

Original Articles

QUANTITATIVE ESTIMATION OF THE ABSORPTION OF AN INGESTED ALLERGEN*

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INTRODUCTION

PREVIOUS papers from this laboratory have described the isolation^{1, 2} and chemical characterization³⁻⁶ of an allergenic polysaccharidic-protein fraction, CS-1A, from cottonseed, that was immunologically distinct from other allergens⁷⁻⁸ and antigens⁹⁻¹¹ present in the cottonseed. The object of this paper is to describe a method for the quantitative estimation of the quantities of allergen absorbed from the gastrointestinal tract when CS-1A and defatted cottonseed are ingested. By this method, reactions in passively sensitized sites provide means of comparing the quantities of allergen that enter the circulation as a result of intramuscular injection and of absorption from the gastrointestinal tract. The procedure involves two steps. First, the quantity of CS-1A, introduced intramuscularly, required to incite a positive reaction and also to neutralize the reagins in a site sensitized with serum from a cottonseed-sensitive subject is determined. Second, the quantity of ingested CS-1A or cottonseed that will likewise incite a positive reaction and also neutralize the reagins in a sensitized site is determined under the same experimental conditions. The quantity of allergen which was absorbed can then be estimated by comparing quantities of ingested CS-1A and cottonseed that induce effects at the sensitized sites equivalent to those produced by definite quantities of CS-1A when introduced intramuscularly. The method may be generally applied to allergens from other sources.

Freeman,¹² in 1925, first recorded the observation that ingested egg allergen passes from the digestive tract into the circulatory system. This was shown by excitation of a site, passively sensitized with serum from an egg-sensitive subject, when egg was ingested. Lewis and Grant,¹³ at Freeman's suggestion, showed that, upon ingestion of herring, an allergen likewise entered the circulatory system. M. Walzer,^{14, 15} working independently, recorded similar observation with both fish and egg allergens. He recognized that the quantity of allergen absorbed upon ingestion of a food was small, but he emphasized the importance to allergists and immunologists of the demonstration that mere traces of unaltered antigen or allergen may pass through the walls of the gastrointestinal tract. Walzer also pointed out that this method, which qualitatively demonstrated absorption, was not of value in determining the actual quantity of allergen absorbed. In 1935, A. Walzer and M. Walzer¹⁶ described a method for determination of the relative quantity of allergen in the circulatory system at intervals after the protein meal. But this method did not determine the

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actual quantity of allergen absorbed. Harten, Gray, Livingston, and Walzer¹⁷ stated that, according to unpublished experiments of Brunner and Baron, when 0.0001 mg. of nitrogen of an unfractionated extract of cottonseed was injected intravenously into man, positive reactions were thereby produced in sites passively sensitized with serum from a cottonseed-sensitive subject. Harten and his co-workers¹⁷ reported that, when this same serum was used to passively sensitize sites on monkeys, intravenous injection of 0.0002 mg. of nitrogen of an unfractionated extract of cottonseed produced positive reactions in the sites. M. Walzer¹⁸ subsequently discussed these and other studies pertaining to this subject.

Demonstration of the absorption of allergen from ingested cottonseed was first reported by Ratner and Gruehl.¹⁹ These authors, as well as M. Walzer,²⁰ adequately reviewed the historical aspects of the subject of the entry into the circulatory system of unaltered protein from the digestive tract, and therefore no further attention to them is needed here.

BLOOD SERUM

A sufficient quantity of blood serum was collected from a cottonseed-sensitive subject (G. W.) so that all passive transfer tests could be conducted with the same lot. Possible variation in reagin content of the serum was, therefore, limited to the gradual decrease in titer which might occur in storage at 2° to 5° C. during the two-month period of the experiments. The potency of the serum was tested by the method of Coca and Grove.²¹ Positive passive transfer reactions were obtained with this serum when used in a dilution of 1:10² but no reaction occurred with a dilution of 1:10.³

TEST SUBJECTS

Subjects were selected at random from the obstetric ward at Gallinger Hospital, Washington, D. C. They were tested with CS-1A to demonstrate lack of sensitivity to cottonseed allergen. The tests to be described were conducted just before or within a few days after parturition, an interval when the subjects could be retained under observation for the duration of the tests.

MATERIAL USED

CS-1A.—The procedure used in the isolation of CS-1A has been described in detail elsewhere.^{1, 2} CS-1A, which represented about 1 per cent of the defatted cottonseed, contained 11.8 per cent total nitrogen and 39.9 per cent polysaccharidic carbohydrate. A stock solution containing 100 mg. of CS-1A (ash-, water-free basis) in 100 ml. of physiologic salt solution was prepared. This solution was sterilized by heating at 100° C. for fifteen-minute periods on three successive days. Suitable dilutions of this solution, in sterile physiologic salt, were prepared to provide the required concentrations of CS-1A. For ingestion experiments, weighed quantities of CS-1A (ash-, water-free basis) were dissolved in 25 ml. of water.

Defatted Cottonseed.—The cottonseed was prepared by thorough ether extraction of "choice quality," dehulled cottonseed meats carefully hand-picked and ground. The extracted cottonseed was pulverized to a fine powder. Residual ether was removed by heating in an autoclave at 100° C. for fifteen minutes. For the ingestion experiments, weighed quantities of the cottonseed were stirred with enough warm water to form a thin suspension. •

DETERMINATION OF THE REACTION-INCITING AND REAGIN-NEUTRALIZING CAPACITIES
OF INTRAMUSCULARLY-INJECTED CS-1A

Determinations were made of the quantities of CS-1A required to induce positive reactions in passively sensitized sites and to desensitize the sites to further test with 100 γ^* of CS-1A.

By a uniform procedure, a single site on the right upper arm of each subject was sensitized with 0.05 ml. of undiluted serum. After an interval of at least twenty-four hours, 1 ml. of one of several dilutions of CS-1A was injected intramuscularly into the left arm. If the amount of CS-1A injected was sufficient to incite a reaction, itching at the sensitized site developed within a few minutes, and a wheal formed in ten to forty minutes. The maximum diameter of the wheal that developed within sixty minutes after the injection was used for measurement of the reaction.

After an interval of at least twenty-four hours, the sites were tested to determine neutralization of reagins. For this purpose 0.25 ml. of a solution containing 100 γ of CS-1A was injected subcutaneously into the right arm 30 to 40 mm. distal to the sensitized site. This technique for testing for neutralization of reagins, rather than injection of the CS-1A solution directly into the sites, was adopted because it eliminated need for a separate control test. Also, false plus-minus reactions owing to trauma of tissue not yet returned to normal were avoided by this indirect injection method. The use of one relatively large dose of CS-1A to test for neutralization was a permissible simplification because the objective of the experiments required only that comparative results be obtained. Therefore the multiple proportion effect²² in reagin neutralization could be neglected.

TABLE I. QUANTITY OF CS-1A, INTRODUCED INTRAMUSCULARLY, REQUIRED TO INDUCE POSITIVE PASSIVE TRANSFER REACTIONS AND NEUTRALIZE REAGINS

SUBJECT*	INITIAL TEST†		TEST FOR NEUTRALIZATION OF REAGINS‡ REACTION (MM.)
	QUANTITY OF CS-1A INJECTED (γ)	REACTION‡ (MM.)	
Z. C.	1	0	15 × 17
O. P.	1	0	15 × 17
E. R.	1	0	40 × 30
L. W.	1	0	16 × 20
A. B.	1	0	25 × 39
E. L.	10	8 × 8	15 × 15
C. M.	10	5 × 5	30 × 20
D. Wh.	10	0	20 × 20
E. B.	10	0	28 × 25
M. R.	100	10 × 10	0
M. B.	100	30 × 30	0
I. P.	100	22 × 22	0
L. S.	100	7 × 10	0
M. T.	100	18 × 15	0
N. B.	100	15 × 15	0
A. Br.	100	18 × 15	0
C. Mc.	100	32 × 32	0
D. Wi.	100	15 × 16	0
M. H.	1,000	14 × 14	0
B. H.	1,000	25 × 19	0
R. J.	1,000	20 × 18	0

*Subjects were uniformly sensitized on the right upper arm with 0.05 ml. of serum from cottonseed-sensitive patient (G.W.).

†The indicated quantity of CS-1A (ash-, water-free basis) in 1 ml. of sterile salt solution was injected intramuscularly in the left arm at least twenty-four hours after sensitization.

‡The numbers refer to the maximum size of the wheal (in millimeters) which formed within sixty minutes after injection of CS-1A.

§Test for neutralization of reagins was uniformly made by subcutaneous injection of 0.25 ml. of a sterile salt solution containing 100 γ of CS-1A about 30 to 40 mm. below the sensitive site. The numbers refer to the size of the wheal (in millimeters) which formed within sixty minutes. Test for neutralization was made at least twenty-four hours after the initial test.

*The symbol γ = 0.000001 gram. This symbol, designated "microgram," has been approved by the Committee on Nomenclature, Spelling and Pronunciation of the American Chemical Society (Chem. & Engin. News 18: 491, 1940).

Results of tests to determine the minimum quantities of CS-1A required to induce positive reactions and to neutralize the reagins at the passively sensitized sites are shown in Table I. One microgram of CS-1A was insufficient to produce positive reactions in any of five tests. Ten micrograms induced positive reactions in two out of four tests, but this quantity did not desensitize the sites. One hundred micrograms produced positive reactions in all nine tests and also neutralized every site. One thousand micrograms of CS-1A induced positive reactions in three tests and neutralized the sites in each case.

DETERMINATION OF THE REACTION-INCITING AND REAGIN-NEUTRALIZING CAPACITIES
OF INGESTED CS-1A

Determinations were made of the quantities of ingested CS-1A that would be required to incite positive reactions in passively sensitized sites and desensitize the sites.

Sites on the upper right arm of subjects were passively sensitized with 0.05 ml. of serum as before. After an interval of at least twenty-four hours a quantity of CS-1A dissolved in 25 ml. of water was administered orally before breakfast. The container was rinsed with an additional 25 ml. of water, and this rinse solution was also ingested. The maximum diameter of the wheals that formed within ninety minutes after the ingestion was used for measuring the reactions. The sites were tested for neutralization of reagins, following an interval of at least twenty-four hours after the ingestion, by a subcutaneous

TABLE II. QUANTITY OF INGESTED CS-1A REQUIRED TO INDUCE POSITIVE PASSIVE TRANSFER REACTIONS AND NEUTRALIZE REAGINS

SUBJECT*	INITIAL TEST†		TEST FOR NEUTRALIZATION OF REAGINS‡ REACTION (MM.)
	QUANTITY OF CS-1A INGESTED (MG.)	REACTION‡ (MM.)	
A. D.	50	0	—
G. C.	50	0	25 × 15
B. S.	100	0	22 × 15
G. B.	100	0	23 × 25
D. H.	100	0	22 × 22
D. M.	100	0	20 × 20
C. G.	100	15 × 18	± Redness
C. H.	100	±	16 × 15
E. W.	100	15 × 15	10 × 15
M. S.	200	Itching	24 × 22
L. M.	200	0	16 × 15
A. C.	200	30 × 25	—
I. K.	200	0	10 × 12
R. B.	200	0	18 × 15
R. M.	200	20 × 18	15 × 10
O. B.	200	25 × 18	17 × 20
O. W.	400	Heat, Redness	10 × 7
L. C.	400	25 × 22	0
M. T.	400	15 × 15	0
M. G.	400	11 × 15	0
E. A.	400	Redness	Redness
E. C.	800	20 × 25	0
E. N.	800	20 × 20	0
B. J.	800	20 × 22	0
W. S.	800	20 × 15	0
T. W.	800	15 × 13	9 × 7
V. B.	1,000	25 × 24	0

*Subjects were uniformly sensitized on the right upper arm with 0.05 ml. of serum from cottonseed-sensitive patient (G.W.).

†The indicated quantity (milligrams) of CS-1A (ash-, water-free basis) dissolved in 25 ml. of water was given orally before breakfast at least twenty-four hours after sensitization.

‡The numbers refer to the maximum size of the wheal (in millimeter) which formed within ninety minutes after ingestion of the CS-1A.

§Test for neutralization of reagins was made at least twenty-four hours after the ingestion of CS-1A.

injection of 0.25 ml. of a solution containing 100 γ of CS-1A by a method similar to that used in the intramuscular tests. Results of this series of tests are shown in Table II. These results, as would be expected, show more variability than those obtained by the intramuscular injection of CS-1A.

Fifty milligrams of ingested CS-1A induced no positive reactions in two tests. One hundred milligrams of ingested CS-1A produced two positive reactions out of seven tests, but in no case was the site desensitized. Two hundred milligrams of CS-1A caused four positive reactions out of seven tests, but again the sites were not neutralized. Four hundred milligrams of CS-1A produced reactions in all tests, and three out of the five sites were desensitized. When 800 mg. of CS-1A were ingested, positive reactions were obtained in all five tests and all sites but one were neutralized. In one test, ingestion of 1,000 mg. of CS-1A produced a reaction, and the site was desensitized. Additional ingestion tests, though desirable, were not conducted owing to the limited quantity of CS-1A available.

DETERMINATION OF THE REACTION-INCITING AND REAGIN-NEUTRALIZING CAPACITIES OF INGESTED COTTONSEED

The quantities of defatted cottonseed necessary to incite positive reactions in passively sensitized sites and to desensitize the sites were determined by the same procedure as employed in the CS-1A ingestion experiments. The powdered cottonseed was given orally in a warm, watery suspension before breakfast. The maximum diameter of the wheal that formed within ninety-minutes after the ingestion was used for measuring the reaction. The sites were tested for neutralization of reagins after an interval of at least twenty-four hours by a subcutaneous injection of 0.25 ml. of a solution containing 100 γ of CS-1A at a point 30 to 40 mm. distal to the sensitized site. Results of this series of tests are shown in Table III. One gram of ingested cottonseed induced a positive reaction in one out of three tests, but the sites were not neutralized. Dosage levels of 2, 5, 10, and 20 Gm. of ingested cottonseed caused positive reactions in every test. The sites were neutralized in all (four) tests, however, only at the 20-Gm. dosage level.

TABLE III. QUANTITY OF INGESTED COTTONSEED REQUIRED TO INDUCE POSITIVE PASSIVE TRANSFER REACTIONS AND NEUTRALIZE REAGINS

SUBJECT*	INITIAL TEST†		TEST FOR NEUTRALIZATION OF REAGINS‡ REACTION (MM.)
	QUANTITY OF COTTONSEED INGESTED (GM.)	REACTION‡ (MM.)	
J. B.	1	28 x 28	Redness, Heat
C. D.	1	0	20 x 16
E. B.	1	0	22 x 20
M. R.	2	20 x 15	25 x 20
R. W.	2	Heat	20 x 14
R. L.	5	20 x 20	28 x 22
O. H.	5	Redness	18 x 16
A. F.	5	11 x 14	24 x 20
C. S.	10	10 x 8	14 x 13
D. S.	10	Redness	20 x 20
H. W.	20	22 x 24	0
J. M.	20	18 x 18	0
E. E.	20	23 x 22	0
W. M.	20	28 x 30	0

*Subjects were uniformly sensitized on the right upper arm with 0.05 ml. of serum from cottonseed-sensitive patient (G.W.).

†The indicated quantity (grams) of defatted cottonseed, suspended in water, was given orally before breakfast at least twenty-four hours after sensitization.

‡The numbers refer to the maximum size of the wheal (in millimeters) which formed within ninety minutes after ingestion of CS-1A.

§Test for neutralization of reagins was made at least twenty-four hours after ingestion of the cottonseed.

DISCUSSION

The method described for the quantitative estimation of the absorption of allergen from ingested CS-1A and defatted cottonseed is based on the principle that reagins in passively sensitized sites are incited to wheal formation and are also neutralized by definite quantities of an allergen. This quantitative relationship is, however, reliable only if the conditions of the tests are rigidly standardized. In this study, effort has been made to reduce variable factors to a minimum consistent with practicable procedure. It is recognized that the results may be influenced by some variables that cannot be evaluated without further study. It has been assumed that, with regard to the total effect of the allergen on the sensitized sites, the intramuscular route of entry of allergen into the circulation is comparable with entry by absorption. The effect on reagin neutralization of differences in the concentration attained by the allergen in the circulation as a result of the more rapid intramuscular administration or as a result of variable rates of absorption in different subjects is not known. Rapid absorption may neutralize the sensitized site more effectively than gradual absorption, even though the total quantity of allergen absorbed in the latter case may exceed that in the former. Conversely, slow absorption may be more effective in reagin neutralization than rapid absorption. Also rapid absorption of an allergen may incite reaction in a sensitized site, whereas slow absorption of an equal or even greater quantity of allergen would not cause a reaction. However, these factors would not be expected to have the same effect in modifying the percentage of absorption as determined by reaction-inciting or reagin-neutralizing capacity. As shown later, the percentage of absorption as determined by both reaction-inciting and reagin-neutralizing capacities agree as closely as could be expected from available data. Therefore, these unknown factors probably do not have undue influence on the results.

Retention of the property of specific reaction of allergen with reagins in passively sensitized sites is generally regarded as evidence of the unaltered nature of the allergen.²⁰ The possibility is recognized, however, that exposure of ingested allergen to the action of digestive enzymes, even briefly, might cause slight alterations in structure of the absorbed allergen which could decrease without destroying the reagin-reacting properties of the allergen.^{4, 23}

In the procedure employed, the minimal quantities of allergen required to produce equivalent reactions in the sensitized sites are determined when the allergen is administered orally and parenterally. Comparison of the results affords a basis for measuring indirectly the quantity of allergen that is absorbed from measured quantities of ingesta. Two criteria were available for comparison of the effect of CS-1A on the sensitized site when introduced either intramuscularly or orally. These criteria were determinations of the minimum amount of CS-1A which incited reaction in the sensitized site and the quantity which neutralized the site. The results in Tables I and II show that 10 γ of CS-1A introduced intramuscularly were approximately equivalent to 100 mg. of CS-1A given orally for the incitation of reactions. The ratio of intramuscular to oral administration in the induction of reactions is therefore 1:10,000. On this basis, it may be deduced that 0.01 per cent of the allergen is absorbed when 100 mg. of CS-1A are ingested. The reagin-neutralizing power of 100 γ of CS-1A given intramuscularly was approximately equivalent to that of 800 mg. of CS-1A given orally. The ratio of reagin-neutralizing power of intramuscularly to orally administered CS-1A is therefore 1:8,000. According to this

method of determination, 0.013 per cent of the allergen is absorbed when 800 mg. of CS-1A are ingested. The calculations from the two criteria are in good agreement when the recognized variability of subjects and the comparatively small number of cases studies are considered. It is recognized that the percentage of absorption may vary with the size of the dose ingested. However, this point cannot be determined from available data.

An allergenic component of CS-1A is readily dialyzable.^{1, 11} This may explain why the CS-1A specific allergen of cottonseed passes through the walls of the gastrointestinal tract. It would be of interest to investigate the absorption of a nondialyzable allergen from cottonseed.⁸

The allergenic fraction CS-1A represents at least 1.1 per cent of the defatted cottonseed on the basis of actual isolation.^{1, 2} However, it is recognized that this is a minimal value because the objective for isolating CS-1A was to obtain the allergen immunologically distinct from other allergens in the cottonseed rather than to effect a quantitative separation of allergen. That CS-1A is not chemically homogeneous has been recorded previously, but the proportion of allergen present in either CS-1A or cottonseed, from which this fraction was isolated, has not been determined. In the present study, the allergic response of sites passively sensitized with serum from a cottonseed-sensitive subject to ingested CS-1A and defatted cottonseed provides a means for estimating the relative concentration of allergen in CS-1A and cottonseed. This comparison is made on the basis of determination of the minimal quantities of ingested CS-1A and cottonseed that will incite positive reactions in passively sensitized sites and that will neutralize the reagins in these sites. The following factors, which, however, cannot be evaluated by available data, must be considered in relation to this method of determining relative concentration of allergen in CS-1A and cottonseed. First, the CS-1A was administered orally in water *solution*, whereas the cottonseed was given orally as a watery *suspension*. The possibility is recognized that allergen in CS-1A might therefore be more readily available for diffusion from the gastrointestinal tract than allergen in the cottonseed suspension. Whether or not the availability of allergen for diffusion in the suspension of cottonseed is a critical factor cannot be determined from available data. Second, the sensitizing serum used in this study contained reagins specific both for CS-1A and another allergen, or other allergens, present in the seed. Although the nature of the unidentified allergen or allergens has been studied previously,^{1, 8} as yet insufficient facts are available to decide whether or not these unidentified allergens contributed to the initial incitation of the reactions under the conditions of this experiment. The possibility is therefore recognized that the positive passive transfer reactions obtained with the cottonseed might result from a summation of reactions induced by CS-1A and the other allergens. This difficulty could be avoided by using serum from a Type II cottonseed-sensitive subject; that is, one sensitive to CS-1A alone.^{1, 7, 8} However, this would have necessitated redetermination of the data for CS-1A in Table II. This has not been possible under present circumstances.

The capacity of ingested cottonseed to neutralize reagins specific for CS-1A, however, is comparable with neutralizing power of CS-1A because CS-1A is immunologically distinct from the unidentified allergens of the cottonseed. Results in Tables II and III show that the CS-1A-reagin-neutralizing power of 800 mg. of ingested CS-1A was approximately equivalent to that of 20 Gm. of ingested cottonseed. Therefore, it can be estimated that CS-1A contains a concentration of this allergen approximately twenty-five times as great as that in defatted cottonseed.

SUMMARY

1. A method is described for the quantitative estimation of the approximate quantities of cottonseed allergen absorbed from the digestive tract when CS-1A and cottonseed are ingested. The method may be generally applied to other allergens and has potential applicability in fundamental studies of the diffusibility of allergens from the gastrointestinal tract.

2. Ten micrograms of CS-1A introduced intramuscularly were approximately equivalent to 100,000 γ of ingested CS-1A in capacity to incite passive-transfer reactions. On this basis, when 100 mg. of CS-1A were ingested, 0.01 per cent of the allergenic fraction was absorbed.

3. One hundred micrograms of CS-1A introduced intramuscularly were approximately equivalent to 800,000 γ of ingested CS-1A in reagin-neutralizing power. According to this criterion, when 800 mg. of CS-1A were ingested, 0.013 per cent of the allergenic fraction was absorbed.

4. One hundred micrograms of CS-1A introduced intramuscularly were approximately equivalent to 20,000,000 γ of ingested cottonseed in reagin-neutralizing capacity.

5. Eight-tenths of a gram of ingested CS-1A was approximately equivalent to 20 Gm. of ingested cottonseed in reagin-neutralizing power.

6. The method described may be applicable to the determination of the relative concentration of an allergen in two substances by comparing the quantities of the ingested substances required to induce equivalent effects in passively sensitized sites. By this method it was estimated that CS-1A contains a concentration of allergen approximately twenty-five times as great as defatted cottonseed.

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