also [3]), the concentration of SVPx does not change significantly after 10 min of incubation.

(6) *Separation of SVPx by paperchromatography.* When a chromatogram was run with *n*-butanol:acetic acid:water (12:3:5), the solvent mixture used earlier[3], the active principle eluted at Rf 0.5 consisted of a mixture of 2 types of substances. As in the case of the unpurified plasma dialysate (Figure 1), one of the factors is DBMC + MEP sensitive, and the other DBMC + MEP insensitive. By use of *n*-butanol:pyridine:water, Figure 3, 2 active substances were separated. One, which peaks around Rf 0.25, is not antagonized by DBMC + MEP. The other, which peaks around Rf 0.4, is blocked by DBMC + MEP. Presumably the latter fraction contains serotonin (see [3]).

Discussion. For historical reasons we felt it might be appropriate to continue to designate the total of the contracting substances of plasma as 'vasoconstrictine', the term introduced in 1905 by BATELLI[2]. In the rabbit it consists of a mixture of serotonin, SVPx, histamine, and barely detectable amounts of NOR-EPI. There then remains an unknown factor (SVPx) which exists in concentrations equivalent in constrictor activity to the concentration of serotonin in plasma. At the present time we can state unequivocally that SVPx exists and can be separated. Its nature, whether a known or a so-far unknown substance, remains to be investigated.

Note. Separation of serotonin from the unidentified 'effective' vasoconstrictor was also achieved by differential dialysis[11], and by countercurrent distribution technique (to be reported in detail)[12].

Résumé. La «vasoconstrictine» (BATTELLI 1905), c'est-à-dire l'ensemble des vasoconstricteurs «effectifs» dissous dans le plasma du lapin, consiste en un mélange de sérotonine, SVPx (substance non-identifiée) et d'histamine. Par l'usage d'une antisérotonine sélective, DBMC, et d'une mépyramine antihistaminique, il a été possible de reconnaître et d'isoler SVPx. La concentration des catécholamines dans le plasma n'est pas suffisante pour produire une contraction de la paroi d'une artère.

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Secondary Antibody Responses of Mice to Bacterial Somatic Antigens

The physical form of the antigen, the interval between multiple injections, and the techniques used to assess the immune response, are known to influence the results and the interpretation of immunizing procedures. We have used the haemolytic plaque technique, indirect haemagglutination and immunoelectrophoresis to study the secondary responses of mice to bacterial somatic antigens.

Female mice, 8 weeks old, were injected i.p. with a mixture of $2.5 \cdot 10^7$ boiled *Salmonella newport* cells and 12.5 μg of purified *S. typhi* lipopolysaccharide (LPS), a Difco product. The antigens were used together so that the responses induced could be compared in individual animals. One group received no further injections; the animals were bled at weekly intervals to determine the course of the primary immune response. 6 other groups were given a second dose of the same mixture by the same route, at intervals of 2–7 weeks after the first injection; 6 days after the second injection, the spleens and blood sera were examined. Spleen cells forming antibodies (IgM or 19S type) to *S. newport*, *S. typhi* or sheep erythrocytes were counted in duplicate by the plaque method described previously[1], using sheep erythrocytes either sensitized with the appropriate bacterial antigens or non-sensitized as required. A correction was applied for the small number of plaques caused by antibody to sheep erythrocytes. Circulating antibodies were titrated in duplicate by haemagglutination of sheep erythrocytes sensitized as above. Susceptibility of the antibodies to reduction was determined by diluting sera 1 in 10 with phosphate buffer of pH 7.4, containing 0.1 *M* 2-mercaptoethanol (ME), and titrating after 1 h at 37 °C. Alkylation of a number of reduced sera by dialysis against iodoacetamide was found not to alter their titres.

Selected sera were subjected to agar gel immunoelectrophoresis, the precipitin arcs being developed with goat antiserum to whole mouse serum or to mouse γ-globulin. Those arcs which contained antibacterial antibody were detected in the gel by treatment with ^{14}C-labelled soluble antigen and autoradiography on X-ray film.

Both spleen plaque numbers and serum antibody titres were highly variable from one mouse to another, as exemplified in Table I, which shows the detailed results obtained from one of the groups after secondary stimulation. In terms of numbers of spleen cells producing haemolytic antibody, the whole bacterial cells were the more potent immunogen and also gave rise to noticeably larger plaques. There was, however, no correlation with serum antibody levels; individual mice sometimes responded well, in terms of serum antibody, to an antigen inducing only minimal reaction in the spleen. On the average, about 50% of the *S. typhi* antibody was reducible but only about 25% of the *S. newport* antibody.

Table II shows the data from the groups of mice which received antigen mixtures at different intervals. There was no consistent change in the numbers of spleen plaques as the primary-secondary interval increased, in contrast to serum antibody which reached a maximum at the 4-week interval then remained approximately constant. That the latter result was a true secondary response (rather than the slow development of antibody

[1] W. J. HALLIDAY and M. WEBB, Aust. J. exp. Biol. med. Sci. *43*, 305 (1965).

from the primary stimulus) is shown by the fact that peak primary titres in a separate group of animals were always much lower than the secondary titres and were falling after 4 weeks. Many of the later sera were completely ME-resistant, while all the 2-week sera were at least partly reducible. The increase in ME-resistance was especially pronounced with antibody to the whole bacteria (*S. newport*).

Representative immune sera, examined by immunoelectrophoresis and autoradiography, gave precipitin arcs corresponding in position and shape to IgG (or 7S γ_2 globulin in the terminology used by FAHEY[2] for mouse immunoglobulins); there was excellent correlation between the density of labelling of these arcs and the titres of ME-resistant antibody. No such labelled arcs were detected in sera highly sensitive to reduction, or in normal mouse serum. We therefore deduce that ME-resistance is a reliable indicator of IgG antibody in these sera, and that mice can produce considerable quantities of this type of antibody in response to bacterial somatic antigens. This is in contradiction to some other investigators, who have found predominantly or exclusively IgM antibody under comparable conditions in mice[3], rabbits[4–6], and man[7,8], even after secondary stimulation.

Table I. Mean specific spleen plaque numbers and mean serum antibody titres

Mouse No.	Spleen plaques per 10^7 cells		Serum antibody titres			
	S. typhi	*S. newport*	*S. typhi* no ME	*S. typhi* + ME	*S. newport* no ME	*S. newport* + ME
1	1	17	80	80	160	160
2	0	210	640	160	320	320
3	3	635	320	160	640	320
4	13	305	160	160	320	160
5	73	62	320	160	160	160
6	5	25	640	160	320	160
7	8	19	1280	320	320	160
8	3	15	40	40	160	320
Average	13	161	250	120	260	190

Results obtained from a group of 8 mice given 2 mixed antigenic stimuli 6 weeks apart. *S. typhi* antigen administered as LPS; *S. newport* antigen as whole bacteria.

Table II. Effect of interval between 2 mixed antigenic stimuli on plaque numbers and antibody titres

Interval (weeks)	Spleen plaques per 10^7 cells		Serum antibody titres			
	S. typhi	*S. newport*	*S. typhi* no ME	*S. typhi* + ME	*S. newport* no ME	*S. newport* + ME
2	11	95	66	< 40	70	< 40
3	3	11	47	< 40	110	40
4	30	58	230	95	280	120
5	2	20	190	130	210	110
6	13	161	250	120	260	190
7	6	12	290	105	210	160

Results are averages from 6 groups of 8 mice.

A recent detailed study[9] has shown both IgG and IgM to be produced in rabbits.

FECSIK, BUTLER and COONS[10] found that 2 doses of diphtheria toxoid in mice should be spaced by at least 6 weeks for maximal response. A similar phenomenon occurs with bacterial somatic antigens (Table II), ME-resistant IgG antibody contributing entirely to the effect. An interval of at least 4 weeks between antigen injections, during which time a process of maturation may occur[10], is thus necessary for the optimal expression of '7S immunological memory'. ME-sensitive antibody (IgM), expressed as differences in titres or as plaque numbers showed no secondary-type response, confirming the suggestion[6,11] that persisting immunological memory is not found in the 19S or IgM antibody system. The conditions required for the demonstration of secondary responses with IgM antibody in rather different circumstances have recently been described[12,13].

The presence of substantial serum antibody titre without concomitant spleen activity, as described above, could have been caused by one or more of the following:

(a) The 2 techniques (hemagglutination and localized hemolysis) are sensitive to different types of antibody, IgG being more efficient than IgM in agglutination of LPS-sensitized erythrocytes[4], and visible plaques being formed by IgM only. (b) Serum antibody titres are a measure of accumulated antibody, whereas plaques are formed only by antibody produced during a brief incubation of cells in vitro. (c) Antibody is formed in extrasplenic sites, which assume a major role in some animals but not in others.

Zusammenfassung. IgG- und IgM-Antikörper wurden mit 2 Injektionen von *Salmonella*-antigenen (*S.-newport*-Zellen und *S.-typhi*-Lipopolysacchariden) in Mäusen erzeugt. Es ergab sich, dass die IgG-Antikörper bei ihrer zweiten Reaktion nur dann ein Maximum erreichten, wenn zwischen der ersten und zweiten Injektion ein Zeitraum von mindestens 4 Wochen verstrichen war. Weiter wurde gefunden, dass die Lipopolysaccharide, im Gegensatz zu den Bakterienzellen, die Bildung der IgM-Antikörper favorisierten.

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