prepared by simply lyophilizing cat saliva (obtained from an anesthetized animal stimulated with pilocarpine to promote salivation) gave a significantly positive skin reaction. The liquids "A.L." and "B.L." and the original cat saliva, simply diluted with equal parts of buffered solution, produced a strong reaction. All other materials applied failed to show any skin reactions.

of the cow, the dog, and the horse would also lend themselves to this method of fractional precipitation, since they too are available in large quantities and yield a high quantity of intrinsic allergens.

A few epidermals, on the other hand, especially those of the cat and the rabbit, provide with a single-step precipitation (as previously described in our own method) a small quantity of highly soluble, strongly allergenic powder which redissolves completely and almost instantaneously. The preliminary step with low-concentration acetone and later use of ammonium sulfate, as used in the preparation of Endo house dust, would not be necessary.

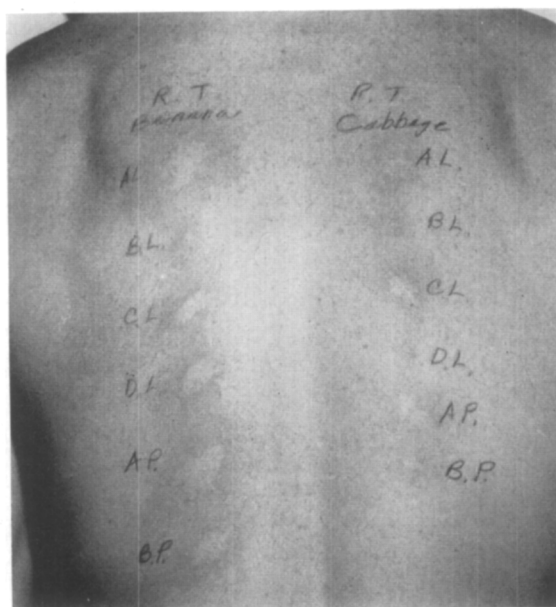


Fig. 10.—Food allergens. Patient R. T., 21-year-old student. Asthma and perennial allergic rhinitis date back to age 4. He was aware that several foods, especially banana, caused gastrointestinal symptoms. Skin testing revealed strong reactions to epidermals, pollens, and many foods.

Comparison of extracts of banana and of cabbage, from various sources, showed lack of allergenicity of several samples. With banana, only the liquid "B.L." failed to cause skin reaction. With cabbage, "B.L." (same commercial source) and "D.L." failed to elicit skin reactions.

It is evident that, for the sake of economy and simplicity, even the method of Boatner and associates for preparing a pure allergen by precipitating the active ingredient using a water-miscible organic liquid, such as acetone, must be adapted to the raw material used. The final allergenic extract will almost inevitably prove satisfactory for clinical use. The dry, powdered material, when proved allergenic, will also be found free from nonspecific irritating substance and can be stored indefinitely under desiccation. Redissolving this material in buffered 50 per cent glycerin, using a standardized method, should on each occasion yield a standard extract which is stable for many months. For those who employ biochemical analyses as presumptive evidence of strength of an allergen, the total-nitrogen and protein-nitrogen studies can be done to confirm the constant quality and stability of these allergens.

CONCLUSIONS

1. The allergenic extracts in common use, obtained in both powdered and liquid forms from various commercial sources, have frequently proved biologically inert, as evidenced by negative skin reactions in known sensitive patients.

2. The use of water-miscible organic liquid, particularly acetone, in the preparation of concentrated extracts of house dust as well as other allergens has proved highly satisfactory. If this method is applied on a larger commercial scale and modified for various allergens, it can provide a method that would not be costly and would not be too cumbersome. It could provide uniform material to all physicians and thus lead to a common language in our discussion of clinical allergy.

3. Since skin testing and desensitizing with specific allergens are important procedures in the practice of allergy, materials of known worth should be made available to those who will employ them.

4. The present method of standardization on the basis of chemical content requires skilled hands and, at best, it still fails to prove specific biologic activity of the allergen.

5. Although no method for standardizing allergens has been universally accepted, the physician should at least be given some assurance that the material has been biologically assayed in that it has strong whealing activity and contains no significant amount of nonspecific irritating substance.

6. The national societies of allergy, together with the regional and local allergy groups, might well accept the responsibility for assaying allergens biologically. In the formative period this may involve the need for making available allergens of known quality to be used in comparative studies with newly prepared material. The commercial laboratories engaged in the preparation of allergens should certainly welcome this. Those clinicians depending on allergens from these commercial sources will, as a result, feel more secure in what they are employing in their daily practice.

REFERENCES

1. Tuft, Louis: Critical Evaluation of Skin Tests in Allergy Diagnosis, *J. ALLERGY* 14: 355, 1943.
2. Boatner, C. H., Efron, B. C., and Dorfman, R. L.: Studies With Antigens. III. The Preparation of Purified Housedust Extract, *J. ALLERGY* 12: 176, 1941.
3. Strauss, M. B., and Spain, W. C.: The Preparation of Active Allergenic Extracts—a Method for the Prevention of Pigmentary Oxidative Products, *J. ALLERGY* 12: 61, 1941.
4. Perlman, Frank: Insects as Inhalant Allergens, *J. ALLERGY* 29: 303, 1958.
5. Hampton, S. F., Johnson, M. C., Frankel, Sam, and Schiele, A. W.: Preparation of Highly Concentrated Allergenic Extracts by Increasing the Concentration of Extraction. I. Timothy and Low Ragweed Pollens, *J. ALLERGY* 26: 16, 1955.
6. Boatner, C. H., and Efron, B. C.: Studies With Antigens. XII. Preparation and Purification of Concentrated Housedust Allergen, *J. Invest. Dermat.* 5: 7, 1942.