

Heat studies with human IgE: A simple method for rapidly distinguishing sera with high total IgE levels

B. A. BALDO, I. D. WELLS *and* J. PEPYS

Cardiothoracic Institute, University of London, Fulham Road, London SW3 6HP

Summary

Human sera, covering a wide range of total IgE concentrations, were subjected to mild heat treatment for varying periods of time and then examined for their abilities to bind to an Fc-specific, radioactively labelled rabbit anti-human IgE serum. As a result of the heat treatment, aggregation of the serum IgE occurred and the aggregated material reacted significantly with the labelled antiserum. Experiments with a standard human IgE serum and over 100 different human serum samples from normal and allergic patients showed that this simple heat method can distinguish high, medium and low levels of IgE in serum. Provided that sufficient examinations are undertaken, the method offers a simple means of rapidly examining large numbers of serum samples and distinguishing those sera with elevated levels of IgE.

Differences observed between individual serum samples may indicate physico-chemical differences in the IgE molecules present in the sera. Such differences may be a further reflection of the heterogeneity of human IgE. The stability of human skin-sensitizing antibodies is briefly reviewed.

Introduction

There is now substantial evidence that antibodies to many allergens belong to the IgE immunoglobulin class (Ishizaka & Ishizaka, 1966, 1967; Ishizaka, Ishizaka & Hornbrook, 1966; Johansson & Bennich, 1967; Stanworth *et al.*, 1967). This finding has led to the desire to examine more closely the concentration of total and allergen-specific IgE in human sera, but this has not always been an easy task since the levels of IgE in normal sera and in the sera of most individuals suffering from allergic diseases are extremely low. In healthy children and adults the levels of total IgE in serum are rarely above 1 $\mu\text{g/ml}$ (Johansson, 1968; Johansson, Bennich & Wide, 1968; Berg & Johansson, 1969; Bennich & Johansson, 1971). The highest levels of serum IgE have been detected in individuals infected with certain parasites (Johansson, Mellbin & Vahlquist, 1968; Hogarth-Scott, Johansson & Bennich, 1969; Ezeoke, Perera & Hobbs, 1973) but, even in such cases, the IgE serum levels rarely reach 1% of the total IgG

Correspondence: Dr B. A. Baldo, Clinical Immunology Unit, Children's Medical Research Foundation, Princess Margaret Hospital, Perth, Western Australia 6008, Australia.

levels found in healthy human adult serum (Bennich & Johansson, 1971). In all but a few cases therefore (Coombs *et al.*, 1968; Heiner & Rose, 1970; Levin, Altman & Sela, 1970; Hoffman, 1973), the procedures used to quantitate serum total and specific IgE have been immunological methods using radioactive iodine (^{125}I or ^{131}I)-labelled IgE or anti-IgE (Wide, Bennich & Johansson, 1967; Johansson *et al.*, 1968; Rowe, 1969; Salmon, 1970; Arbesman *et al.*, 1972; Gleich, Averbek & Svedlund, 1971; McLaughlin *et al.*, 1971; Smith, Ozkaragoz & Gokcen, 1972).

While using the radioallergosorbent test (RAST) with patients' sera and specific allergens and, bearing in mind the known tendency of IgE to aggregate (Stanworth, 1963; Ishizaka & Ishizaka, 1968; Ishizaka *et al.*, 1970), we became aware that the RAST procedures might be modified to examine the levels of total IgE in human sera. Although the lability of reagins has been frequently emphasized, it is now apparent that heating human sera containing IgE to 56°C does not completely disrupt the structure of the IgE molecule (Ishizaka, Ishizaka & Menzel, 1967). Using serum from a goat immunized with the Fc fragment of human IgE, Ito, Wicher & Arbesman (1971) demonstrated the presence of antibodies which reacted with heat aggregated human IgE. This paper presents a preliminary account of the heat studies carried out with human sera covering a wide range of total IgE concentrations and the subsequent reactions of these sera with a ^{125}I -labelled Fc-specific anti-IgE.

Materials and methods

Human sera

Blood obtained from patients attending the routine allergy clinics at the Brompton Hospital, London and samples of cord blood from the Princess Beatrice Hospital, London were centrifuged within 4 hr of collection and the sera stored at -20°C . Some samples initially stored at -20°C , were later thawed and kept for periods of from 1 to 20 weeks at 4°C in the presence of 0.05% sodium azide. One hundred and twelve serum samples covering the range 50–16,000 u/ml total IgE were selected for examination.

IgE reference serum

Medical Research Council Research Standard A 68/341 for human serum immunoglobulin E consisting of vials containing pooled, lyophilized human serum were obtained from the Medical Research Council, Division of Biological Standards, National Institute of Medical Research, Holly Hill, London. After reconstitution with distilled water, solutions containing 10,000 u/ml were obtained (Rowe *et al.*, 1970). For the studies described in this paper, one WHO reference standard unit (u) was taken to be equal to 2.42 ng of IgE (Bazaraal & Hamburger, 1972).

Anti-IgE

Purified antibody, specific for the Fc portion of human IgE was a gift from Dr L. Wide, Kliniskt-Kemiska Centrallaboratoriet, Akademiska Sjukhuset, Uppsala, Sweden. The antiserum was prepared by injecting rabbits with IgE myeloma protein ND (Johansson & Bennich, 1967; Wide, Bennich & Johansson, 1967).

Labelled anti-IgE

IgE-specific rabbit antibody (10 μl) was labelled with ^{125}I to a specific activity esti-

mated to be 50–100 $\mu\text{C}/\mu\text{g}$ using the McCahey & Dixon (1966) modification of the Greenwood, Hunter & Glover (1963) method. The ^{125}I used was purchased from Amersham, Buckinghamshire, England. Chloramine T and sodium metabisulphite were both purchased from Hopkins and Williams Ltd, Essex, England. Labelled protein was separated from free radioactive iodine by passage through a 1.3×38 cm column of Sephadex G-25 (Pharmacia). Prior to the addition of the labelled materials, phosphate buffered saline pH 7.3 (PBS) containing Sigma human serum albumin fr. V (HSA) 0.1% was passed through the column. The labelled antibody was stored in PBS containing 0.5% HSA and 0.05% sodium azide. Labelled antibody was stored for no longer than 10 weeks.

Estimation of total IgE levels in sera

The concentrations of IgE in human sera were measured by the radioactive single-radial-diffusion technique of Rowe (1969) using sheep anti-human IgE and ^{131}I -labelled rabbit anti-sheep IgG.

Serum complement

Lyophilized guinea-pig serum (lot number K 4935), for use as a source of complement was obtained from Wellcome Research Laboratories, Beckenham, England.

Heating experiments with human sera

0.05 ml volumes of each of the sera to be examined were added to 3 ml clear polystyrene LP/3 test tubes, obtained from Luckham, Burgess Hill, Sussex. Each serum sample was examined at least in duplicate. Tubes were closed with Luckham polythene press-on LP/3S stoppers and placed in a Grant Instruments (Cambridge, England) type SB2 water bath and maintained at 56°C with an accuracy of $\pm 0.2^\circ\text{C}$ for periods of from 30 min to 24 hr. After heating for the desired time, tubes were removed from the bath, brought to room temperature and 0.5 ml of PBS added to each tube. Tubes were then capped and rotated for from 1 to 12 hr before washing three times with $2\frac{1}{2}$ ml volumes of 0.1 M tris-buffered saline pH 7.3 containing 0.5% Tween 20. This wash buffer will subsequently be referred to as tris-buffered saline. Washing was carried out by adding the tris-buffered saline to each tube with the aid of a model AB Filamatic Vial Filler (National Instruments Co., Inc., Baltimore). Tubes were centrifuged at 3000 rev/min and then aspirated using an attachment on the suction tube which reached a fixed distance almost to the bottom of the tubes. This procedure left 0.2 ml of solution in the tubes after each aspiration. After the third wash, 0.1 ml of ^{125}I -anti-IgE diluted to contain 60,000–140,000 ct/min, was added to each tube. Tubes were rotated at room temperature for 12 hr and washed 5 times with $2\frac{1}{2}$ ml volumes of tris-buffered saline. After the final centrifugation, the radioactivity remaining was measured by counting the tubes in a Packard model 3001 Auto-gamma Spectrometer.

Results

In the initial series of experiments, sixty human serum samples comprising fifty adult and ten cord samples were studied. Duplicate samples (0.05 ml) were heated at 56°C for 30 min and then tested for their reaction with ^{125}I -labelled anti-human IgE using the procedure described under Materials and Methods. Labelled antiserum was also added to unheated samples of the same sera and the results compared. When freshly thawed, unheated sera were examined, no significant differences were usually noticed

between the counts for the tubes which contained the cord and the adult samples. Three different adult samples, however, produced counts which were from $1\frac{1}{2}$ to 2 times those obtained with cord sera. With the heated samples, differences in the amount of radioactivity bound by some of the adult and cord samples were much more pronounced. Each of the cord samples showed approximately the same counts as the corresponding unheated sample but twenty-four of the fifty adult samples gave significantly increased counts when compared to the corresponding unheated sample and

Table 1. Reaction of unheated and heated human sera with ^{125}I -labelled anti-human IgE

Serum	Total IgE concentration* (i.u./ml)	Counts/30 sec					
		Unheated	Unheated + EDTA	Heated 56°C, 30 min	Heated 56°C, 3 hr	Heated + guinea-pig complement†	Heated‡ + anti-IgE
Air	1000	1278	1384	2566	3995	2769	1472
God	4000	1403	1553	3339	5254	3172	1824
Cal	200	1115	1197	1285	2115	1478	1366
Kno	150	820	844	817	854	848	976
Sug	5800	1197	1227	3451	5792	4451	1488
Jon	5000	1498	1437	2873	5169	3135	1412
Pli	4800	1331	1432	3164	4470	4067	1820
Cap	4500	1395	1455	2982	4612	3467	1395
Tur	340	1365	1326	1677	2437	1758	1703
Alb	1000	1355	1392	2030	3094	1567	1573
Bry	1000	1571	1555	2793	4103	2547	1654
Wad	1000	1508	1166	1846	3147	2035	1639
Spe	200	1418	1231	1678	1668	1286	1596
Mal	150	725	825	744	723	740	756
Hen	8300	1735	1663	6334	8445	5952	2130
Cord 1	< 100	686	881	563	834	691	826
Cord 2	< 100	825	947	712	713	792	858
Cord 3	< 100	631	643	847	753	598	986
Cord 4	< 100	840	613	646	776	693	687
Cord 5	< 100	564	549	619	715	602	818

* Determined by the method of Rowe (1969). See Materials and Methods.

† Heated at 56°C for 30 min before addition of complement.

‡ Heated at 56°C for 30 min, washed and pre-incubated with unlabelled anti-IgE before addition of ^{125}I -labelled anti-IgE.

these counts were from 2 to 12 times higher than the mean of the cord counts. The remaining twenty-six adult, heated samples gave counts which were either the same or slightly higher than the corresponding unheated samples.

After the above preliminary experiments, sodium azide (0.05%) was added to each of the sera and the samples were then stored at 4°C. Three weeks later the sera were again studied in a series of examinations with ^{125}I -labelled anti-IgE. The results of a typical experiment with twenty sera are set out in Table 1. Each of the examinations detailed in Table 1 were carried out concurrently using the same batch of labelled anti-IgE.

Examination of heated and unheated serum samples

Unheated adult serum samples produced counts which were significantly higher than the counts recorded for the cord sera and, again, heating at 56°C for 30 min caused an increase in the amount of label remaining in the tubes which contained the adult samples (Table 1). When the heating time at 56°C was increased to 3 hr, even higher counts were obtained (Table 1).

To determine whether or not the labelled material remaining in the tubes was bound to the inside surfaces, the tubes were washed out with tris-buffered saline and the washings retained. When the tubes and the corresponding washings were counted, the greater proportion of the radioactivity was found in the washings (Table 2). Repeated washings reduced the counts in the tubes still further demonstrating that the label was attached to insoluble material and not to the inside surfaces of the tubes.

Table 2. Examination of tubes and tube-washings after reaction of ^{125}I -anti-IgE with heated sera*

Serum	Counts/30 sec	
	In tubes	In first wash†
Kno	817	611
Sug	3451	2867
Ion	2873	2105
Cap	2982	2098
Tur	1677	1208
Cord 1	563	393
Cord 2	712	519

* Heated at 56°C for 30 min (see also Table 1).

† After heating, tubes treated as described in Materials and Methods, then washed out with 2 ml buffered saline. Washings retained and counted.

Investigation of the possible involvement of complement in the heated serum- ^{125}I -labelled anti-IgE reaction

Heating human serum at 56°C for 30 min inactivates complement. In order to check if complement inactivation was somehow enhancing the binding of ^{125}I -anti-IgE to heated and washed human serum components, experiments were carried out using heated and unheated sera, EDTA and guinea-pig complement. To 50 μl of the twenty unheated adult and cord sera being examined was added 50 μl of a 0.02 M solution of EDTA, in PBS pH 7.3. To 50 μl samples of heated (56°C for 30 min) sera from the same individuals was added 50 μl of guinea-pig serum freshly reconstituted from the lyophilized state. The tubes containing the unheated sera and EDTA and those containing the heated sera and guinea-pig complement were then compared with unheated and heated (56°C for 30 min) sera to which neither EDTA nor guinea-pig complement had been added. Table 1 shows that after the addition of labelled anti-IgE and the subsequent washing, no marked differences were observed between the counts recorded for the tubes which contained unheated serum or unheated serum with EDTA. Likewise,

no significant differences were seen between the tubes which contained heated serum or heated serum plus guinea-pig complement.

Effect of prior incubation with unlabelled anti-IgE

In order to examine the specificity of the reaction between heated, washed human serum and ^{125}I -labelled anti-IgE, heated samples were preincubated with unlabelled anti-IgE before the addition of the labelled anti-IgE. Sera ($50\text{ }\mu\text{l}$ /tube) were heated at 56°C for 30 min, cooled, washed with PBS and tris-buffered saline and to each tube was added $50\text{ }\mu\text{l}$ of a 1 in 5 dilution of unlabelled rabbit anti-human IgE. Tubes were rotated at room temperature for 12 hr, washed three times with tris-buffered saline and labelled

Table 3. Effect of washing on the reaction between heated human sera and ^{125}I -labelled anti-human IgE

Serum	Counts/30 sec	
	Washed* with PBS before addition of ^{125}I -anti-IgE $_{\dagger}$	Washed $_{\dagger}$ with PBS and ^{125}I -anti-IgE $_{\dagger}$
213G	1267	224
216G	1128	205
247G	1060	609
280G	2578	200
321G	831	194
325G	1958	195
329G	1359	286
334G	1734	230
335G	1328	203
428G	2089	269
429G	4776	396
Cord serum	659	190

* Tubes rotated in presence of PBS for 12 hr, washed with tris-buffered saline, ^{125}I -anti-IgE added, rotated for 12 hr, washed and counted.

$_{\dagger}$ Tubes rotated in presence of PBS and ^{125}I -anti-IgE for 12 hr before washing and counting.

$_{\ddagger}$ 65,000 counts/30 sec added to each tube.

anti-IgE added. After a further 12 hr rotation at room temperature each tube was washed five times with buffered saline and counted. Table 1 shows that preincubation with unlabelled anti-IgE significantly inhibited the binding of ^{125}I -anti-IgE to nine of the adult heated serum samples. Each of the cord and six of the adult samples, however, showed no significant change from the counts obtained when the corresponding unheated sera were not pretreated with unlabelled anti-IgE.

Effect of washing before and after addition of labelled anti-IgE

To determine whether or not the initial wash with PBS was necessary for successful binding of anti-IgE to heated sera, labelled anti-IgE was added to some heated serum samples together with the PBS. Tubes were rotated for 12 hr, washed five times with tris-buffered saline and the counts compared with tubes treated in the normal way—i.e., sera heated and washed with PBS and tris-buffered saline before labelled anti-IgE

was added. The results of one such experiment are shown in Table 3. It is clear that washing the heated sera prior to addition of the IgE is necessary if the antiserum is to bind successfully to heated serum components.

In other experiments, heated serum samples were rotated with PBS for $\frac{1}{2}$, 1, 2, 4, 8 and 12 hr before addition of the label. These studies showed that similar counts were obtained in all cases and, for this reason, 1 hr rotation times with PBS were used in all subsequent experiments.

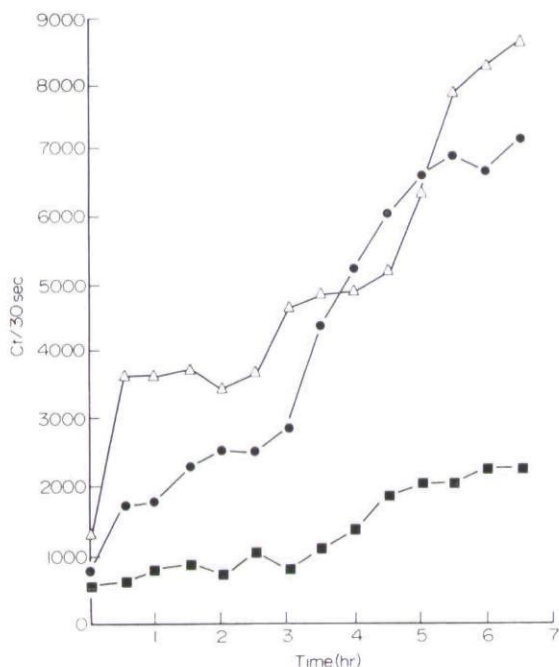


Fig. 1. Reaction of some heated human sera with ^{125}I -labelled rabbit anti-human IgE. Two samples of adult sera containing total IgE concentrations of (Δ) 12,100 i.u./ml and ($\bullet$) 6400 i.u./ml and one sample of ($\blacksquare$) cord serum containing < 100 i.u./ml, were heated in uncapped plastic tubes at 56°C for periods of from 30 min to $6\frac{1}{2}$ hr. Insoluble material left in the tubes after heating was then examined for reactivity with the labelled anti-human IgE using the procedure described in Materials and Methods.

Effect of prolonged heating on the reaction between human sera and labelled anti-IgE

In the first experiment, human adult sera known to contain high levels of IgE (> 5000 u/ml) and one sample of human cord serum (< 100 u/ml) were heated in uncapped tubes at 56°C for periods of up to $6\frac{1}{2}$ hr. At 30 min intervals, tubes containing $50\ \mu\text{l}$ of each sample were removed from the water bath and placed at 4°C . After the last tube had been removed from the bath, tubes were treated as described in Materials and Methods and then counted. Figure 1 shows that the amount of label taken up by the two adult sera rose rapidly at 30 min, increased gradually up to 3 hr and then increased sharply up to $6\frac{1}{2}$ hr. By contrast, the uptake of label by the cord serum was small until 3 hr after heating commenced. Uptake then increased sharply up to $4\frac{1}{2}$ hr and then tapered off between $4\frac{1}{2}$ and $6\frac{1}{2}$ hr.

Some sera were heated in capped tubes for periods of up to 24 hr. Figure 2 shows the results of heating three adult sera each containing high levels of total IgE, and one cord serum, at 56°C for 1, 3, 5, 6, 15 and 24 hr. This and subsequent experiments showed that heating can distinguish those sera containing high, medium and low levels of total IgE and that maximum binding of anti-IgE occurred when sera had been heated for from 12 to 18 hr. For most of the sera examined, maximum binding of label occurred 15 hr after heating commenced. For sera of high IgE content, after the peak binding activity was reached at around 15 hr, heating beyond this point produced marked reductions in the amount of ^{125}I -anti-IgE bound (Fig. 2). This effect was less noticeable when sera of low total IgE content were examined. Some cord sera showed maximum binding of anti-IgE 20–24 hr after heating commenced (Fig. 2).

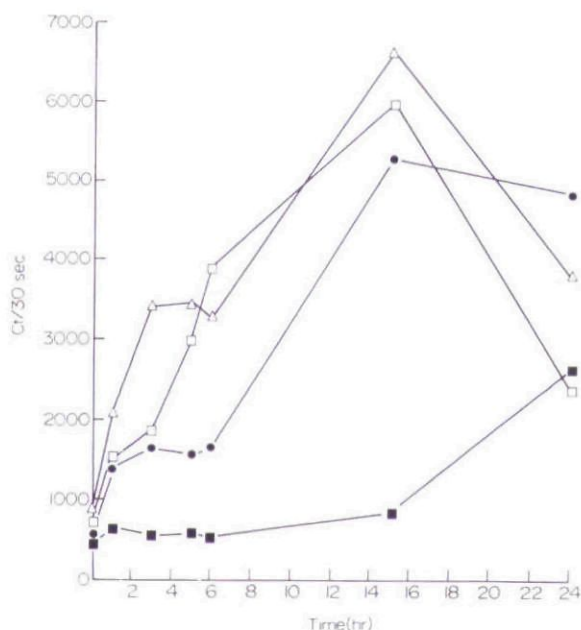


Fig. 2. Reaction of some heated human sera with ^{125}I -labelled rabbit anti-human IgE. Three samples of adult sera containing total IgE concentrations of (Δ) 12,100 i.u./ml, (◻) 10,000 i.u./ml and (●) 6,400 i.u./ml and a sample of (■) cord serum containing < 100 i.u./ml total IgE, were heated at 56°C for periods of from 1 to 24 hr. Insoluble material left in the tubes after heating was then examined for reactivity with the labelled anti-human IgE using the procedures described in Materials and Methods.

Effect of heating at different temperatures on the reaction between human sera and labelled anti-IgE

Seven different sera, covering a wide range of total IgE concentrations, were pipetted into plastic tubes and held at 4, 21, 37, 45, 50, 56, 60 and 65°C for 4 hr. After rotation with PBS, washing with tris-buffered saline and addition of ^{125}I -anti-IgE (70,000 ct/30 sec/tube), tubes were incubated for 12 hr, washed five times and the amount of radioactivity remaining measured. Figure 3 shows that no significant changes in the amount of ^{125}I -anti-IgE bound occurred with any of the sera up to a temperature of

45°C. After 4 hr at 50°C all of the sera except the mixed cord and Cal samples showed a marked increase in the amount of label taken up. Between 50 and 60°C the amount of anti-IgE bound by sera Pri, Phi, 250G, 280G and 429G continued to increase but serum Cal showed only a slight increase in counts and the cord serum was unchanged. At 65°C the amount of radioactivity bound by the cord and Cal sera rose sharply and sera Pri, Phi and 250G each reached their highest points at this temperature. By contrast, the maximum amount of label bound by sera 280G and 429G reached a maximum at 60°C and marked reductions occurred at 65°C (Fig. 3).

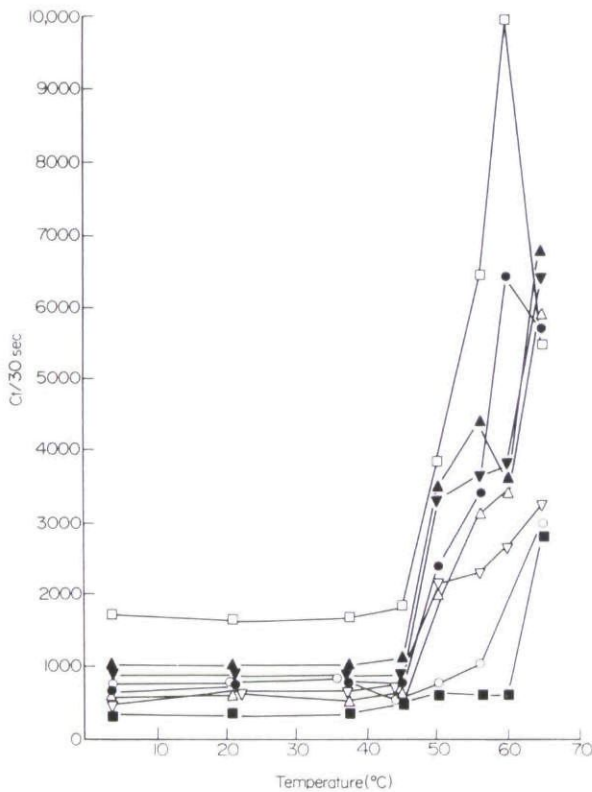


Fig. 3. Reaction of some human sera with ^{125}I -labelled rabbit antihuman IgE after sera had been heated for 30 min at different temperatures in the range 4–65°C. Total IgE concentrations of sera examined: (□) 429G 16,800 i.u./ml, (▲) 826F 9850 i.u./ml, (▼) 250G 8200 i.u./ml, (●) 280G 7600 i.u./ml, (△) Phi 6400 i.u./ml, (▽) Pri 1050 i.u./ml, (○) Cal 200 i.u./ml, (■) cord serum < 100 i.u./ml. After 30 min at the desired temperature, insoluble material left in the tubes was examined for reactivity with labelled antihuman IgE using the procedure described in Materials and Methods.

Attempt at estimating total IgE levels in human sera

Using a WHO IgE serum standard 68/341 and human sera covering a wide range of total IgE concentrations, heating experiments were carried out to try to establish whether or not the heating procedure described above could be used as a simple method for estimating the total IgE levels in different human serum samples. In a typical experiment, serum 68/341 was used full-strength and in a series of dilutions

with cord serum down to a dilution of 1–128. These were each pipetted into 6 tubes (50 μ l/tube) and the tubes were capped and heated at 56°C for 6 hr. Samples of undiluted adult and cord sera which had been examined for total IgE content by the Rowe (1969) method were treated similarly. After cooling, all tubes were treated as already described. The counts obtained for the tubes containing serum 68/341 were used to construct a standard curve and from this curve (Fig. 4) the total IgE content of the other sera being examined was estimated. Although Lang-Levy pipettes were used to accurately measure the 50 μ l quantities of standard sera, variations of up to 20% were sometimes seen in the counts recorded for the various dilutions of standard serum. This scatter of the readings is shown in Fig. 4. The effect of the presence of Tween 20 and protein on the reproducibility of the experiment was examined by washing with tris-buffered saline with or without Tween 20 (0.5%) and HSA (0.5%). No marked

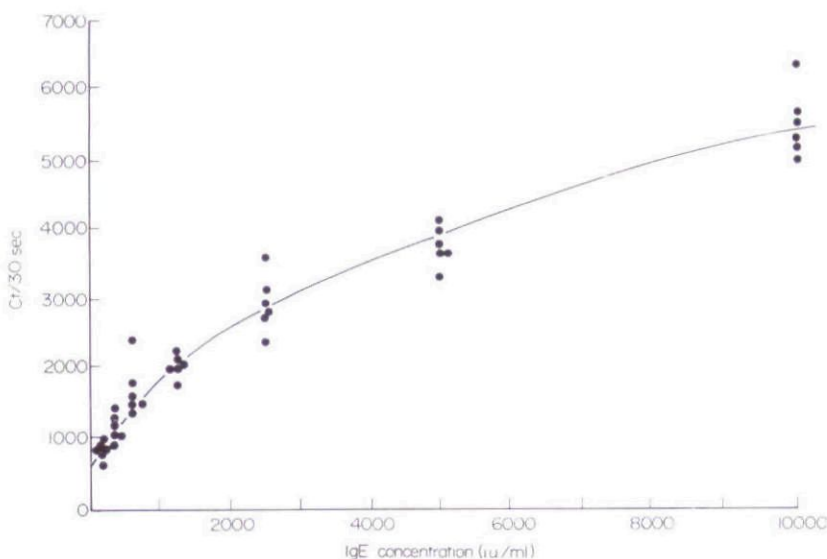


Fig. 4. Reaction of heated human WHO standard serum 68/341 with ^{125} I-labelled rabbit anti-human IgE. Undiluted serum 68/341 containing 10,000 i.u./ml of IgE and serial two-fold dilutions of this serum, were heated at 56°C for 6 hr and examined for reactivity with anti-human IgE as described in Materials and Methods.

improvements in reproducibility were seen with the tubes which had been washed with buffer containing Tween, buffer containing HSA or buffer without Tween and HSA.

A comparison of individual total IgE values for forty-two different sera, by the above method and the Rowe (1969) method, did not reveal a very close correlation. In general, a broad correlation was found with the sera which contained high (for example, >1500 u/ml), medium and low (<200 u/ml) IgE levels but, within these three broad groups, values obtained by the two different methods for individual serum samples often did not show close agreement. The results of one such study are shown in Table 4.

Table 4. Total IgE levels in human sera determined by radioactive single-radial-diffusion and by reaction of heated sera with ^{125}I -labelled anti-human IgE

Serum	IgE (u/ml) estimated by single-radial-diffusion*	IgE (u/ml) estimated after heating and reaction with ^{125}I -anti-IgE†
Cord 1	< 100	< 100
Cord 2	< 100	< 100
Cord 3	< 100	< 100
Cord 4	< 100	< 100
Cord 5	< 100	< 100
Bow	< 100	< 100
Nix	< 100	< 100
Sta	< 100	< 100
Teb	< 100	150
Bax	100	< 100
Cla	125	< 100
Ram	145	500
Mal	150	< 100
Ohn	170	< 100
Muz	175	950
Cal	200	150
Pop	200	100
Row	200	400
Spe	200	100
Woo	265	150
Chi	270	1550
Bee	320	1200
Tuc	340	< 100
Tur	340	100
Dia	490	150
Win	500	100
Bir	525	400
Tab	620	1200
Arg	680	450
Hol	720	600
Ben	820	1550
Air	1000	900
Bry	1000	1150
Wae	1080	550
Ger	1140	2700
Sap	1150	2000
Whi	1150	1000
Wad	1770	750
Bra	2500	4800
Cap	4500	2900
Phi	6400	7400
429G	16,800	> 10,000

* Method of Rowe (1969).

† Sera heated for 2 hr in capped tubes, then treated as described in Materials and Methods.

Discussion

Although the heat lability of reagins has been known for many years (Coca & Grove, 1925) and frequently emphasized (Loveless, 1940; Stanworth, 1963; Layton *et al.*, 1963; Ishizaka, Ishizaka & Menzel, 1967; Reid, 1970), it is now clear that human IgE is relatively stable under various mild conditions of storage and processing. Loveless (1940) found that heating for 2–5 hr at 56°C or for 30–60 min at 60°C was necessary to destroy the skin-sensitizing activity of sera from ragweed-sensitive patients. Using quantitative P-K tests based on the accurate measurement of weal sizes, Stanworth & Kuhns (1965) showed that when serum from a horse dandruff-sensitive individual was heated at 56°C for 30 min, 75–90% of the skin-sensitizing activity of the serum was destroyed. Heating at 56°C for a further 30 min completely eliminated the residual activity. Although freezing or lyophilization of whole human reaginic serum appears to have no effect on skin-sensitizing potency (Harter, 1961; Stanworth, 1963; Perlman & Layton, 1967; Feinberg, Feinberg & Lee, 1968), reductions in potency have been noticed after freezing highly diluted sera (Feinberg *et al.*, 1968) and isolated reaginic-rich serum fractions (Sehon, Harter & Rose, 1956; Stanworth, 1963). Measurements of the stability of purified IgE and reaginic sera after storage at 4°C, room temperature and 32°C have demonstrated little or no reductions in potency (Harter, 1961; Perlman & Layton, 1967; Feinberg *et al.*, 1968; Bazaral & Hamburger, 1972). Johansson & Stanworth (unpublished observations, see Foucard, Aas & Johansson, 1973) found that sera stored for 2 days at room temperature showed a 4–8 fold decrease in PCA titre but that the same serum produced RAST values which were almost unchanged. This result suggests that certain adverse storage conditions can affect the biological action of reagins but the combining sites of the antibodies remain intact and functional. The latter conclusion is supported by the earlier finding of Ishizaka *et al.* (1967) who demonstrated that heat inactivation (56°C for 2–4 hr) of IgE did not destroy the antigen-binding properties, but it did inactivate those structures on the IgE molecules which were involved in fixation to tissue. These experiments also showed that the heated IgE would not precipitate with anti-IgE, suggesting that the antigenic determinants on the γ E globulins are heat labile. Our experiments showed that anti-IgE did react with IgE in heated human sera but we measured the amount of radioactivity bound in solution unlike Ishizaka *et al.* (1967) who used radioimmunodiffusion. The ability of our Fc-specific, labelled rabbit anti-IgE to react with heat aggregated IgE may be due to anti-carbohydrate-specific antibodies present in the rabbit anti-serum. The Fc portion of human immunoglobulin E has a high carbohydrate content (Bennich & Johansson, 1971) and, unlike protein determinants, carbohydrate structures are likely to resist heat treatment without marked disruption of their antigenic configurations.

In the experiments described in this paper, experiments were frequently done using different batches of labelled anti-IgE which had been stored for varying periods of time. This makes comparisons between sera examined on different days difficult but, even so, some general features clearly emerged from the studies. The labelled anti-IgE consistently reacted with a large number of human sera after the sera had been heated at 56°C for from 30 min to 24 hr. As the rabbit antiserum used was specific for the Fc portion of human IgE, the most likely explanation for the observed reaction seems to be that the IgE in the human sera was aggregated by heating at 56°C and the aggregated immunoglobulin reacted with the labelled antisera. This explanation is supported by three main experimental findings. Firstly, preincubation of heated and

washed human sera with unlabelled anti-IgE before addition of ^{125}I -anti-IgE caused a significant reduction in the amount of label left in the tubes (Table 1). After labelled anti-IgE had been added to heated sera and the tubes washed, almost all of the remaining radioactivity could be easily flushed out of the tubes. This indicated that after the final washing, the label left in the tubes was bound to aggregated material (Table 2). Finally, the reaction observed between labelled antiserum and increasing concentrations of standard serum 68/341 indicated that the antiserum was reacting with IgE (Fig. 4).

Ito, Wicher & Arbesman (1971) also observed a reaction between heat aggregated IgE and a goat Fc-specific anti-IgE. In their gel diffusion experiments, two precipitin lines were seen with IgE which had previously been heated for 1–4 hr. Only one line appeared with unheated and 8–24 hr heated IgE. The authors concluded that the goat antiserum contained different antibodies with activities directed towards determinants on the heated and unheated IgE molecules. Ito *et al.* (1971) injected two rabbits with the same material used to immunize the goat but, both rabbit antisera contained antibodies only for the native, unheated IgE determinants. The authors acknowledge that the apparent absence of rabbit antibodies to determinants characteristic of heat denatured IgE may merely have been due to the experimental test method used and point out that the rabbits, unlike the goat, were injected with freshly prepared Fc fragment. Material used to inject the goat was first stored at -20°C for 10 weeks and storage may have produced partial denaturation of the protein. The authors suggest that this may explain the differences observed between the goat and rabbit antisera. In our experiments only one anti-human IgE serum was used. It would be interesting to examine other ^{125}I -labelled anti-human IgE sera to see if they also react with heat aggregated human IgE.

The effects on IgE after heating human serum over an extended period and at different temperatures up to 65°C needs further study. The differences observed between individual sera are intriguing and may indicate differences in the IgE present in the serum samples. For example, the temperature at which most uptake of label occurred varied with the different sera. This is best illustrated by the differences observed in the region $50\text{--}65^{\circ}\text{C}$ between sera Phi, 826F and 250G on the one hand and sera 280G and 429G on the other (Fig. 3). These results may be a reflection of physico-chemical differences in the IgE molecules present in the sera being examined. Human antibodies of the IgG, IgM and IgA classes form an extremely heterogeneous group of proteins (Cohen & Porter, 1964; Grey & Kunkel, 1964; Kabat, 1966, 1968; Vaerman & Heremans, 1966; Kunkel & Prendergast, 1966; Schur, 1972) and there seems no reason to suppose that immunoglobulins of the IgE class will be any less heterogeneous. Reaginic antibodies have been found to be chromatographically heterogeneous (Stanworth, 1963; Goodfriend, Perelmutter & Rose, 1966; Reid, Minden & Farr, 1966; Ishizaka & Ishizaka, 1967) and these and other reports (Fireman, Boesman & Gitlin, 1967; Perlman & Layton, 1967; Parish, 1969; Varga & Ceska, 1972) emphasize the heterogeneity and/or stability differences of some skin-sensitizing antibodies.

Using the heating procedures described in this paper, further studies are being undertaken in an attempt to investigate the potential usefulness of this method for estimating serum total IgE levels.

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