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Immunological Studies in Sickle Cell Crisis in Ghana

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Summary. We have investigated thirty patients in sickle cell crisis and forty-six healthy controls. IgG, IgA and IgM immunoglobulins and β_1A globulin were measured with radial immunodiffusion; antistreptolysin 0, C reactive protein and rheumatoid factor were tested in the plasma with latex slide text technique.

It was found that there is no decrease in the major immunoglobulin levels during sickle cell crisis. The ASO test was positive in almost equal numbers of controls and patients, whereas the C reactive protein was positive in almost half of the sicklers and negative in all the controls. Rheumatoid factor was positive only in two controls.

There was, however, a significant decrease in the β_1A globulin level in patients. This globulin is a degradation product of the complement factor C'3. The view was expressed that, perhaps during crisis an absolute or relative deficiency of complement may be responsible for the decreased opsonizing function and this could explain the higher incidence rate of infection in sickle cell crisis.

Résumé. Nous avons mesuré les immunoglobulines IgA, IgM, IgG et β_1A avec la methode immunodiffusion radiale chez 30 patients souffrants de la crise drepanocitaire et chez 46 individues saines. Antistreptolysin 0, protein reactif C et facteur rheumatique ont été aussi examiné avec la methode precipitation du latex.

Nous avons trouvé que la concentration de IgG, IgA et IgM n'a pas diminué pendant la crise drepanocitaire. L'antistreptolysin 0 était positif dans les deux groupes presque au meme nombre. Au contraire, le protein reactif C n'était positif que dans le sang de la moitié des malades et negatif dans tout les controls.

Le taux de β_1A globulin a diminué significativement dans le sang des malades. Ce globulin est le produit de degradation de facteur complement C'3. A notre avis il existe la possibilité d'un déficit absolu ou relatif de complement avec le consequence de l'opsonization pauvre; cette derniere peut expliquer l'apparition des infections en plus grande proportion pendant la crise drepanocitaire.

INTRODUCTION

Infections are frequently associated with sickle cell crisis in Ghana (Konotey-Ahulu, 1965,

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1969). Caggiano & Holden (1968) in the United States found higher immunoglobulin levels than normal in the steady state of the disease and they speculated that infections which predispose to crisis may play a role in determining these high values. Evans & Reindorf (1968) also found elevated IgG, IgM and IgA levels in children having sickle cell disease. Little is known about the causes of the frequent occurrence of infection in sickle cell crisis. One can assume that a fall in the concentrations of immunoglobulins may enhance infection. We are presenting data on immunoglobulin concentrations, β_1 globulin, a derivate of C'3 complement factor in plasma, and the results of some latex tests in sickle cell crisis.

PATIENTS AND METHODS

Thirty patients admitted with the diagnosis of sickle cell crisis to the Department of Medicine and Therapeutics in Korle Bu Teaching Hospital, Accra, were investigated. Among the patients, twenty-one were males and nine females; seventeen had Hb SS, eleven SC and two S-thalassaemia genotype. Forty-six healthy secondary school students, all with AA haemoglobin type served as controls. The majority of the patients had been admitted previously and followed-up and treated in the Sickle Cell Clinic, where they were given chloroquine as an antimalarial prophylactic; their Hb electrophoresis was already known so the diagnosis on admission was based on clinical findings. The previously unknown sicklers were diagnosed on admission on the basis of clinical symptoms and later confirmed by laboratory tests. Criteria for the clinical diagnosis for sickle cell crisis were severe pains in the vertebral column, legs or arms, high temperature and fast pulse. Blood was taken from an arm vein with heparin as an anticoagulant before the treatment started, within 2 hr of admission.

TABLE 1. Clinical data on sickle cell disease patients tested

	Males	Females
Number of cases	21	9
Malaria parasite in blood	6	1
Antimalarial prophylaxis		
Regular	11	5
Irregular	4	3
None	6	1
Complications		
Osteomyelitis (*S. typhi*)	1	—
Osteomyelitis, no pathogen	3	—
Pneumonia	—	1
Urinary tract infection (coliform)	—	1
Leg ulcer	—	1
Alcoholic intoxication	1	—
Died in crisis	1	—
Liver enlarged	5	1
Spleen enlarged	4	2
Haemoglobin genotype:		
SS	12	5
SC	7	4
S-Th	2	—

Plasma immunoglobulin levels were quantified, using the commercially available immunoplates (Partigen, Behringwerke) and Standard Human Serum (Behringwerke). Batches of patients' and control sera were measured simultaneously for immunoglobulin levels, to avoid errors, the serum between blood taking and measurement was kept at −20°C. The error of estimation for the IgG was ±8%, for IgM, IgA and β_1A globulin ±5%; C reactive protein, ASO and rheumatoid factor were tested for with latex slide techniques (Behringwerke).

TABLE 2. Immunoglobulins and β_1 globulin in plasma of thirty sickle cell disease patients and forty controls

Samples	Sex	No.	Age (years)	IgA (mg/100 ml)		IgG (mg/100 ml)		IgM (mg/100 ml)		β_1A globulin (mg/100 ml)	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD
Patients	M	21	23·7	254	129	1908	507	116	43	59	19
	F	9	19·2	250	131	1773	410	147	67	58	23
Controls	M	26	20·6	228	124	1648	404	152	66	72	15
	F	20	17·3	155	117	1872	418	201	86	73	12

TABLE 3. Laboratory results. Latex tests on thirty sickle cell disease patients and forty-six normal controls

	Sex	No.	Positive	ASO		CRP		Rf	
				M	F	M	F	M	F
Patients	M	21	4+:	2	2	4	—	—	—
	F	9	3+:	—	—	1	—	—	—
			2+:	1	1	3	3	—	—
			1+:	1	—	2	—	—	—
Total		30	—	4	3	10	3	—	—
Controls:	M	26	4+:	2	—	—	—	—	—
	F	20	3+:	1	2	—	—	—	—
			2+:	—	1	—	—	1	—
			1+:	—	—	—	—	—	1
Total		46	—	3	3	—	—	1	1

RESULTS

The average age in the control group was 20·6 years for the males and 17·3 years for the females and in the patient group males 23·7 years, females 19·2 years. Although the majority of the patients were on regular antimalarial prophylactic treatment, falciparum malaria parasites were found in the blood of six males and one female. This is not an unusual finding in individuals living in a hyperendemic area and does not automatically imply clinical malaria. Osteomyelitis was found in three cases, among these *Salmonella typhi* was isolated in one case. Among the female patients there was one case of pneumonia and one of urinary

tract infection. Enlargement of the liver was present in five males and one female and of the spleen in four males and two females. One male patient had alcoholic intoxication on admission and one died during the crisis (Table 1).

In the male controls the IgA value of 228 mg was higher than the 155 mg found in the females, although in the latter group the scatter of the mean was very large. The IgG level was higher in the female controls (1872 mg) than in the males (1648 mg). The IgM globulin concentration was 152 mg in the male controls and 201 mg in the females.

During crisis the IgA was higher in both males and females and the IgG (1908 mg in males and 1773 mg in females) was also higher in males but not in females. The IgM was lower in both males and females in crisis, 116 and 148 mg respectively. None of the differences were found to be statistically significant.

The β_1A globulin was lower in both males and females in the crisis group than in the controls and this was significant at the 95% level in females and 99% in males.

C reactive protein was negative in every control but it was positive in thirteen patients (42%). Antistreptolysin 0 was positive in five controls and seven patients, five of the latter also had positive CRP tests. Rheumatoid factor was demonstrable in two of the controls but in none of the patients (see Tables 2 and 3).

DISCUSSION

Malaria, pneumococcus, *Salmonella typhi* and urinary tract infections are often found in sickle cell crisis. It is not clear whether these infections always precede the crisis due to decreased host defence reaction and whether they invariably have any triggering action on crisis. American authors observed fatal pneumococcal meningitis and septicaemia in crisis (Kabins & Lerner, 1970; Hallock, 1970; Robinson & Watson, 1968), and it was suggested that diminished immune response is due to engorgement of the reticulo-endothelial system by red cell debris subsequent to fragmentation of sickling cells (Robinson & Watson, 1968; Kabins & Lerner, 1970). It was also thought that in patients with a haemoglobinopathy there is an impaired antibody formation in response to stimulation with *Salmonella typhi* antigen (Robinson & Watson, 1968), however, no such impairment was observed by Robbins & Pearson (1965).

It has been found, however, that the opsonizing index in patients with sickle cell anaemia against pneumococci is decreased, but not against *Salmonella typhi* (Winkelstein & Drachman, 1968). In the present study the antistreptolysin latex agglutination test was positive in almost equal numbers of patients and controls, indicating that *Streptococcus haemolyticus* infection does not occur more often in patients in sickle cell crisis than in members of the population in the same age group.

The IgG and IgM level in our controls in Ghana was higher than in healthy individuals in non-tropical countries (Stiehm & Fudenberg, 1966; Fahey & McKelvey, 1965; Allansmith *et al.*, 1968, Storiko, 1968) and this agrees with the findings of Turner & Voller (1966) and McFarlane (1968) in Nigeria. The IgA level was slightly lower than in controls in a temperate climate. The higher IgG and IgM levels in negroes living in tropical countries are attributed to environmental factors and racial characteristics.

There was a slight increase of IgA in patients. The IgG was in higher concentration in the male sickle group than in the control. In both males and females the IgM was higher in crisis without the difference being statistically significant. Caggiano & Holden (1968)

and Evans & Reindorf (1968) found in American negroes higher IgG and IgM values in the steady state in sickle cell anaemia patients than in normals. It seems that the main antibody carrier, IgG is not diminished during crisis. We do not attribute any importance to the small lowering of the IgM level during crisis so we conclude that there is no apparent decrease in the major immunoglobulins in sickle cell crisis.

The rheumatoid factor is a special type of IgM which occurs in rheumatoid arthritis. However, there is a 'rheumatoid like globulin' which is present in many blood samples originating from the tropics ('M antiglobulin', Houba & Allison, 1966) and this agglutinates latex particles; however, this is not pathognomonic for rheumatoid arthritis. None of our patients showed positivity; there were, however, two positive reactions in the control group. The C reactive protein serum reacts with the somatic C polysachharide antigen of the pneumococcus, its positivity is also a good indicator of activity of rheumatic fever and of other infections, but it is also positive when extensive tissue damage is present. In almost half of our patients the CRP latex test was positive indicating infection and/or inflammation in a proportion of the patients.

The β_1A globulin concentration in controls in Europe was found to be between 80 and 140 mg (Storiko, 1968) and $143 \pm 35{\cdot}8$ (Grob & Jemelka, 1971); in our controls it was lower, between 33 and 100 mg. In both male and female sickle groups this globulin was significantly lower.

β_1A globulin is the immuno-electrophoretic equivalent of C'3 complement factor; however, in fresh serum this factor is present as β_1C globulin. The latter will be transformed to β_1A globulin *in vitro* due to activation; *in vitro* the transformation takes place subsequent to treatment with zymosan or hydrazin or by allowing to stand the serum in a plastic tube at 37°C for a few hours or at 4°C for a few days. For *in vitro* measurement of C'3 fraction the use of β_1A globulin is preferable because it is more stable than the β_1C globulin (Grob & Jemelka, 1971). It is noteworthy that the C'3 component is involved in the opsonization and chemotactic function and as was mentioned above, in sickle cell anaemia patient the only abnormality in the immune defence mechanism found so far is in the reduced opsonizing function. The splenic hypofunction in sickle cell anaemia may have some additional effect on the weakened host defence. 'Autosplenectomy' is not being considered as responsible primarily for fulminant pneumococcaemia in sickle cell crisis (Robinson & Watson, 1968; Kabins & Lerner, 1970); however, the spleen has a role in phagocytosis of non-opsonized agents.

Various causes can precipitate C'3 deficiency syndrome: inherited partial C'3 factor defect (Alper, Propp & Klemperer, 1969); acquired deficiency of synthesis (Asofsky & Thorbecke, 1961; Gotoff *et al.*, 1965); increased decomposition (Hermann *et al.*, 1970) and consumption by formation of immunocomplexes (Blaker, Fischer & Witte, 1969; Korngold, 1966) may all lead to low β_1 globulin level in serum. One can speculate that in sickle cell crisis and perhaps in the steady state there is an absolute or relative deficiency of the C'3 fraction and this is responsible for decreased opsonization which together with splenic hypofunction may enhance certain infections. Further studies are necessary to evaluate the role of the immunological defence system in the pathogenesis of infections in sickle cell anaemia.

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The Release of Pharmacologically Active Substances During Antigen–Antibody Reaction in the Rat Peritoneal Cavity

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Summary. In rats' peritoneal washings, after antigen–antibody reaction, pharmacologically active substances have been obtained; these include histamine, bradykinin and SRS (a slow reacting substance).

The slow reacting substance has been found to be similar to SRS-A obtained from guinea-pig lungs. The presence of bradykinin-like material has also been established.

Tyrode de Jalon solution is observed to give better control values than saline in rats.

Résumé. L'histamin, bradykinine et SRS (une substance de l'activité retardée) ont été obtenues des lavage peritoneal de rats après l'antigène anticorps réactions. La substance de l'activité retardée a été trouvée d'avoir même qualitée comme le SRS-A du poumons de cobaye. On a aussi constaté la prèsence d'une substance avec/l'activité de bradykinine.

La solution Tyrode de Jalon a été observée de produir aux rats les resultats plus meuilleurs que la solution salinée.

It is a well established fact that during antigen–antibody reaction, histamine, SRS-A (slow reacting substance of anaphylaxis) 5-hydroxytryptamine as well as bradykinin are released from actively sensitized animals such as guinea-pigs, rats, dogs and calves (Brocklehurst, 1960; Uvnas & Thon, 1959; Brocklehurst & Marquis, 1972).

The present work was prompted by the observations of Rapp (1961) who stated that apart from histamine released, a slow reacting substance (SRS) was also released *in vivo* in the rat which had similar pharmacological profile as SRS-A obtained from sensitized guinea-pig lungs. Earlier studies by Brocklehurst (1958, 1960) failed to show the presence of SRS-A *in vivo* in the rat.

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Slow reacting substances have been reported to be formed following anaphylaxis (or antigen–antibody reactions) in many tissues and in several species (Brocklehurst, 1958, 1960; Charkravarty, 1960). Brocklehurst advanced the idea that the substrate giving rise to the slow reacting substance must be particularly abundant in blood vessels, whereas Uvnas & Thon (1959) and Charkravarty (1960) suggested that mast cells may be the source. Work by Boreus & Charkravarty (1960) reported that the yields of both histamine and slow reacting substance following antigen–antibody reaction in guinea-pig lungs are related to the number of mast cells involved.

The work of Rapp (1961) cast some doubt on the significance of mast cells in respect of the release of slow reacting substance. It has been shown that little or no slow reacting substance was produced when histamine was freed from guinea-pig mesentery by anaphylaxis '*in vitro*' (Austen & Humphrey, 1961) and in omentum (Rapp, 1961).

All the SRS-A preparations that have hitherto been used (Brocklehurst, 1955, 1960, 1962; Berry & Collier, 1964) were obtained '*in vitro*', so this report on '*in vivo*' release of a SRS was of special interest and prompted a series of experiments to determine whether this material was indeed similar to SRS-A (slow reacting substance of anaphylaxis).

EXPERIMENTAL

(1) *Anti-sera preparation and purification*

Guinea-pig anti-ovalbumin sera were prepared and the Y_1- and Y_2-globulins were purified by preparative electrophoresis followed by DEAE cellulose chromatography as described by Colquhoun & Brocklehurst (1965).

(2) *Passive sensitization and the collection of peritoneal washings*

Male and female rats (100–200 g body weight) were injected intraperitoneally with 0·6 μg of purified globulin-containing antibody. Rabbit antibovine serum albumin or guinea-pig anti-ovalbumin was given intraperitoneally in 0·2 ml of Tyrode or de Jalon solution. 4 hr later, 2·0 mg antigen in 5–7 ml of the same solution was injected intraperitoneally. The rats were killed 5 min later, the abdomen was opened and the edges retracted and inverted to avoid contamination with blood, or the peritoneal fluid removed by means of a polythene pipette. On some occasions a small midline slit was made in the abdominal wall, and the fluid collected in a polythene funnel without exposing the viscera. For control purposes, some animals were injected with antigen only, but no antibody, some others were injected with antibody only, and no antigen, and some with the Tyrode solution only.

(3) *Assay and identification of active substances*

The recovered peritoneal fluids were kept on ice until estimated for histamine-like activity, SRS-like activity, and bradykinin-like activity, all of which were assayed on guinea-pig ileum. Assays for bradykinin were also performed on the rat uterus in oestrus, and rat blood pressure or rat duodenum. All the estimations were either carried out on the same day or within 24 hr of collection. In all but the very earliest experiments the fluid collected was spun at 4°C to remove any cells and debris.

RESULTS

(a) *Purity of Y-globulins*

When preparations were tested by immuno-electrophoresis, only a single area, corresponding with the slower part of the Y-globulin of whole serum, was visible. When stored for a few weeks at 4°C, Y-globulin solutions were altered, an additional area appearing on immuno-electrophoresis. The solutions were routinely sterilized by passage through cellulose acetate filters (Oxoid Ltd, London) and stored in sealed ampoules. Such solutions kept well at 4°C for at least 1½ years.

FIG. 1. Typical assay of histamine on guinea-pig ileum. Atropine 10^{-7} M was present at all times. U', U'', Y', Y'' are peritoneal washings collected from animals. :, High dose of histamine (4·0 ng/ml); •, low dose of histamine (2·0 ng/ml).

(b) *Histamine assay*

A 2 plus 1 assay was performed, using the terminal piece of guinea-pig ileum bathed in Tyrode solution containing atropine 10^{-7} g/l at a temperature of 37°C.

(c) *SRS-A like activity assay*

This was performed before and after chymotrypsin digestion of the aliquot. This was to eliminate any contribution which bradykinin-like material may make to the contractions elicited from the guinea-pig ileum during assay. The assay was usually performed on the same piece of ileum as used for the histamine assay. The Tyrode solution contained both atropine 10^{-7} g/l and mepyramine 10^{-6} g/l.

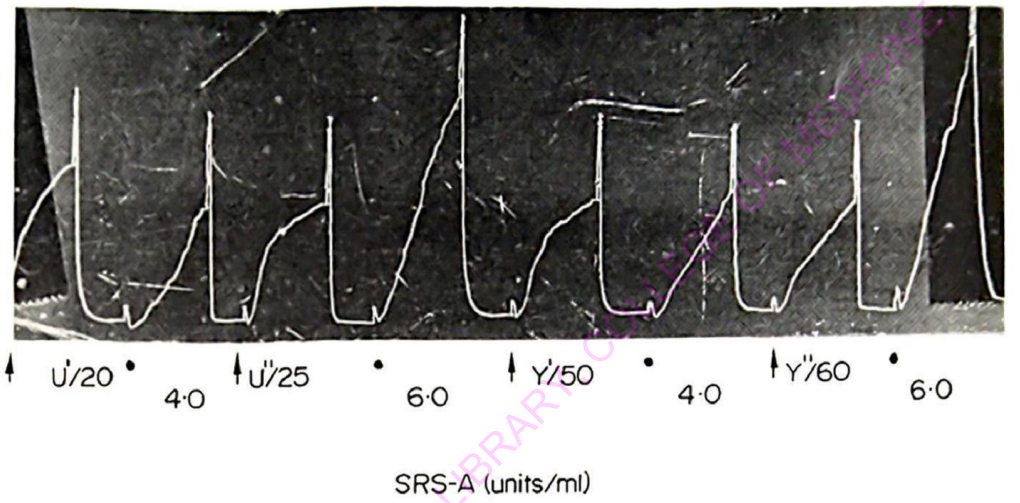

FIG. 2. Typical assay of SRS/(SRS-A) on isolated guinea-pig ileum. Atropine 10^{-7} M and mepyramine 10^{-6} M were present in bathing Tyrode solution.

TABLE 1. SRS-like material obtained after 5 min challenge (8–12 units/ml)

No. of rats	Average volume collected from each rat (ml)	Mean total units of SRS	SD
6	5	8·0	±0·4
6	6	13·0	±0·65
6	5	10·0	±0·42

(d) *Bradykinin-like activity*

Aliquots (0·5 ml) were poured into 1·5 ml of cold absolute alcohol to inactivate enzymes, stood for 30 min in ice to give full precipitation of protein and then boiled for 5 min. After being centrifuged at 2500 g, the supernatant was removed and evaporated under reduced pressure and below 30°C temperature using a rotary evaporator. Uteri from stilboestrol-treated rats of 200–300 g were used to assay the activity against synthetic bradykinin (Sandoz) as standard. Some of these samples were incubated with salt-free chymotrypsin (0·5 mg/ml of sample) for 30 min at 37°C to destroy bradykinin. The reaction mixture was then boiled for 1 min and cooled ready for assay. Chymotrypsin alone, treated in the

same way, and present in amounts in which it occurred in the reaction mixture did not have any oxytocic activity on the isolated guinea-pig ileum preparation.

As may be seen in Table 1, these 5 min samples contained too little SRS to be sure that it was SRS-A, but there was bradykinin, which Rapp did not report and some histamine was

TABLE 2. Histamine release in antigen–antibody reaction in the rat peritoneal cavity

No. of animals	Solution used intraperitoneally	Antibody	Antigen	Histamine-like activity obtained (ng/ml)
2	Saline	–	–	660·0 ± 36·0
2	Tyrode	–	–	42·0 ± 3·0
2	Tyrode	+	–	50·0 ± 4·9
2	Tyrode	–	+	62·0 ± 5·3
4	Tyrode	+	+	312·0 ± 4·8

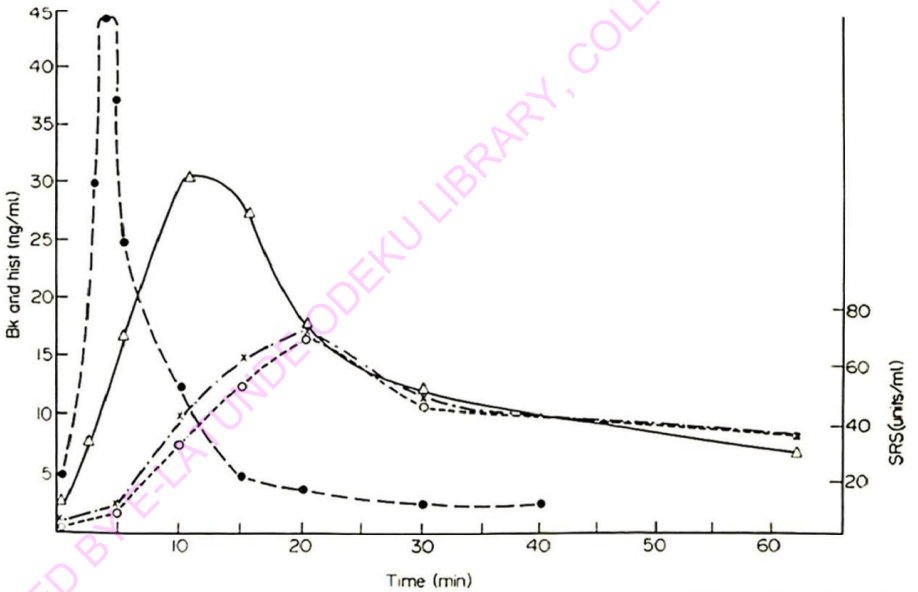

FIG. 3. Diagram showing time-course of release of histamine, bradykin and SRS during antigen–antibody reaction in rat peritoneal cavity. ●---●, Histamine; △——△, bradykinin; ×—·—·—×, SRS-like (before chymotrypsin); ○-----○, SRS-like (after chymotrypsin).

also present. Tyrode or de Jalon solution gave only a very small spontaneous yield of histamine and it was therefore possible to show the modest release of histamine which occurred during the local anphylaxis. It seemed possible that a larger yield of SRS-A would be obtained by allowing the antigen–antibody reaction to continue for a period longer than 5 min.

(e) *Time-course of release of active substances*

The details of this experiment are similar to those described above. The additional conditions are as follows:

Three rats were used for each period of time: 0, 2·5, 5, 10, 15, 20, 30 and 60 min.

Chymotrypsin 0·5 mg/ml was used to hydrolyse both standard and control samples of bradykinin.

Three such experiments were performed and the results obtained are shown in Fig. 3. It was found that the maximum yield of histamine was obtained at about 3 min, whereas the bradykinin maximum was at 15 min and the SRS maximum was 20 min. This SRS strongly resembled SRS-A pharmacologically. It was reasonable to suppose that when the guinea-pig ileum preparation was used any bradykinin present might lead to an over-estimate of SRS-A present, hence the use of chymotrypsin.

DISCUSSION

This work was undertaken in the belief that SRS-A plays a wider role in allergic and inflammatory conditions than had previously been shown. Also, it has been the contention of many workers (Hogberg & Uvnas 1958; Charkravarty, 1960; Boreus & Charkravarty, 1960) that SRS was released only from mast cells. The '*in vivo*' studies of Rapp (1961) had shown, though not conclusively, that SRS he obtained did not originate from mast cells.

On investigating the fluid collected, it was discovered that saline washes released a great deal of active material irrespective of antigen–antibody reactions (this was not reported by Rapp (1961)). However, Tyrode or de Jalon solution were both found to be satisfactory giving modest control values of biological activity. The use of polythene receptacles were found to prevent the loss of the spasmogenic substances and reproduceable results were obtained. Bradykinin was found to be present in the peritoneal washings along with SRS and histamine. This is an additional find to that of Rapp (1961).

The systematic study of the time-course of release of SRS and other spasmogens (Fig. 3) showed that the maximum yield of histamine was obtained at about 3 min after challenge, whereas the bradykinin was at 15 min and SRS maximum was 20 min.

This aspect confirms the trend in antigen–antibody reactions in the rat, in that histamine, is first released which then activates other enzymatic reactions which lead to bradykinin release which is immediately followed by SRS release (Brocklehurst, 1962). The SRS-like and bradykinin-like activities were assayed before and after chymotrypsin digestion, this was to eliminate any contribution which either material may make to contractions elicited from the guinea-pig ileum during assay.

The quantity of bradykinin varied with the purity of the antibody used. Less bradykinin was obtained when purified guinea-pig IgA-containing anti-ovalbumin (Colquhoun & Brocklehurst, 1965) was used. The reason for this is not known and it is beyond the scope of this investigation. It may be that antibodies other than IgA are responsible for the release of bradykinin and it may be significant that the purified guinea-pig IgA-containing anti-ovalbumin used contained some IgG in it.

The SRS-like material obtained from these washings strongly resembles SRS-A when assayed on guinea-pig ileum. Furthermore, there was no apparent difference when the substance was purified by the method used for SRS-A from guinea-pig lung and SRS-A from cat paws in spite of the different methods by which the substances were originally

obtained (Marquis, 1966). The present work strengthens the conclusion reached by Rapp (1961) that the SRS material obtained from peritoneal washings of the rat was similar to SRS-A as supplied to him by Brocklehurst.

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