# REVIEW ARTICLE

## THE ROLE AND FUNCTION OF THE RETICULO-ENDOTHELIAL SYSTEM IN IMMUNOLOGICAL PROCESSES*

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Our knowledge of the role and functions of the reticulo-endothelial system is very imprecise and uncertain. However, there is considerable interest in immunological processes, that is to say in the resistance of host to infection, and our knowledge is increasing rapidly. That certain types of cells are implicated in the production of immunity was first suggested by Metchnikoff[1]. It is to him that we owe the first comprehensive and plausible theory of the role played by what he named "phagocytosis" in protection of the host against pathogenic invaders. But the existence of an integrated system of cells fulfilling a particular bodily function was postulated by Aschoff and his school. In 1913, Aschoff

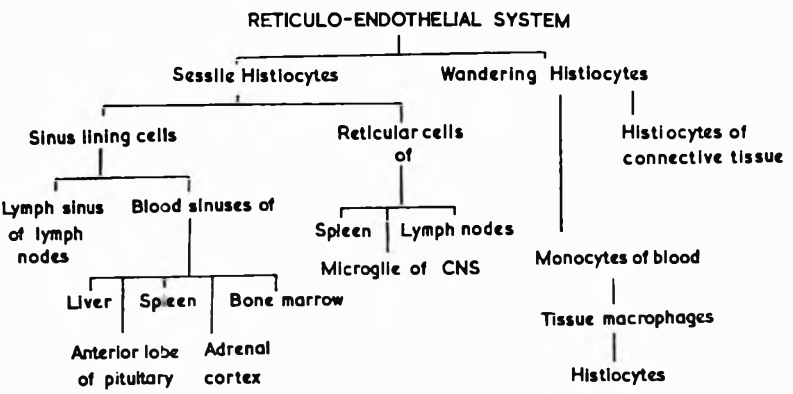


Fig. 1. Aschoff's classification of the R.E. cells.

and Kiyono[2] reported the histological and physiological autonomy of this important system, to which they gave the name reticulo-endothelial system (R.E.S.). Whether Aschoff's hypothesis of the functional unity of this system of cells, based on their ability to ingest and accumulate dyes, will stand the test of time is difficult to predict. His conception has been seriously challenged by some distinguished contemporary histophysiologists with valid arguments[3]. However, for clarity, I shall accept Aschoff's conception of the R.E.S. which is represented in Figure 1.

The histiocytes, which form the R.E.S., are widely but irregularly scattered through almost all tissues and organs. According to the classical conception, they are divided in *sessile* and *wandering* cells.

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Methods for studying the function of all the R.E. cells are not available at present, and in this review, I shall restrict my subject to the study of sessile histiocytes. I propose to ignore the wandering macrophages, irregularly scattered in the connective tissues, which certainly play an important part in the local and general defensive reactions, but which have eluded all attempts at quantitative evaluation.

Sessile histiocytes are concentrated in some important strategic areas of the vascular bed, where they are attached to the endothelia of the sinuses of various organs; liver, spleen, bone marrow and some other glands.

These cells, being in direct contact with the blood stream seem to be particularly active in taking up both inert and living particles from the circulating blood. To stress the importance and the role of this group of phagocytes, Miles[4] proposed to designate it the "systemic defences".

If immunity is defined as the resistance of a body to the effects of pathogenic invaders, the R.E. cells should be considered as one of the most important sources of protection to the host. They act by a double mechanism: (i) phagocytosis, followed by digestion and destruction of bacteria and other foreign debris; (ii) a humoral process, as yet not well understood but which is reflected in an increase of resistance to infections, and which involves, among other factors the production of specific antibodies.

Discussion of phagocytosis and immune reactions mediated by the R.E.S. will form the two main parts of this review.

## The Dynamics of the Phagocytic Function

The property of engulfing particles of inert or living material is present even in the simplest animals. Metchnikoff[1] conceived that this process, which in the amoeba, for example, serves primitive nutritional needs, had become, in the course of evolution, an essential process of defence by which pathogenic invaders are combatted.

The importance of phagocytosis is considerable and it is believed by Boyd[5] to be "the greatest single mechanism contributing to natural resistance". Moreover, phagocytosis is not restricted to protection against bacterial infections. Modern physiology points out that the R.E. cells play an important part, by their remarkable capacity of clearance and of storage, in the metabolism of lipids[6], lipoproteins[7], cholesterol[8], haemoglobin[9] and iron[10].

These considerations emphasise the interest of the investigations made on the patterns of the phagocytic function, not only against bacteria, but also against inert particles. Also, the mechanism of phagocytosis of two such different substrates shows some intriguing discrepancies.

The patterns of phagocytosis are studied, in principle, by measuring clearance rates of the particles in the blood and their accumulation in the R.E. cells at specific times after the intravenous injection of a standard colloidal suspension of particles known to be phagocytosed by these cells.

Certain very definite criteria should be strictly observed in the choice of the particulate substances chosen to study the phagocytic activity of the cells[10].

(1) The particles should be phagocytosed by the cells of R.E.S. in contact with the blood and should not be taken up by other cells.

(2) They should normally not cross the capillary barrier.

(3) They should be homogenous in size.

(4) The substance should be non-toxic for the R.E. cells and the host organism.

(5) The substance should be stable *in vitro* and *in vivo* and should not be changed in physical or chemical properties by contact with the blood or after intravenous injection.

(6) The substance should be accurately measurable in the blood and tissues.

*The General Patterns of Clearance of Inert Particles*

The intravenous injection of a colloid meeting the desired criteria into an animal or a man is followed by a more or less rapid decrease of the particles from the blood stream and their concomitant accumulation in the R.E. cells, mainly in those of the liver and spleen. Figure 2 illustrates the disappearance rates of a suspension of carbon injected at various doses into albino rats. When presented in semilogarithmic co-ordinates by plotting the clearance as the log of the blood concentrates with respect to time, this phenomenon can be expressed by an exponential function (Fig. 3): $C = Co \cdot 10^{-KT}$

or by the equation: $$\frac{\log C_1 - \log C_2}{T_2 - T_1} = K$$

in which C = concentration of the foreign colloid in blood expressed in mg./100 ml., $C_1$ and $C_2$ the colloid concentration in the blood at times $T_1$ and $T_2$ minutes respectively.

TABLE I

DISTRIBUTION OF COLLOIDS INJECTED INTRAVENOUSLY IN THE RAT

| Colloid | Dose mg./100 g. | Per cent recovered | | | | K |
|---|---|---|---|---|---|---|
| | | Liver | Spleen | Lung | Total | |
| Carbon . . . . | 4 | 92 | 4 | — | 96 | 0·062 |
| | 8 | 90 | 4·7 | — | 94·7 | 0·026 |
| | 16 | 79 | 9 | — | 88 | 0·013 |
| | 32 | 78 | 10 | 2 | 90 | 0·006 |
| | 48 | 70 | 17 | 1·6 | 88·6 | 0·004 |
| C.A.G.$^{131}$I . . | 2 | 89 | trace | — | 89 | 0·118 |
| | 4 | 84 | 1·5 | — | 85·5 | 0·064 |
| | 8 | 83 | 1·9 | — | 84·9 | 0·031 |
| | 12 | 75 | 3 | trace | 78 | 0·022 |

The constant K of these equations, which defines the slope of the clearance curve in semilogarithmic co-ordinates, is therefore a measure of the clearance of particles and thereby of phagocytosis of the R.E. cells. We have called K the phagocytic index.

The data represented in Figures 2 and 3 show clearly that the main characteristic of the phagocytosis by the R.E.S. of the particles circulating in the blood is that the kinetics of phagocytic activity are affected by the quantity of colloid injected. The mathematical analysis indicates that the phagocytic index K varies inversely with the dose of colloid injected (D). In general, the relationship $K \times D = CT$ is true for different colloidal suspensions except when relatively small numbers of particles are injected[11].

The relationship $K \times D = CT$ has been verified in various animal species and seems to be a general principle. By comparing the phagocytic activity of the R.E.S. in various animal species, it has been found that the R.E.S. of the mouse is the most active; then the rat, guinea pig and the rabbit in order of decreasing activity[12].

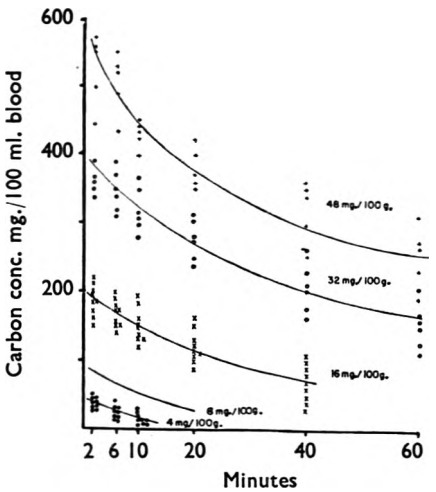


FIG. 2. Blood clearance of various doses of carbon in the rat.

The colloidal particles disappearing from the circulating blood are removed by the cells which line the sinuses of the major organs containing fixed R.E. cells. It may be of some interest to analyse the distribution of the colloidal matter between these organs. The liver is the most important organ, by far, in this respect. As seen from the figures in Table I, the Kupffer cells phagocytise 70 to 90 per cent of injected colloids. The role of spleen is much less important but, in general, the percentage recovered from the spleen increases with the dose. Thus for very small doses of colloids, which are rapidly cleared, the Kupffer cells take up nearly all the injected colloid; however, with higher doses which remain in circulation for hours, the spleen contains more than twice as much per gram as the liver. The reasons for these differences between the liver and spleen may be due to the total quantity of the phagocytes or, even more plausibly, to the circulatory patterns of the two organs.

In addition, species differences are rather important in this respect. While in rats, mice and rabbits, the spleen uptake is rarely as high as 10 per cent, a spleen deposition of over 20 per cent is common in dogs, which have a relatively large spleen compared with the other animals[13].

The role of the bone marrow in the uptake of colloids is similar to that of the spleen. With the usual colloids at average doses, the quantity found in the bone marrow is less than 10 per cent. But, when small particle colloids which disappear slowly from the blood stream are used, the deposition in the bone marrow may be much more considerable, increasing to 50 per cent of the injected material according to Dobson[13]. It should

be added that the high concentrations in the bone marrow have been found only in rabbits.

There is controversy about the part played by the lungs as a clearing organ. In our experiments, using various colloids, no significant deposition in the lungs has been observed, except when the material used was not stable. For example the use of commercial Indian inks for measurements of clearance, produces a gross error. The shellac they contain has potent thromboplastic properties and by the production of microthrombi, causes an accumulation of the carbon in the lungs. In this instance, we are dealing with a simple mechanical filtering effect of the lung capillaries and not with true and active phagocytosis[14].

This same mechanism may explain the observations of Wright[15] concerning the retention of bacteria in the lungs of strongly immunised animals. The bacteria aggregated by the specific antibodies are simply trapped in the pulmonary capillaries. It has been shown that particles, whether inert or living, once trapped in a fibrin net work in lung capillaries, are subsequently phagocytised by the polymorphonuclear cells and carried by them to the R.E. depots in the spleen and liver where they are phagocytised by the R.E. sessile cells.

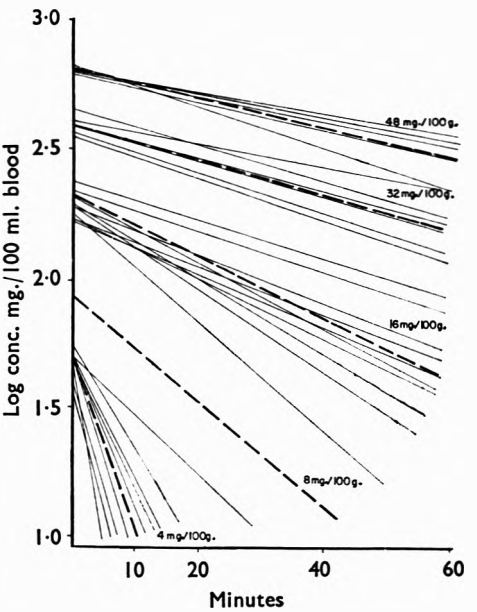


FIG. 3. Blood clearance of various doses of carbon in the rat in semi-logarithmic plot.
Dotted lines indicate the mean values.

$$C = Co.10^{-KT} \quad \frac{\text{Log } C_1 - \text{Log } C_2}{T_2 - T_2} = K$$

Studies with various colloids in different animal species have proved that the kinetics of phagocytosis of particulate substances introduced into the blood stream obey the same general laws, and the differences observed are quantitative only. However, several factors may influence the patterns of the clearance rates, namely the nature of the colloid, the size of the particles, the velocity of the blood flow and the functional conditions of the cells[16,17].

I now propose to discuss the saturating effect of repeated injections of colloids; discriminative phagocytosis and the importance of the stability of the injected colloids in the circulating blood.

*The Saturating Effect of Repeated Injections*

When the same dose of colloid is repeatedly injected into the same animal, after the particles of the preceding injection have been cleared,

evidence of a saturating effect of the phagocytes is observed (see Fig. 4). These results show clearly that the particles already phagocytised exert a depressive effect on the clearance capacity of the phagocytes towards the same substrate. This saturating effect of the phagocytised colloid is directly related with the so called "blockade" of the R.E.S.

*The "Discriminative Phenomenon" in Phagocytosis*

When particles of different sizes are injected together, the presence of some colloids in the circulation markedly modifies the rate of clearance of others by the R.E.S. This effect may be, of course, conditioned by the respective concentrations of the two colloids, but the experimental findings suggest that the clearance rate is also influenced by the physico-chemical nature of each colloid[18,19].

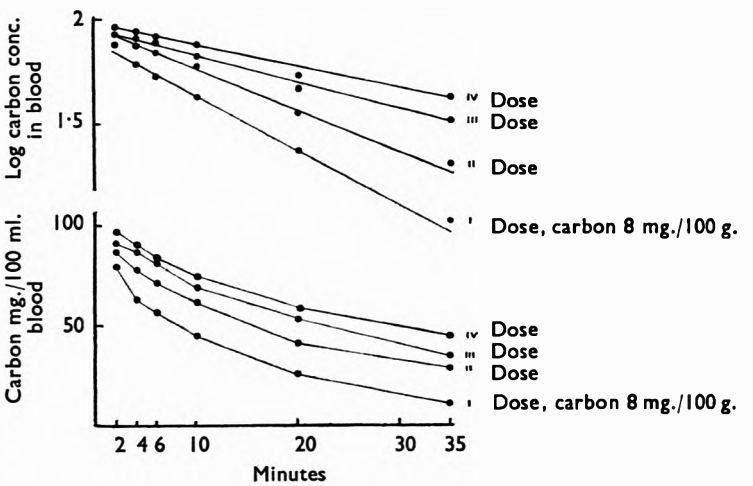


FIG. 4. Blood clearance of 4 subsequent doses of carbon injected intravenously in the rat.

These observations introduce the concept of discriminative phagocytosis, indicating that the avidity of the phagocytes for substances offered simultaneously, may vary widely according to the nature of the colloid. Figure 5 illustrates the phenomenon of discriminative phagocytosis where two different colloids are injected. A carbon suspension (16 mg./100 g.) is injected intravenously and the clearance rate established (K = 0·020). About twenty minutes later, a colloidal heat denatured serum protein suspension (C.A.G.) labelled with $^{131}I$ is injected (1·5 mg./100 g.). The clearance of carbon almost ceases, as the phagocytes take up the C.A.G. particles rather than carbon. As soon as clearance of the C.A.G. particles is completed, the phagocytosis of carbon particles is resumed at about the same rate as before the injection of the denatured proteins.

The phenomenon of discriminative phagocytosis appears to be a very important and fundamental process. Probably, it governs the turn-over of the lipids, lipoproteins, cholesterol, the clearance of the erythrocytes

and of many other normal and pathological substrates. The experimental example reported here, with a system of two colloids, gives some idea of the complexity of this clearance process when several substrates compete simultaneously. It also provides an explanation of certain pathological processes, resulting from the appearance of a pathological compound, or to the alteration of the clearance capacities of the phagocytes.

*Importance of the Stability of the Suspension in the Circulating Blood*

In the phagocytosis of erythrocytes, heterologous or denatured homologous erythrocytes are cleared from circulating blood by the R.E. cells,

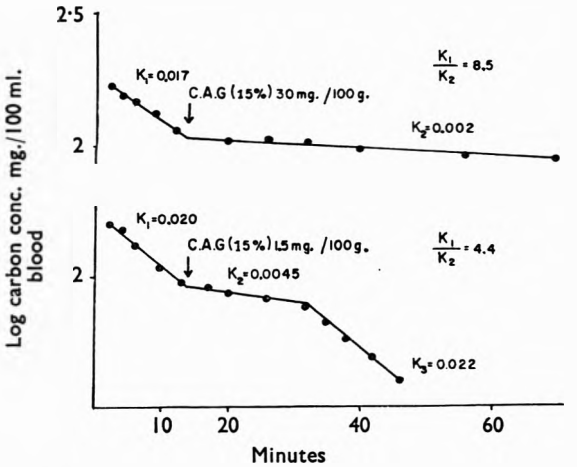


FIG. 5. Effect of the intravenous injection of two doses of rabbit C.A.G. on the clearance of carbon particles in mice for the dose of 16 mg. per 100 g. bodyweight.

mainly those localised in the liver and spleen[20]. In Figure 6 is shown the kinetics of clearance of pigeon erythrocytes labelled with $^{38}P$ injected into mice and rats, in doses of $\frac{1}{4}$, $\frac{1}{2}$, 1, 2 and 5 billions of cells per 100 g. of body weight. The logarithms of the numbers of erythrocytes per ml. of blood are plotted as ordinates against time as abscissae.

In the mouse, the pigeon erthrocytes are cleared by the R.E. cells according to the general principles already discussed. In rats, however, the kinetics of the clearance are by no means the same. The cause of this strikingly different behaviour of the two animal species, towards the same substrate, is due to immunological factors. Rat serum contains natural agglutinins against pigeon erythrocytes, while such antibody does not exist in detectable amounts in the mouse. The evidence that this interpretation is correct is provided by immunisation of mice against pigeon erythrocytes and thereby creating an identical situation which occurs spontaneously in rats (see Fig. 6).

These findings stress the precautions necessary in interpreting the results of clearance rates for the assessment of the phagocytic dynamics of the R.E.S.

*The Clearance Patterns of Bacterial Substrates*

The question now arises whether the principles which govern the clearance of inert particles may be applied without restriction to the phagocytosis of bacterial substrates.

In the ingenious studies of Wright[15] on experimental infections, suspensions of various strains of pneumococci were injected intravenously into

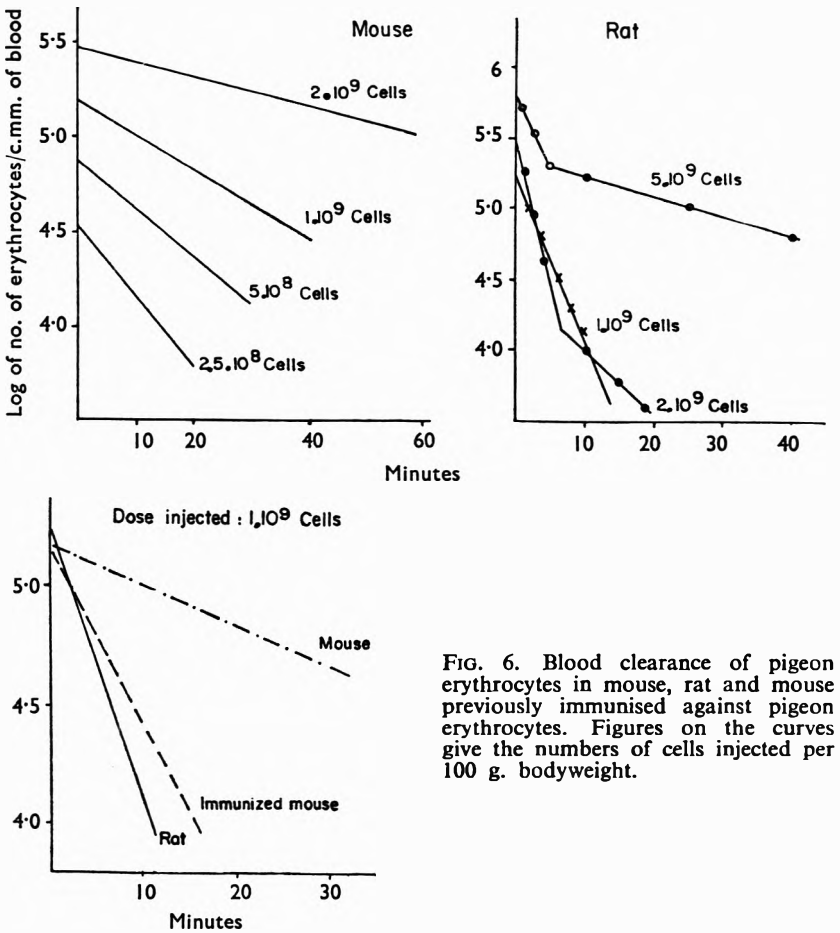


FIG. 6. Blood clearance of pigeon erythrocytes in mouse, rat and mouse previously immunised against pigeon erythrocytes. Figures on the curves give the numbers of cells injected per 100 g. bodyweight.

rabbits and the numbers of the bacteria in the blood counted. In Figure 7, where Wright's results are summarised, logarithms of the number of bacteria per ml. of blood are plotted as ordinate against time as abscissa.

It is evident from these data that the patterns of phagocytosis of living bacteria, while they are grossly similar to the clearance of inert particles, also show significant differences. With living bacteria, clearance is a resultant of phagocytic activity, but also of the virulence of the germs, of their multiplication power, and probably of some other unknown biological factors. To avoid some of these complications, we investigated

the clearance rates of dead bacteria, which being deprived of their virulence and of their capacity of multiplication, can be legitimately likened to inert particles. We used heat killed *Salmonella enteritidis* labelled with $^{51}Cr$. The results are in Figure 8, which shows that their clearance follows a rather complex pattern. The curve is likely to be a resultant of at least two different kinetics. Our feeling is that, with both dead or alive bacteria, the lung interferes by liberating some thromboplastic agents. The reason why this is suggested is the peculiar effect of heparin on the clearance rates of bacteria. Pretreatment of animals with heparin, which does not alter the kinetics of clearance of inert colloids, consistently affects the clearance of bacteria. At the present time, these investigations are in hand and the explanation of the particular clearance patterns of dead bacteria can only be an hypothesis.

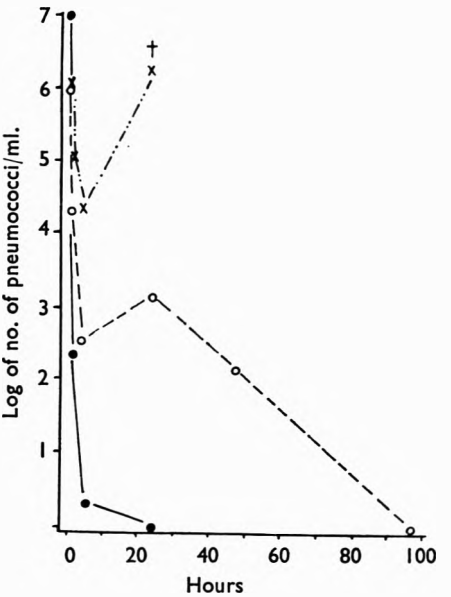


FIG. 7. Blood clearance of living pneumococci according to their virulence in the rabbit. After Wright[15].
●—● Avirulent. o—o Slightly virulent.
×—× Highly virulent. † Died.

*The Clearance Patterns of Endotoxins*

It has been shown by Bennett and Beeson[21] that endotoxins administered intravenously are rapidly removed from the blood and accumulated in the liver and spleen. More recently, Howard[22] proved that they are phagocytised by the R.E. cells of these organs.

Figure 9 illustrates the clearance rates of intravenously injected endotoxins extracted from Gram-negative bacteria and labelled with radioactive phosphorus. The results indicate that although lipopolysaccharides are rapidly removed from the blood, the clearance does not follow the usual exponential function.

Various investigations suggest that this phenomenon is likely to be conditioned by several factors: (i) the toxicity of the substrate to the phagocytes. After the injection of endotoxin, the R.E. cells are likely to be damaged and impaired. (ii) Thomas[23] has shown that the injection of endotoxins produces a modification of the physical state of the circulating fibrinogen. This is evidenced by the appearance in the plasma, about one hour after the injection of endotoxin, of a large amount of protein which is precipitated in the cold by heparin. This heparin-precipitable protein, is not present in normal rabbit plasma and Benacerraf[24] has recently shown that this denatured fibrinogen is phagocytised by

the R.E. cells. The slowing down of the clearance of endotoxins is perhaps related to the appearance in the blood of the fibrinogen aggregates which are phagocytised preferentially to endotoxins. (iii) The third factor is the heterogenous composition of the lipopolysaccharides. Endotoxin contains several constituents which differ from others in their molecular size. The larger molecular constituents are removed quite rapidly while about 30 per cent of the injected lipopolysaccharides were found to remain in the blood and to be only gradually eliminated in the course of 24 hours.

*Factors which Stimulate the Activity of the R.E. Cells*

The R.E.S. seems to be autonomous. In general, the activity of the R.E.S. escapes the control of the central and vegetative nervous systems. In fact, it has been shown by perfusion of isolated liver[25] that the phagocytic activity of the R.E. cells obeys grossly the same principles as *in vivo*. Owing to their anatomical situation, the cells are dependent upon the blood supply which is one of the main factors affecting phagocytosis. It has been claimed in the literature that other substances, like histamine or antihistamine drugs[26], exert more or less specific effects on the phagocytosis of the R.E. cells. In our investigations, we have been unable to ascertain such an effect except when, by gross vascular changes, the blood supply to the phagocytic organs has been impaired. On the other hand, the removal of the various endocrine glands has never produced any consistent action. Cortisone at high doses, depresses the recuperation phase of the R.E.S. after injection of a blocking dose of colloid, very likely due to its antiproliferative effects, so does nitrogen mustard[27]. Among the numerous substances and hormones investigated, certain steroids belonging to the oestrogen group were found to have an unequivocal stimulant effect on the R.E.S.[28–30]. Surprisingly enough the enhancing properties of oestradiol and diethylstilboestrol on the R.E. cell activity could be only ascertained in a simple animal species, the mouse. All the attempts to prove a similar effect in other laboratory animal species failed. The reason of this species specificity remains unknown.

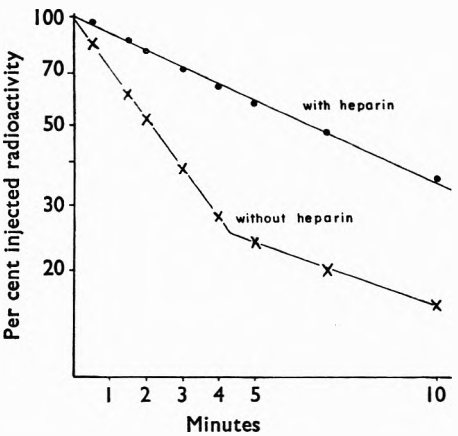


FIG. 8. Blood clearance of radiolabelled *Salmonella enteritidis* in normal and heparinised mice. Dose injected $1 \cdot 10^9$ organisms per 20 g.

At this point, I would like to mention one of the very curious properties of the R.E. cells which is related to the process of stimulation.

I discussed previously the injection of a high dose of a substrate phagocytised by R.E. cells, which is followed, for a short while, by a reduction

of the phagocytic activity. If the colloid does not contain an ingredient toxic to the cells, the animal recovers the normal phagocytic function in 2–3 days. Generally, the phagocytic activity of R.E.S. remains overstimulated during that period. This recovery and overstimulation are always associated with a hypertrophy of the main phagocytic organs namely the liver and spleen. A subsequent injection of the colloidal substrate produces a new and stronger stimulation of the R.E. cells and, by repeating the injections of the colloid, this phenomenon can be remarkably amplified (Fig. 10). This characteristic action on the R.E.S. is even more striking with certain bacteriological constituents especially endotoxins extracted from Gram-negative bacteria.

As shown by Howard and his colleagues[22], a dose as low as 10 $\mu$g. of purified lipid produces a typical effect. As with other colloids, a biphasic action is observed with endotoxins, especially when they are administered intravenously. During the first 8 hours, a definite decrease of activity is noticed, followed by a return to normal values. Subsequent injection of endotoxin will enhance the functional activity of the R.E.S. We found recently that more powerful and durable stimulation can be obtained with certain bacilli belonging to the family of mycobacteria[31]. The data in Figure 11 illustrate the action of 1 mg. of B.C.G. in mice. The maximal effect is observed about 2 to 3 weeks after the injection and enhanced rates of clearance are still evident 30 to 40 days later.

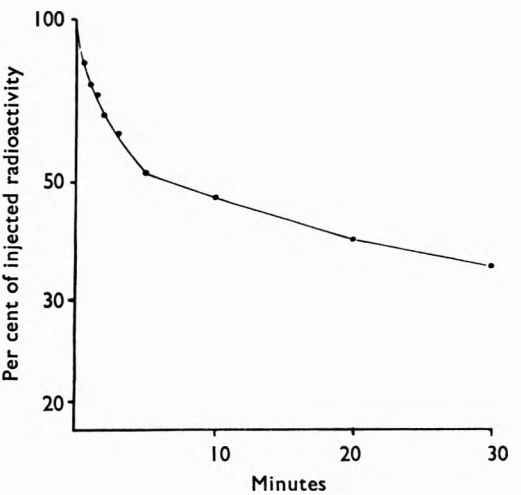


FIG. 9. Blood clearance of radiolabelled ($^{32}$P) *E. coli* polysaccharide endotoxin in the mouse. Dose 40 $\mu$g. per 20 g.

There are two main points which should be emphasised. The modifications of the phagocytic activity are paralleled with the changes of the metabolic activity of the R.E. cells. The metabolic activity of the Kupffer cells has been measured by a new technique which has been recently devised in my laboratory and which consists in the determination of the rate of breakdown of a labelled denatured colloidal protein phagocytised by these cells[33]. We are therefore entitled to infer that changes of the phagocytic function reflects changes in the metabolic (enzymatic) activity of the cell. The second point is the hypertrophy of the liver and spleen always associated with the increased activity of the R.E.S. The gain in weight is not a simple vascular effect but an increase in the protoplasmic mass of the organ. It is very unlikely that the increase in weight which

attains sometimes 50 to 100 per cent or even more is attributable to the multiplication of the R.E. cells only. We are dealing apparently with a special type of regulation of histogenicity which ensures an equilibrium between the number of the R.E. cells and the cellular mass of the organ. The nature and mechanism of this regulation remains unknown.

The discovery of substances capable of stimulating the metabolic and phagocytic activity of the R.E. cells will make it possible to correlate the enhancement of these functions and the other immunological features in

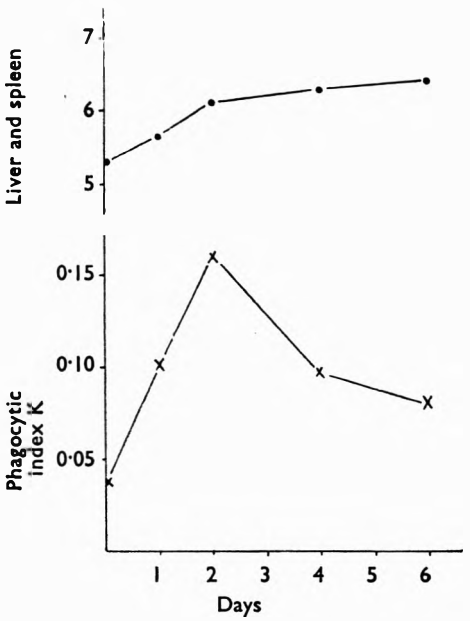


FIG. 10. Effect of several injections of rabbit C.A.G. on the weight of liver and spleen (g./100g.) and on the phagocytic activity of the R.E.S. of the rat measured by the index K for the dose of 8 mg. of carbon per 100 g. in the days following the treatment.

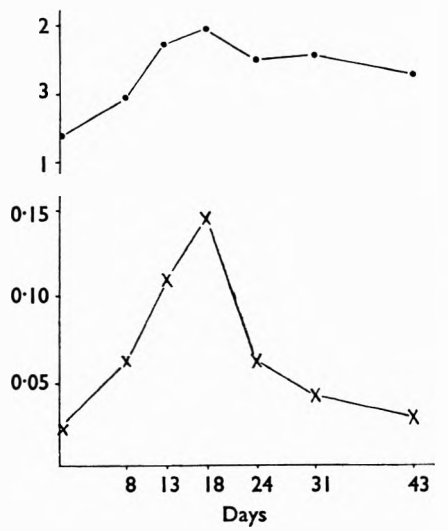


FIG. 11. Effect of infection with living B.C.G. on the weight of liver and spleen (g./20g.) on the phagocytic activity of the R.E.S. measured by the index K for the dose of 16 mg. of carbon per 100 g. in mice.

which the R.E.S. is supposed to be involved. This brings me to the discussion of the second point of my subject, the immunological implications of the R.E.S.

## THE RETICULO-ENDOTHELIAL SYSTEM AND IMMUNITY

The sessile R.E. cells may play an important part in the resistance against bacterial infections either by systemic phagocytosis, or through other immunological processes.

### *Phagocytosis and Immunity*

As long as we regard bacteria merely as inert particles, the principles discussed previously concerning the clearance of colloids need only little modification. But, living bacteria are capable of multiplication and it is

the balance between their capacity to multiply and the capacity of tissues to clear them that determines the fate of the host.

It seems that the avirulent bacteria are digested and killed by intracellular enzymes but there is a great deal of evidence that virulent bacteria, when phagocytised continue to multiply vigorously in the cells and it is the cell which dies, liberating living virulent bacteria in the blood stream. In such situation, the sessile phagocytes constitute the ultimate elements available for the defence of the organism.

Furthermore the decisive role of the sessile phagocytes has been established by evidence showing that their efficiency in protection of the organism against infection is greater than that of other tissues, as shown by Dutton[34] recently. He measured the infectivity for mice of graded doses of bacteria introduced by various routes of injection. At critical infecting levels, the dose required to kill the animal by intravenous injection was, in most instances, larger than the subcutaneous killing doses. With *Pneumococci* and *Salmonella typhimurium*, for example, the difference was over 100 fold. The superior defensive power of the sessile phagocytes is probably a matter of number. But whatever the relative defensive value, cell for cell, for a given germ, the defence afforded by the system of the sessile phagocytes is much more effective than that of local tissue lodgement. I fully agree with Miles when he states[4] that systemic phagocytosis is an integral part of the defence from the earliest stage of infection onwards and not only a mechanism which comes into play when local defences have definitely failed.

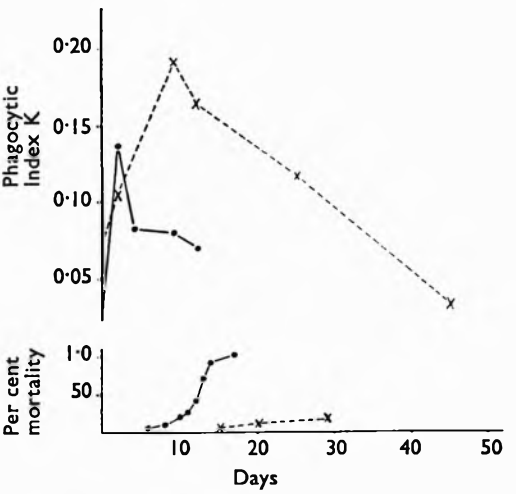


FIG. 12. Effect of an infection with *S. typhimurium* ($10^8$ organisms intraperitoneally) in control rats and rats treated with horse C.A.G. on the mortality and on the phagocytic index K for the dose of 8 mg. of carbon per 100 g. C.A.G. = 5 injections (50 mg.) in three days intravenously. The animals are infected 24 hours after the last injection.

●—● Control. ×—× Treated.

*The Functional State of the R.E.S. and the Resistance to Infections*

I shall attempt to provide evidence that experimentally induced modifications of the functional condition of the R.E. cells is related to a change in the resistance of animals to severe infections. Furthermore, the changes of the activity of R.E.S. are reflected in a change in the susceptibility of the animal to certain endotoxins.

As mentioned before, the injection of some colloidal substrates produces an enhancement of the phagocytic and metabolic activity of the R.E. cells. Experiments have been carried out to investigate whether animals whose R.E.S. has been so stimulated, show differences in resistance to severe experimental infections.

Stimulation of the R.E.S. has been accomplished with different substances.

(i) *C.A.G.* In the first group of experiments the R.E.S. was stimulated with a colloidal protein, obtained by heating serum proteins at 60°. This

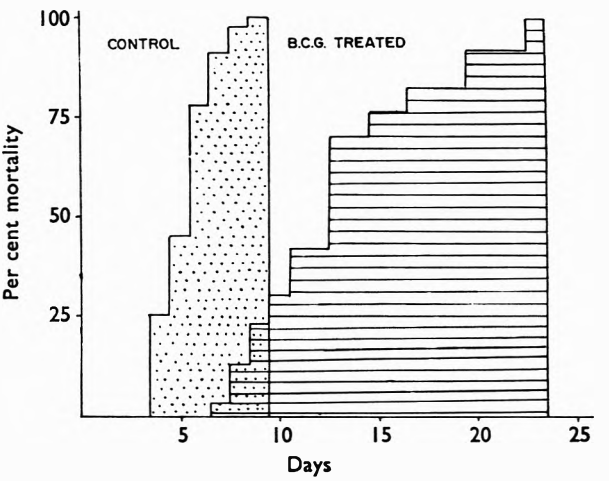


FIG. 13. The effect of B.C.G. infection on the survival time of mice infected with *Salmonella enteritidis*.

colloid is selectively phagocytised by the R.E. cells and repetition of injections causes a significant stimulation of the R.E.S.[35]. The results are shown in Figure 12.

A group of treated and control rats were infected with the same dose of a suspension of *Salmonella typhimurium*, injected intraperitoneally, and the sequences of the infections studied. It is evident from Figure 12 that animals whose R.E.S. has been stimulated previously were able to resist infection much better than the controls, as on the 17th day nearly all control animals died, while 95 per cent of the treated animals were alive. On the other hand, in the treated group, the phagocytic activity of the R.E.S. was greatly stimulated during the course of infection and this hyperactivity lasted until recovery was achieved, while in the control group, a vigorous stimulation of the R.E.S. was observed in the first days, but was shortly followed by a decrease in the phagocytic index until death occurred.

(ii) *Stimulation with B.C.G.* The infection of mice with living B.C.G. leads to a more intense and lasting stimulation of the phagocytic and metabolic activity of the R.E. cells[31]. To assess the development of a mortal infection in treated animals in comparison with the control group,

*Salmonella enteritidis* (var. *Danysz*) was used. The infecting dose was administered intravenously and was the LD100 for the control group[32].

From Figure 13 it can be seen that although there were no survivors amongst either control or treated animals due to the severity of the infection, the mean survival time was greatly increased in the later group.

By what mechanism does the over-stimulation of the R.E.S. increase the resistance of the animals to infections?

The remarkable studies of Wright put beyond doubt that the increase in phagocytic intensity is at least partly responsible for the increased immunity.

It is also likely that the increase in survival time of animals pretreated with B.C.G. is due to the fact that the multiplication of the phagocytized germ is either prevented or significantly retarded, as B.C.G. enhances not only phagocytosis but also the metabolic rate of R.E. cells[32].

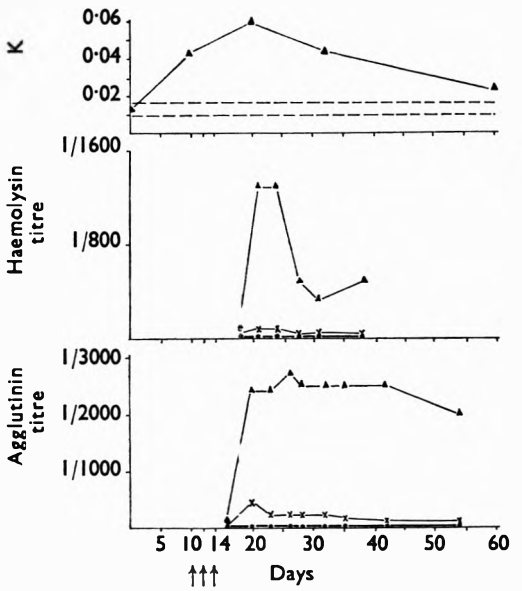


FIG. 14. Phagocytic function of the R.E.S. in mice infected with B.C.G. Antibody production in X—X, control mice, in ▲—▲ mice treated with B.C.G. and in ●—● mice splenectomised and treated with B.C.G. The arrows indicated the injections of antigen.

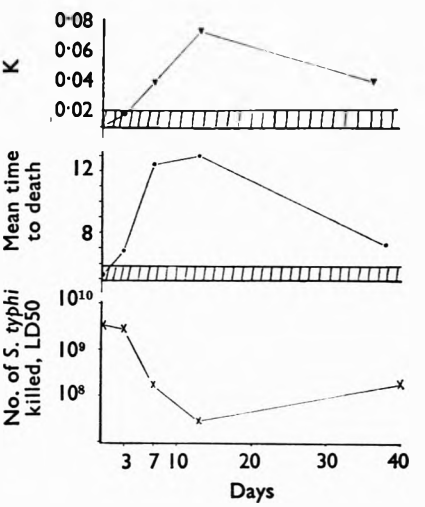


FIG. 15. Change in phagocytic index K of the R.E.S. in resistance to endotoxin LD50 of killed *Salmonella typhi* per 20 g. and resistance to infection with *Salmonella enteridis* (mean survival time after 1000 organisms intra-intravenously) at different times after infection with B.C.G. in mice. The standard deviation of control mice is indicated.

But enhancement of resistance in animals to severe infection may imply the presence of another mechanism: the production of antibody. Experiments in mice, treated with B.C.G., show clearly that the rate of antibody production is also significantly increased[36].

In the experiments summarized in Figure 14, the rates of production of two different types of antibodies—agglutinins and hemolysins—have been studied in normal animals and in animals treated with B.C.G. It is obvious from the data represented that a remarkable increase in antibody titres is observed in the B.C.G.-treated animals, compared with the controls. The antibody response to the two different antigens is enhanced and this observation suggests that this reaction is a general feature.

The role of the R.E.S. in the production of the antibody is evidenced by removal of a part of the R.E.S. by splenectomy. It is obvious from data presented in Figure 15 that the titre of agglutinins and hemolysins is considerably reduced in splenectomized animals, even in those which have been treated with B.C.G.

I am aware that the spleen contains besides R.E. elements many other cells, to which an important role has been conferred in the production of antibody. But a glance at the modern literature shows that the morphologists are far from being in agreement on the genealogical relationships between the R.E. cells and the other types of cells, such as immature plasmocytes, to which a main role in production of antibody has been attributed, and I do not propose to involve myself in such a discussion.

*Correlation Between Stimulation of the R.E.S. and Resistance to Endotoxins*

Endotoxins, as we have seen, are removed from the circulation by the R.E. cells. They have, *per se*, a potent stimulating action on the R.E.S. and resistance to endotoxins[23] has been considered by several authors to develop as a result of this action. Endotoxins, on the other hand, increase the resistance of animals to infections, as proved recently by Rowley and his colleagues[37].

Whether and to what extent resistance to infections, that has been enhanced by administration of B.C.G. to animals, has some parallel increase in resistance to endotoxins has not yet been clarified. The results obtained show that such a correlation does not always exist[38].

As shown in Figure 15, B.C.G.-treated animals, which have an enhanced phagocytic activity, and also an increased resistance to infections, become highly sensitive to endotoxins. The lethal dose of endotoxin in B.C.G.-treated animals is about 100 times lower than in normal controls. These findings are apparently in conflict with current opinion about the relation between the functional state of the R.E.S. and sensitivity to endotoxins. But the opposition is more apparent than real.

Our recent investigations suggest that the increased susceptibility of B.C.G.-treated animals to endotoxin, results from a particular state of immunity caused by the tubercle bacillus. The phenomenon of enhanced susceptibility to endotoxins is related to the increased vulnerability of the blood vessels observed in animals infected with tubercle bacilli and is similar to a generalised Shwartzman phenomenon.

As a matter of fact this susceptibility to endotoxins is only observed when stimulation of the R.E.S. is induced with the tubercle bacillus, but not when oestrogens or other colloids are used.

Thus from the work discussed in this article it is apparent that the R.E. cells form the keystone of immunity. By their powerful and practically unlimited phagocytic capacity, the sessile phagocytes form the fundamental system for clearing bacterial endotoxins from the circulating blood. In turn, the phagocytised substrates stimulate the activity of the cells, inducing a kind of self-stimulating process, the possibilities of which are limited only by the toxic effects of the ingested material on the phagocytes and their proliferative capacity.

Stimulation of the phagocytes may be associated with an increase of the metabolic enzymatic activity of the R.E. cells. It may be deduced that intracellular antibacterial mechanisms, still largely unknown, may be affected in similar manner. This may provide the explanation of the increased resistance to infections found in animals whose R.E.S. has been over-stimulated.

If phagocytosis is beyond doubt one of the means by which the R.E. cells are involved in the enhancement of immunity, our recent investigations show pertinently that animals whose R.E.S. has been stimulated are able to respond by an increased antibody production. Our results indicate that, in the laboratory animals, when antigens are administered intravenously, the spleen is the main organ involved in antibody production, while the Kupffer cells form essentially the clearance system.

For many of the experimental observations reported here I am as yet unable to offer a firm or definite explanation. These are completely new problems and no doubt some of our present hypotheses may be questioned by to-morrow's findings.

However, it is my conviction that the R.E.S. is of considerable importance and I believe that the now widely conducted and intensive investigations will underline this.

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# RESEARCH PAPERS

## THE EFFECT OF SALICYLATES ON THE THYMUS GLAND OF THE IMMATURE RAT

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Salicylic acid and its derivative, acetylsalicylic acid, induced thymic atrophy not only in intact immature rats but also in adrenalectomised immature animals when administered by mouth in the diet, or by subcutaneous injection. A straight line with a negative slope was obtained by plotting the log dose of salicylate against the relative thymus weight. This linear relation was used to compare quantitatively the thymolytic activities of acetylsalicylic acid, ethyl salicylate, and salicylamide with those of salicylic acid. The results of this investigation suggested that salicyl compounds act directly on the thymus gland and not by stimulating the pituitary-adrenal system. No evidence was found to indicate that salicylates potentiate the thymolytic action of adrenal corticosteroids.

Salicylates have been used for the treatment of rheumatic diseases since 1876[1]. When corticotrophin and the cortisone-like steroids were found to possess antirheumatic properties, it was assumed that salicylates acted by stimulating the pituitary-adrenal system. Support for this theory regarding the mode of action of the salicylates was obtained by the observation that salicylates induced depletion of adrenal ascorbic acid in the intact rat[2–7], but not in hypophysectomised animals[8–13]. However, the finding that therapeutic doses of salicylate failed to increase the level of 17-hydroxycorticosteroids in both urine[14] and plasma[15–18] cast some doubt on the validity of this hypothesis. A study of the action of salicylates on carbohydrate metabolism led Smith[1] and later Feeney and others[19], to the conclusion that the effect of salicylate on rheumatic diseases was not mediated through the pituitary-adrenal system, but through some other unknown mechanism.

Salicylates may have a direct adrenal corticoid-like action on some peripheral tissues and exert their therapeutic effect in this manner. Since it is well known that involution of the thymus gland is a manifestation of glucocorticoid-like activity[20–23], a study was made of the action of salicylate and other salicyl derivatives on this target organ. Salicylates were found to cause thymus involution in both adrenalectomised and intact immature rats. The results of this investigation have suggested that (*a*) salicyl compounds act directly on the thymus gland of the immature rat, (*b*) do not specifically influence the pituitary-adrenal system, and (*c*) do not potentiate the effect of endogenous adrenal corticoids. The linear relation which exists between the log dose of the salicyl derivative and the relative thymus weight was used to compare the relative thymolytic activity of several of these compounds.

## Experimental Methods

The test animals were 23 to 28 day-old albino rats derived from the Wistar strain. The compounds under investigation were administered either orally or by subcutaneous injection. In the oral test, the salicyl derivatives were mixed thoroughly in the standard laboratory diet*, and when a free acid was studied, sufficient sodium bicarbonate was added to neutralise the acid and thus prevent any change in the acid-base balance of the test animals. In the studies involving subcutaneous administration, the compounds were dissolved in isotonic saline adjusted

TABLE I

Thymolytic activity of various salicyl compounds administered orally to immature rats

| Compound | Concentration of salicyl compound in the diet per cent | Thymus weight per cent |
|---|---|---|
| Control | 0 | 100·0 |
| Sodium salicylate | 0·29 | 89·5* |
| | 0·58 | 69·4* |
| Salicylic acid | 0·25 | 88·4* |
| | 0·50 | 66·0* |
| Acetylsalicylic acid | 0·25 | 92·5 |
| | 0·50 | 81·7* |
| Ethyl salicylate | 0·35 | 92·9 |
| | 1·00 | 70·5* |
| Salicyl alcohol | 0·25 | 96·4 |
| | 0·50 | 91·3 |
| Salicylamide | 0·25 | 107·0 |
| | 0·50 | 101·0 |
| | 1·00 | 105·0 |
| | 4·00 | 87·2* |
| Sodium *p*-aminosalicylate | 0·25 | 100·5 |
| | 2·00 | 102·1 |

* Significant decrease in relative thymus weight (P = 0·95)

to approximately pH 7. A total of 8 to 10 rats was assigned at random to each dose group, with the restriction that the average body weight was the same for all dose groups in any one experiment. The rats were caged in pairs and the quantity of food offered per day was adjusted so that the caloric intake of each pair of animals was approximately the same at all dose levels of the salicyl derivative. The actual amount of food consumed daily by each pair of rats over the 2 to 3 day test period was recorded to provide an estimate of the average dose of the compound under investigation. The animals were allowed access to the food up to the time of death. On the other hand, in the tests in which the salicylates were injected subcutaneously in divided doses over a 2 to 3 day period, the thymus glands were removed and weighed approximately 20 hours after the last injection.

When adrenalectomies were done, the glands were removed by the lumbar route under ether anesthesia. These animals were given 1 per

* Master Fox Starter Meal—Toronto Elevators Ltd.

cent saline to drink and the stock laboratory diet *ad libitum* as well as a daily subcutaneous injection of 0·1 mg. of desoxycorticosterone acetate in aqueous suspension for a period of 1 week before the test. By following this regimen the adrenalectomised rats appeared to be in good physical

TABLE II

EFFECT OF SALICYLATE ON THE THYMUS GLAND OF THE IMMATURE RAT

| Salicylate in the diet* per cent | Average total intake per rat. mg. | Relative thymus wt. mg./100 g. of rat | Reduction per cent in thymus wt. |
|---|---|---|---|
| 0 | 0 | 426·5 ± 16·0 | 0 |
| 0·0625 | 14 | 407·6 ± 6·6 | 4·4 |
| 0·125 | 27 | 401·3 ± 9·7 | 5·9 |
| 0·25 | 47 | 352·3 ± 9·2† | 17·4 |
| 0·50 | 67 | 286·4 ± 13·8† | 32·8 |

* Salicylic acid mixed with an equal quantity of sodium bicarbonate in the diet.
† Significant at 1 per cent level.

condition and maintained their body weight. The rats were checked for adrenal remnants when the thymus glands were removed at the end of the experiment. Previous work has shown that this dose of desoxycorticosterone acetate had no effect on the thymus gland of the immature rat[23].

## RESULTS

The data in Table I show the effect of salicylate and other salicyl derivatives on the weight of the thymus gland of the immature intact rat, when administered in the diet for several days. Salicylamide did not induce thymic atrophy when fed at a concentration of 1 per cent of the diet but caused significant involution when the amount reached 4 per cent of the daily food. In contrast, salicylate and acetylsalicylate

TABLE III

RELATION BETWEEN THE LOG DOSE OF SALICYLIC ACID AND THE RELATIVE THYMUS WEIGHT IN THE IMMATURE RAT

Let Y = the relative thymus weight, and X = the log dose of salicylic acid.
Equation of the regression line = Y = 825·7 − 290·8 X

ANALYSIS OF VARIANCE TABLE

| Source of variation | d.f. | s.s. | m.s. | "F" | P |
|---|---|---|---|---|---|
| Between dose groups | | | | | |
| (1) linear regression .. .. | 1 | 63850 | 63850 | 51·868 | 0·01 |
| (2) deviation from regression .. | 1 | 2637 | 2637 | 2·142 | 0·05 |
| Within dose groups (error) .. .. | 27 | 33226 | 1231 | | |
| Total .. .. .. .. | 29 | 99713 | | | |

decreased the thymus weight significantly when given at a concentration of less than 0·5 per cent of the diet. Although salicyl alcohol only showed a tendency to lower the thymus weight when the diet contained 0·5 per cent of this compound, it is probable that thymus involution would have occurred if a larger quantity had been administered. On the other hand, sodium *p*-aminosalicylate did not reveal any thymolytic activity even at the 2 per cent concentration.

The results in Table II demonstrate that the decrease in thymus weight induced by the oral administration of salicylate for 3 days, is proportional to the dose. By using the values for 0·125 per cent, 0·25 per cent and 0·5 per cent shown in Table II, it was possible to calculate a regression line relating the log dose of salicylic acid and the weight of the thymus gland. The analysis of variance, with regression, given in Table III

TABLE IV

RELATIVE POTENCY OF SALICYLATES IN THE IMMATURE RAT

| Compound | Thymus involution assay* | | | |
|---|---|---|---|---|
| | Weight basis | | Equimolar basis | |
| | Potency | Fiducial limits† | Potency | Fiducial limits† |
| Salicylate .. .. .. | 100 | | 100 | |
| Acetylsalicylate .. .. | 70·4 | 63–78 | 91·8 | 82–101 |
| Ethyl salicylate .. .. | 49·8 | 40–69 | 60·0 | 49–83 |
| Salicylamide .. .. .. | 7·7 | 6–10 | 7·8 | 6–10 |

* Oral administration for 5 days; thymectomy in the sixth day. † P = 0·95.

illustrates that this relation is linear and that the slope of the regression line is highly significant. A modification of the thymus involution assay for adrenal corticoids[23] was employed to determine the potency of several salicyl derivatives relative to salicylate using intact rats and administering the compounds orally in the diet at two dose levels. Each of the values shown in Table IV represents the weighted mean of at least two valid

TABLE V

THYMOLYTIC ACTION OF ACETYLSALICYLIC ACID (ASA) IN THE IMMATURE RAT

| ASA per cent in the diet* | Normal rats | | | Adrenalectomised rats† | | |
|---|---|---|---|---|---|---|
| | Average total intake of ASA per rat mg. | Relative thymus wt. mg./100 g. of rat | Reduction per cent in thymus wt. | Average total intake of ASA per rat mg. | Relative thymus wt. mg./100 g. of rat | Reduction per cent in thymus wt. |
| 0 | 0 | 419·6 ± 13·1 | 0 | 0 | 550·0 ± 19·5 | 0 |
| 0·25 | 71·4 | 368·2 ± 9·2 | 12·2 | 72·2 | 457·7 ± 12·3 | 16·8 |
| 0·50 | 137·0 | 302·6 ± 11·3 | 27·9 | 125·3 | 404·4 ± 12·4 | 26·5 |

* Acetylsalicylic acid mixed with an equal quantity of sodium bicarbonate in the diet.
† Maintained for 7 days on 1 per cent saline as drinking water and 0·1 mg. desoxycorticosterone acetate daily before feeding the acetylsalicylic acid.

assays by the thymus involution method. On an equimolar basis, acetylsalicylate was about 90 per cent and ethyl salicylate approximately 60 per cent as active as salicylate. In contrast, salicylamide was less than 10 per cent as potent.

The next step in this study was an investigation of the mode of action of salicylate in causing thymic involution in the intact rat. The effect of acetylsalicylate administered orally for 5 days to intact and adrenalectomised rats was investigated and the data in Table V show clearly that this compound had approximately the same action in both groups. This experiment was repeated by administering the acetylsalicylate subcutaneously over a 2-day period. Again it was possible to demonstrate

in both groups atrophic changes in the thymus gland. The data in Table VI indicate that the per cent reduction in thymus weight was somewhat greater in the intact group than it was in the adrenalectomised rats at the higher dose levels of acetylsalicylate. A significant involution was obtained at both the 10 and 20 mg. dose levels in the intact rat, but only at the 20 mg. level in the adrenalectomised animal. This slight

TABLE VI

THYMOLYTIC ACTION OF ACETYLSALICYLIC ACID AFTER SUBCUTANEOUS INJECTION IN THE IMMATURE RAT

| | Normal rats | | | Adrenalectomised rats | | |
|---|---|---|---|---|---|---|
| Total dose of ASA* mg. | No. of rats | Relative thymus wt. mg./100 g. of rat | Reduction per cent in thymus wt. | No. of rats | Relative thymus wt. mg./100 g. of rat | Reduction per cent in thymus wt. |
| 0 | 25 | 392·2 ± 5·5 | 0 | 15 | 433·5 ± 8·5 | 0 |
| 5 | 5 | 378·2 ± 9·8 | 3·6 | 5 | 417·4 ± 19·2 | 3·7 |
| 10 | 15 | 368·7 ± 7·8† | 6·0 | 10 | 415·8 ± 20·9 | 4·1 |
| 20 | 45 | 346·0 ± 4·7† | 11·8 | 20 | 401·1 ± 6·5† | 7·5 |

* Acetylsalicylic acid (ASA) was neutralised with sodium bicarbonate and administered in five subcutaneous injections over a 2-day period.
† Significant reduction in thymus weight.

difference in the effect was not observed when the acetylsalicylate was given orally. Previous experience with the thymus involution assay of adrenal corticosteroids has shown that the small amount of stress which occurs as a result of local irritation at the site of injection can have an effect on the thymus weight. Hence this slight stimulation of the pituitary-adrenal system in the intact rat can reasonably account for this difference in response between the two groups.

TABLE VII

EFFECT OF SALICYLATE ON THE THYMOLYTIC ACTION OF HYDROCORTISONE IN THE IMMATURE RAT

| Treatment | | Average per cent reduction in relative thymus wt.* | |
|---|---|---|---|
| | Total dose mg. | Intact rats per cent | Adrenalectomised rats per cent |
| Control .. .. .. .. .. | 0 | 0 | 0 |
| Sodium salicylate .. .. .. | 40 | 24·3 ± 3·1† | 16·0 ± 11·3† |
| Hydrocortisone .. .. .. | 0·3 | 32·3 ± 7·5 | 31·3 ± 2·5 |
| Sodium salicylate + hydrocortisone .. | 40 + 0·3 | 44·0 ± 1·7 | 42·8 ± 3·0 |

* Average of three replicate experiments. † Standard deviation of individual replicates.

A series of three experiments was carried out in both intact and adrenalectomised rats to investigate the possibility that salicylates exert their effect on the thymus gland of the immature rat by potentiating the action of adrenal corticoids. The compounds employed in this study were dissolved in a neutral aqueous medium containing 10 per cent of ethanol and 0·9 per cent of sodium chloride. The total doses described in Table VII were administered in a series of five divided subcutaneous

injections over a 2-day period. Table VII shows the average percentage reduction in the relative thymus weight in the intact and adrenalectomised rats. The standard deviations given in Table VII provide only an estimate of the variation observed between the replicate tests and do not represent the variation in the individual reponses of the rats. These data give no support to the hypothesis that the thymolytic action of sodium salicylate depends on the presence of adrenal corticoids like hydrocortisone because the percentage reduction in the relative thymus weight obtained when the compounds were given together was less than the sum of the individual effects.

## Discussion

According to Hart[24] salicylamide is considerably less toxic than acetylsalicylic acid in the rat when repeated doses are given at daily intervals. Evidence to support this observation was obtained by Seeberg, Hansen and Whitney[25] who reported that while salicylamide was readily absorbed from the gastrointestinal tract of the rat, the serum concentration was lower than that of other salicyl derivatives when the compounds were administered at equal dose levels. In addition salicylamide was found to be less irritant than acetylsalicylic acid to the gastric mucosa of the rat.

Our results on the thymolytic activity of salicylamide relative to salicylate tend to confirm these findings. It is possible that the concentration of salicylamide in the blood at doses comparable to those of salicylate may have been too low to cause involution of the thymus gland. It was also noted that the rats would not tolerate levels of salicylate in the diet much beyond 0·5 per cent, whereas salicylamide could be fed at a concentration of 4 per cent of the diet without any significant impairment of the daily food intake.

However, the supposedly low serum concentration of salicylamide and its less irritant properties may not be entirely responsible for the relatively low thymolytic activity. The results shown in Tables I and IV suggest that compounds with a free carboxyl group such as salicylic acid and its derivatives, with the exception of sodium *p*-aminosalicylate, were the most active in causing thymic atrophy. Neither salicylamide nor salicyl alcohol are true salicylates because they lack a carboxyl group in the position ortho to the hydroxyl group. While the formation of the sodium salt had no effect on the thymolytic activity, esterification of salicylic acid with ethanol reduced the potency by approximately 40 per cent.

The data presented in Tables V, VI and VII suggest that salicylate acted directly on the thymus gland of the immature rat. Salicylate did not appear to primarily induce thymus involution by stimulating the pituitary-adrenal system, although it is reasonable to assume that handling and irritation at the site of injection caused some endogenous production of adrenal corticosteroids. The results given in Table VII show that the percentage reduction in the relative thymus weight obtained when sodium salicylate and hydrocortisone were injected together was less than the sum of the individual effects. Therefore it can be concluded that

salicylate did not act by potentiating the thymolytic action of hydrocortisone. The results presented in Table VII suggest that a slight antagonism may even exist between the two compounds., This could be interpreted as evidence of a possible competition between hydrocortisone and salicylate for certain receptor sites on the cells of the thymus gland.

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# THE PHYSICAL PROPERTIES OF LYSOLECITHIN AND ITS SOLS

## PART IV. SOLUBILISATION

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The solubilisation of triolein, cholesterol and monostearin in water by lysolecithin has been investigated by the optical density method. These substances solubilised to a considerable extent, increasing in the order—triolein, cholesterol and monostearin. An increase in temperature of 15° had little effect on solubilisation of any of the three substances. The change in optical density with concentration of the solubilised component showed a similar pattern for the three substances, but the mixed sols formed had different stabilities. Lysolecithin-triolein sols contained only small proportions of triolein and were fluid and stable for no longer than 12 hours whilst lysolecithin-monostearin sols containing high proportions of monostearin formed gels and were stable for at least a month. Lysolecithin-cholesterol sols were unstable showing an initial rise and then a steady decrease in optical density over a period of 72 hours for given concentrations as slow separation of cholesterol was taking place. Factors operative in the solubilisation of each of these three substances by lysolecithin have been suggested.

The clearing action of lysolecithin on aqueous homogenates of whole rat brain and other biological substances has recently been reported[1]. The property of lysolecithin to solubilise in heterogeneous systems might be very different from its action on the pure components in these systems. We thought it desirable, therefore, to investigate more closely the solubilisation of certain pure biological substances by lysolecithin in an aqueous medium and some factors operative in bringing about this solubilising action. The following substances were examined—cholesterol, chemically reactive and insoluble in water; triolein, representative of a typical fat, chemically inactive (except at ethenoid linkages) and also insoluble in water; monostearin, chemically reactive and dispersible in water.

### EXPERIMENTAL

Methods of preparation of the sols are described elsewhere[2]. All mixed sols contained a constant amount of 0·5 per cent w/v of lysolecithin. The sols were centrifuged before each optical density reading.

The optical densities of the sols were measured in a Spekker Absorptiometer (Hilger 506) incorporating H508 neutral density filters and a filament lamp. The sols and pure solvent were contained in matched cells of 5 cm. path length. The absorption spectrum of an aqueous 4 per cent w/v pure lysolecithin sol and the absorption spectra of 4 per cent w/v cholesterol, triolein and 0·4 per cent w/v monostearin solutions, using chloroform as solvent, were measured at room temperature with a Hilger Uvispek.

## Results

The variation of optical density of lysolecithin-triolein sols with concentration of triolein is shown in Figure 1. The variation of optical density of lysolecithin-cholesterol sols with concentration and time is shown in Figures 2 and 3, and for lysolecithin-monostearin sols in Figures 4 and 5.

Pure 0·5 per cent w/v lysolecithin sols (approximately $10^{-2}$ molar) themselves showed an optical density of less than 0·001 in the 5 cm. cell.

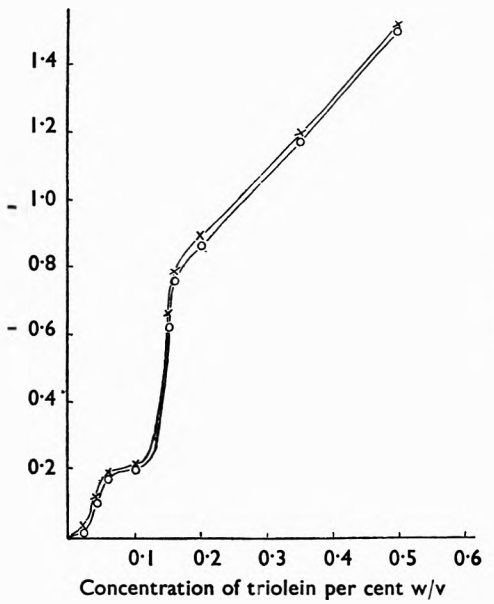


FIG. 1. Lysolecithin-triolein sols. ×, 25°: ○, 40°.

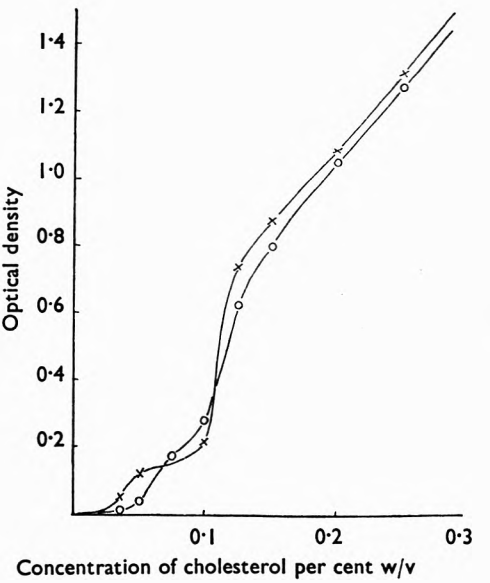


FIG. 2. Lysolecithin-cholesterol sols. ×, 25°: ○, 40°.

Spectrophometric readings for a 4 per cent w/v aqueous pure lysolecithin sol contained in a 4 cm. cell are shown in Table I. Readings for 4 per cent w/v cholesterol and triolein and 0·4 per cent w/v monostearin solutions using chloroform as a solvent are also given. The absorption spectrum within the visible region of the lysolecithin sol showed that light was not predominantly absorbed at any particular wavelength; the spectra of cholesterol, triolein and monostearin showed a similar behaviour.

## Discussion

Solution of the three substances examined must be mainly attributed to the solubilising property of lysolecithin since they do not themselves dissolve appreciably in water. The systems showed an increasing tendency to solubilisation by lysolecithin in the order triolein, cholesterol and monostearin. The ageing of the sols varied and did not follow a pattern; these effects and possible mechanisms of solubilisation are discussed below.

*Lysolecithin-Triolein.* Triolein showed the least tendency to solubilisation by lysolecithin, requiring, for example, a seven-fold ratio of lysolecithin to obtain an arbitrary optical density value of 0·2. The optical

TABLE I

EXTINCTION COEFFICIENTS (*E*) FOR DIFFERENT WAVELENGTHS WITHIN THE VISIBLE REGIONS FOR EACH COMPONENT. TEMP. 20°

| λ(Å) | Aqueous lysolecithin sol* | Cholesterol in chloroform* | Triolein in chloroform* | Monostearin in chloroform† |
|---|---|---|---|---|
| 4200 | 0·242 | 0·097 | 0·382 | 0·024 |
| 4400 | 0·212 | 0·088 | 0·274 | 0·022 |
| 4600 | 0·192 | 0·079 | 0·206 | 0·020 |
| 4800 | 0·172 | 0·074 | 0·152 | 0·018 |
| 5000 | 0·155 | 0·069 | 0·117 | 0·017 |
| 5200 | 0·140 | 0·066 | 0·092 | 0·016 |
| 5400 | 0·128 | 0·059 | 0·074 | 0·015 |
| 5600 | 0·117 | 0·056 | 0·059 | 0·014 |
| 5800 | 0·108 | 0·053 | 0·049 | 0·013 |
| 6000 | 0·097 | 0·050 | 0·038 | 0·012 |

* Concentration 4 per cent  † Concentration 0·4 per cent

density thereupon increased rapidly for increasing concentrations of triolein (Fig. 1). The solubilising power of lysolecithin in this system was influenced very little by an increase in temperature of 15°, and attempts to solubilise more triolein by shaking the sols for periods of up to 18 hours

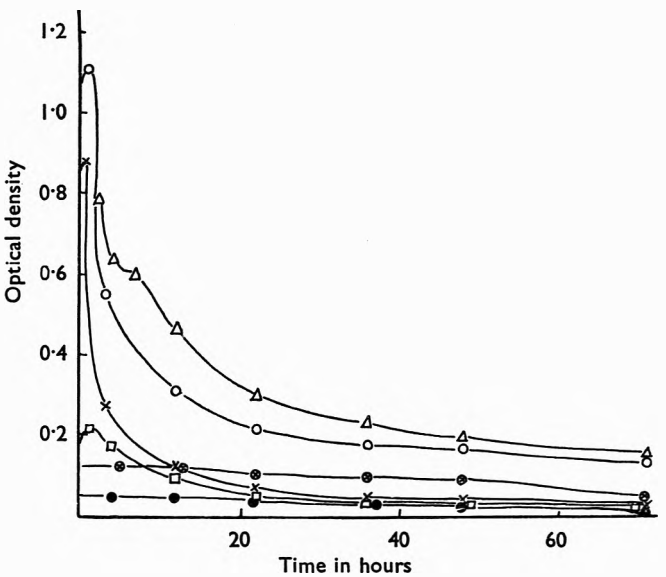


FIG. 3. Lysolecithin-cholesterol sols. Temp. 25°.
●—0·025 per cent w/v cholesterol
⊗—0·05 " " "
□—0·10 " " "
△—0·125 " " "
×—0·15 " " "
○—0·20 " " "

at 40° were unsuccessful. All sols were stable for 12 hours but a film of emulsified triolein formed on the surface of sols containing more than 0·1 per cent w/v triolein after 24 hours, although there was no marked change in optical density during this period and the following 48 hours.

It is probable that steric factors mainly prevent ready solubilisation of triolein in the micelles of lysolecithin. Double bonds in each fatty acid chain of the molecule will inhibit formation of a close-packed micelle and weaken hydrophobic association with the hydrocarbon chain of lysolecithin. The unsaturated linkages situated midway along the hydrocarbon chains of the triolein molecule are probably not suitably placed to undergo dipole-dipole or dipole-ion interaction with the head group of lysolecithin within the micelle.

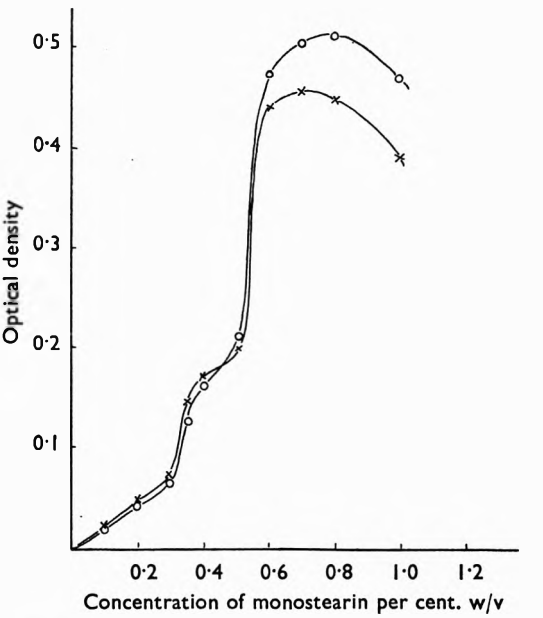


FIG. 4. Lysolecithin-monostearin sols. ×, 25°: ○, 40°

Although both substances were intimately mixed after evaporation of the mutual solvent it is probable that some particles of the triolein were many times bigger than a lysolecithin micelle and formed an emulsified phase which, on shaking and allowing to stand, produced the film observed after 24 hours.

*Lysolecithin-Cholesterol.* Cholesterol required only four times its molar quantity of lysolecithin to give the optical density value of 0·2 quoted for triolein. Again the optical density rose sharply for increasing quantities of cholesterol, an increase of 15° in temperature having small effect (Fig. 2). The ageing of lysolecithin-cholesterol sols was different, showing an initial rise in optical density within the first 5 hours followed by a gradual decline indicating slow separation which continued for another 67 hours (Fig. 3).

Strong dipole forces between the hydrophilic groups of each substance probably helped the lath-shaped cholesterol molecule to orient itself parallel to a lysolecithin molecule with its free hydroxyl group outwards, but its bulkiness ($7{\cdot}5 \times 4{\cdot}5 \times 20$ Å$^3$) would prevent close packing within a spherical micelle. In this mixed sol it is not possible to distinguish clearly between the lysolecithin as a solubilising substance and as a protective colloid.

The interfacial tensions between triolein and water and cholesterol and water are high and it is to be expected that a large excess of lysolecithin would be required to lower the interfacial tensions sufficiently to solubilise these substances completely. The latent heat of fusion involved in the transition of cholesterol from the solid into the liquid state when solubilised in the aqueous sol will also oppose its solubilisation.

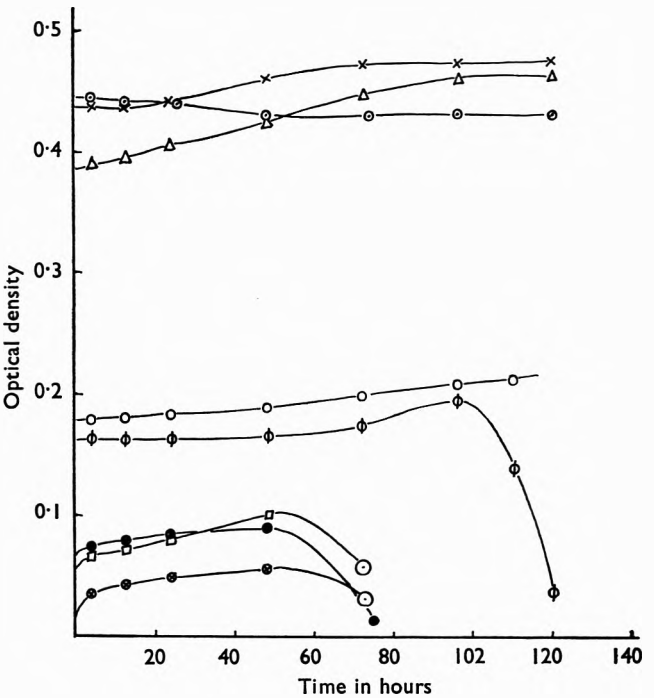


FIG. 5. Lysolecithin-monostearin sols. Temp. 25°.
⊗—0·1 per cent w/v monostearin
□—0·2 ,, ,, ,,
●—0·3 ,, ,, ,,
ϕ —0·4 ,, ,, ,,
○—0·5 ,, ,, ,,
×—0·6 ,, ,, ,,
⊙—0·8 ,, ,, ,,
△—1·0 ,, ,, ,,
⊙—denotes separation of monostearin.

*Lysolecithin-Monostearin.* Monostearin was solubilised in a mol ratio of 1·34 to 1 of lysolecithin at the optical density value of 0·2, the optical density subsequently increasing rapidly to a maximum for a mol ratio of 2:1. In this region the amount solubilised was slightly decreased by a 15° rise in temperature (Fig. 4). Sols containing small fractions (below 0·5 per cent) of monostearin were unstable after 48 hours when the monostearin separated quite rapidly (Fig. 5), but sols of higher proportions of monostearin formed gels which increased their stability and no separation was observed after a month.

Monostearin possesses a hydrophilic head group and a lipophilic region which gives the substance some degree of amphipathic character. In lysolecithin, which has a similar lipophilic region, this property is considerably increased by the presence of the phosphate-choline group giving the molecule complete water solubility and high surface activity due to the balance between the lipophilic and hydrophilic regions. The similarity in structure of these substances enables the monostearin molecules to orient themselves within the micelle, parallel and adjacent to the lysolecithin molecules, with maximum hydrocarbon chain adhesion by van der Waal's forces and dipole-dipole and dipole-ion interaction of the head groups exerting attractive Coulombic forces.

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# DETERMINATION OF LOW CONCENTRATIONS OF SOME ANTIBACTERIAL SUBSTANCES IN SOLUTIONS AFTER CONTACT WITH BACTERIA

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A liquid-liquid extraction procedure is described for the separation of certain antibacterial substances (hexylresorcinol, chloroxylenol, oxine and chloramphenicol) from their solutions after contact with bacteria, before spectrophotometric analysis. The choice of a suitable organic solvent for a particular drug-bacteria system is discussed. The use of the proposed extraction method permitted concurrent determination of the drug and cell exudate ($\lambda$ max approximately 260 m$\mu$) in a single solution. The accuracy and validity of the method is demonstrated.

Ultra-violet spectrophotometric methods of assay of low concentrations of antibacterial substances in solutions obtained after drug-bacteria contact are usually accurate and quick provided the drug has significant light-absorbing properties beyond the 300 m$\mu$ region. Problems arise if the drug absorbs only between 220–300 m$\mu$ because the contact of the drug with bacteria causes the release of a complex mixture of water-soluble constituents with light absorbing properties in this region.

In certain studies of drug-bacteria interaction, it was necessary to determine low concentrations of antibacterial substances, having absorption maxima at wavelengths between 220–300 m$\mu$, in solutions obtained after simple drug-bacteria contact and after growth experiments. The materials released from the bacteria under these conditions are not necessarily identical and the following designations will be made. "Cell exudate" will be used to describe the substances released from bacteria by heat or chemicals, and which absorb light in the ultra-violet region ($\lambda$ max about 260 m$\mu$) due to the presence of purines, pyrimidines and nucleotides[1–4]. The term "cell exudate" is preferred to "bacterial lysis" since the latter has also been used to describe the optical clearing of bacterial suspensions[5]. Substances released simultaneously which do not absorb ultra-violet light will not be considered in the present paper. The permeability and osmotic properties associated with the cytoplasmic membrane[6] will control the release of cell exudate; the cell walls would not constitute a barrier to the escape of substances having relatively low molecular weights (see Mitchell and Moyle[7]). The term "growth exudate" is used to describe the ultra-violet absorbing substances released from bacteria during growth.

A suitable method for the quantitative separation of the drug from the bacterial exudates is described; its application to a study of the interaction of *Staphylococcus aureus* with oxine has been published previously[8].

## Experimental Methods

### *Materials*

*Organic solvents.* Reagent grade solvents were used. (Not all commercial samples of chloroform proved satisfactory in this work.) Diethyl ether (1 l.) was washed with successive portions (600, 300, 300 ml.) of distilled water and redistilled before use. It remained free from peroxides (B.P., 1953, test) for about ten days and gave satisfactory "blank" determinations up to a week after preparation.

*Hexylresorcinol.* Commercial *p-n*-hexylresorcinol was recrystallised from light petroleum (b.p. 40–60°) as colourless needles, m.p. 69° (uncorr.), (Cox[9] gave 68–70°) and log $\epsilon$ 3·42 at $\lambda$ max 280 m$\mu$ in distilled water.

*Chloroxylenol.* Commercial material was recrystallised from light petroleum (b.p. 100–120°) as colourless needles, m.p. 115° (uncorr.), (Vogel[10] gave 114–116°) and log $\epsilon$ 3·11 at $\lambda$ max 279 m$\mu$ in distilled water.

*Oxine.* The physical constants of the material used have been described previously[8].

*Chloramphenicol.* Commercial material was recrystallised from water as pale cream needles, m.p. 150° (uncorr.) and log $\epsilon$ 3·99 at $\lambda$ max 278 m$\mu$ in distilled water [Rebstock and others[11] gave m.p. 150·1° and $E$(1 per cent, 1 cm.) 298 at 278 m$\mu$].

*Bacteria. Aerobacter aerogenes* and *Staph. aureus* as described previously[12,8] and *Escherichia coli*, originally N.C.T.C. 5933, were employed.

*Culture conditions. A. aerogenes* was grown in a simple glucose-inorganic salts medium at 40° for 16 hours with positive pressure aeration. *Staph. aureus* and *E. coli* were cultured on nutrient agar slopes for 24 hours at 37°.

*Absorption measurements.* These were made using matched 1 cm. cuvettes and a Hilger H 700 spectrophotometer.

### *General Extraction Procedure*

After drug-bacteria contact, the bacteria were removed by centrifuging and 25 ml. of the supernatant solution was shaken with portions, usually 25 ml. and 4 × 15 ml., of a water-immiscible solvent. The organic layers were combined, washed with water and evaporated to dryness under reduced pressure. The residue was dissolved in water, using heat if necessary, the solution diluted to 50·0 ml. and examined spectrophotometrically; the drug constituted the only ultra-violet absorbing species in this solution (cf. the results for hexylresorcinol presented in Table I and those for chloramphenicol presented in Fig. 1). The combined aqueous layers containing the cell or growth exudate obtained after extraction were boiled to remove the organic solvent, cooled and diluted to 50·0 ml. and examined spectrophotometrically.

### *Check on the Non-extraction of Exudates by Organic Solvents*

25 ml. volumes of cell and growth exudates derived from bacterial suspensions, corresponding to tube 3 of Brown's opacity tubes, were extracted as described above.

*Growth exudate* was obtained from a 16-hour culture of *A. aerogenes* by centrifuging at 18,000 *g* for 10 minutes to remove the bacteria. *Cell exudate from* E. coli *and* Staph. aureus. A bacterial suspension was obtained by harvesting the bacteria in 0·02 M phosphate buffer at pH 7·0. The bacteria were washed twice and resuspended in the same medium

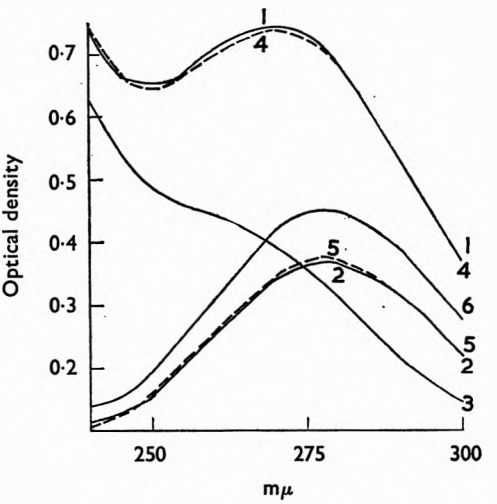


FIG. 1. Ultra-violet absorption curves of solutions obtained before and after extraction of chloramphenicol from a solution containing *A. aerogenes* growth exudate.

1. Solution before extraction.
2. Aqueous solution of the chloramphenicol extracted.
3. Aqueous solution remaining after extraction of the original solution.
4. Calculated curve for the initial solution obtained by addition of curves 2 and 3.
5. Calculated curve for chloramphenicol obtained by subtraction of curve 3 from curve 1.
6. Typical curve for an aqueous solution of chloramphenicol.

before keeping the suspension at 70° for 30 minutes, or alternatively at 100° for 10 minutes. The cell exudate was obtained after removing the bacteria by centrifuging.

*Blank Check of Organic Solvents*

Water was substituted for the drug solution in the general extraction procedure described above. Both the aqueous layer, after removal of the organic solvent by heating, and the aqueous solution obtained from evaporation of the solvent and extraction of the residue were examined spectrophotometrically.

Solvents were rejected if the optical density of the appropriate blank solution exceeded 10 per cent of the optical density of the absorbing species at the absorption peak of the latter.

## TABLE I

QUANTITATIVE SEPARATION AND DETERMINATION OF MIXTURES OF HEXYLRESORCINOL AND EXUDATE OBTAINED FROM HEAT-KILLED *E. coli*

Optical densities

| λ in mμ | Initial readings on the cell exudate | Readings* on the cell exudate separated from the mixture† | | | Initial readings on the hexylresorcinol solution | Readings* on the extracted hexylresorcinol in aqueous solution‡ | | | Sum of the initial readings for cell exudate and hexylresorcinol | Experimental readings for initial mixture | Sum of the average readings for the separated components of the mixture |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | | 1 | 2 | 3 | | | |
| 230 | 0·117 | 0·113 | 0·122 | 0·108 | 0·480 | 0·495 | 0·492 | 0·498 | 0·597 | 0·594 | 0·606 |
| 240 | 0·087 | 0·088 | 0·083 | 0·880 | 0·094 | 0·094 | 0·084 | 0·096 | 0·181 | 0·178 | 0·174 |
| 250 | 0·110 | 0·110 | 0·103 | 0·100 | 0·047 | 0·048 | 0·041 | 0·050 | 0·157 | 0·154 | 0·149 |
| 260 | 0·126 | 0·128 | 0·122 | 0·116 | 0·095 | 0·097 | 0·092 | 0·102 | 0·221 | 0·218 | 0·219 |
| 270 | 0·113 | 0·112 | 0·109 | 0·104 | 0·227 | 0·227 | 0·224 | 0·231 | 0·340 | 0·337 | 0·335 |
| 280 | 0·077 | 0·078 | 0·075 | 0·068 | 0·345 | 0·347 | 0·341 | 0·352 | 0·422 | 0·420 | 0·421 |
| 290 | 0·043 | 0·041 | 0·039 | 0·035 | 0·151 | 0·154 | 0·154 | 0·157 | 0·194 | 0·194 | 0·193 |
| 300 | 0·022 | 0·019 | 0·017 | 0·014 | 0·015 | 0·019 | 0·019 | 0·021 | 0·037 | 0·034 | 0·036 |

* The readings were obtained using the general extraction procedure on three aliquot portions of the mixture.

† Corrected for the blank obtained by shaking the appropriate volume of chloroform with water and removing the solvent from the aqueous layer (readings ranging between 0·010 and 240 mμ and 0·007 at 300 mμ).

‡ Corrected for the blank obtained by evaporating the appropriate volume of chloroform to dryness and adding the appropriate volume of water as in the test experiment (readings ranging between 0·040 at 240 mμ and 0·020 at 300 mμ).

*Colorimetric Determination of Hexylresorcinol*

The method originally described by Gibbs[13] and later modified by Singer and Stern[14] was found to be satisfactory. *Reagents*: 0·32 per cent w/v 2:6-dibromo-*p*-benzoquinone-4-chloroimine in acetone-free ethanol. Buffer solution, pH 8·3, containing 12·369 g. boric acid, 14·911 g. potassium chloride and 1·60 g. of sodium hydroxide in 1 litre of distilled water. 1·0 and 5·0 per cent w/v sodium hydroxide solutions. *Method*: 4·0 ml. of hexylresorcinol solution in distilled water, containing up to 2000 $\mu$g., was transferred to a volumetric flask and 1·0 ml. of 5·0 per cent solution of sodium hydroxide and 10·0 ml. of buffer solution added. The reagent solution, in the proportion of 1·0 ml. per 500 $\mu$g. of hexylresorcinol, was added and the solution set aside for 15 minutes to allow colour development. 3·0 ml. of 1·0 per cent solution of sodium hydroxide was added and the volume adjusted to 100 ml. with distilled water. The absorption maxima of the solutions obtained were at 510 m$\mu$. Calibration curves were prepared for solutions containing hexylresorcinol, 2–20 $\mu$g./ml., alone and with added cell exudate; the latter made little difference to the results.

## Results

*Non-extraction of Exudates by Organic Solvents*

Extraction of the cell or growth exudates as described under the general extraction procedure left the concentration of the ultra-violet absorbing constituents in the aqueous phase virtually unchanged. For example, see Table I and Figure 1. The identical shape of the ultra-violet absorption curve of chloramphenicol (curve 6) and curve 2 indicates that the ultra-violet absorbing constituents in the exudate have not been transferred to the solvent layer, since distortion of curve 2 relative to curve 6 would otherwise occur, especially in the region 220–250 m$\mu$. Even using eight extractions instead of four as described in the general procedure, the concentration of ultra-violet absorbing components in the exudate was left unchanged.

As examples of the applications of the extraction procedure, the results obtained using four drug-bacteria systems will now be presented.

*Hexylresorcinol*—E. coli *System*

Portions of known mixtures of cell exudate and hexylresorcinol were extracted with chloroform; spectrophotometric measurements were made of the aqueous layer, after removal of the solvent, and of the solution of extracted hexylresorcinol in water. Reference solutions of hexylresorcinol, cell exudate and the mixture were also examined at comparable dilutions. In Table I, the results obtained for three determinations on a single mixture of the phenol and cell exudate illustrate the accuracy of the method even under unfavourable conditions in which the blank densities constitute 10 per cent of the observed readings. The absorption curves of aqueous solutions of the extracted material were identical with those derived by subtracting the spectrophotometric readings of the aqueous solutions after extraction from those for the solution from hexylresorcinol

—*E. coli* contact; all were identical with the absorption curves for pure hexylresorcinol in water.

The validity of the extraction method was further established by colorimetric determination of hexylresorcinol; this reaction was unaffected by the presence of cell exudate. Results for the determination of hexylresorcinol by both procedures are shown in Table II. An investigation of the interaction of hexylresorcinol with *E. coli* will be presented in subsequent papers.

TABLE II

COMPARISON OF RESULTS OBTAINED FOR SOLUTIONS OF HEXYLRESORCINOL BY THE EXTRACTION AND COLORIMETRIC PROCEDURES

| Experiment number | Hexylresorcinol concentration in μg./ml. | |
|---|---|---|
| | Extraction procedure | Colorimetric procedure |
| 1 | 240·0 | 243·0 |
| 2 | 239·0 | 244·0 |
| 3 | 239·0 | 244·0 |
| 4 | 239·0 | 237·5 |

*Chloroxylenol* — E. coli *System*

The curves obtained by subtracting the spectrophotometric measurements of the aqueous layers after chloroform extraction from those of known mixtures of the phenol and exudate before extraction were identical with those of chloroxylenol in water. Experiments with solutions after drug-bacteria contact gave similar results. The colorimetric procedure, as described above for hexylresorcinol, and the subtraction method gave identical figures of chloroxylenol content in solutions obtained after drug-bacteria contact.

Evaporation of the chloroform extract of chloroxylenol led to losses due to the slight volatility of the latter under these conditions. Attempts to prevent losses of the phenol by evaporation of the organic solvent in the presence of sodium hydroxide solution failed to give optically clear solutions.

Methylene chloride also quantitatively extracted chloroxylenol and other phenols from aqueous solutions; the resultant aqueous solutions of cell exudate were unsatisfactory because of high blank readings.

*Oxine*—Staph. aureus *System*

Losses of oxine also occurred on evaporation of a chloroform solution, although complete separation from cell exudate could be readily effected. For example, the standard deviation of the optical density of the cell exudate at 260 mμ for eight determinations of a single known mixture mixture with oxine was 0·00378 and the coefficient of variation 1·05 per cent. The mean of these results was 0·359, whereas, the corresponding optical density of the original cell exudate was 0·362. Comparison of the appropriate absorption curves, as in the previous examples, again confirmed the identity of the extracted material.

*Chloramphenicol*—A. aerogenes *System*

The extraction of chloramphenicol from growth exudate was required in certain investigations. Diethyl ether and ethyl acetate effected quantitative separations of the drug from aqueous solutions; chloroform, methylene chloride and hexane were unsatisfactory. Ethyl acetate partially extracted ultra-violet absorbing constituents from the growth exudate. Ether effected a satisfactory separation of chloramphenicol from a mixture containing growth exudate; it was necessary to wash the solvent thoroughly and re-distil before use.

Figure 1 shows the results obtained using ether as solvent. The curves for the separated components could be superimposed upon those obtained initially for the chloramphenicol and growth exudate, again establishing the identity of the extracted material and the accuracy of the method.

## Discussion

It seemed improbable that a simple liquid-liquid extraction could effect the desired separation, nevertheless the partition coefficients of many drugs relative to those of the ultra-violet absorbing constituents of the exudates permit such separations. The possible irrelevant absorption due to solvents, which may occur in the same region as the absorption due to the drug, and the danger of light scattering from slightly turbid solutions can be readily overcome.

Before the adoption of a simple liquid-liquid extraction procedure, chromatographic and ion exchange separations were investigated but found to be unsuitable because of incomplete recovery of the drug and cell exudate. Conventional spectrophotometric techniques, including the use of the Morton-Stubbs correction, cannot, for obvious reasons, be applied to the analysis of solutions derived from drug-bacteria contact.

The development of the extraction procedure involved not only the choice of solvents suitable for the quantitative separation of the drug from the cell exudate, but also the selection of those suitable for the subsequent preparations of the drug and exudate for spectrophotometric analysis. Solvents satisfactory for the former requirements were frequently unsatisfactory for the latter, e.g. the aqueous layer, after shaking with the organic solvent and heating to remove the dissolved solvent, may remain turbid even after centrifuging at 18,000 *g*.

Water-insoluble trace residues were also obtained in certain of the organic extracts despite all precautions to exclude grease, for example, Teflon sleeves were used at all ground glass joints and no lubricant was applied to the taps of separating funnels. This problem of turbid aqueous solutions, derived subsequent to evaporation of the organic solvent, may be avoided by spectroscopic measurements on the extracted drug in the separated organic solvent after centrifuging to remove suspended water. Aqueous solutions of the extracted drug, or metabolic product, were generally required in the present work. Despite the difficulties associated with this type of assay, we have found that reliable and consistent results can be obtained.

In conclusion, a re-appraisal of the well-known effects of drug concentration and temperature on the bactericidal action of phenolic compounds in relation to the amount of drug bound under these conditions now becomes feasible.

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# THE INTERACTION OF PHENOLIC COMPOUNDS WITH BACTERIA

## PART I. HEXYLRESORCINOL AND *ESCHERICHIA COLI*

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Various quantitative aspects of the interaction of hexylresorcinol with *E. coli* suspensions are described. The amounts of hexylresorcinol bound by the bacteria from solutions of varying concentrations were determined and the speed of this reaction and the influence of temperature on the extent of binding examined. The release of cellular constituents from the bacteria and the changes in the light-scattering properties of *E. coli* suspensions on addition of hexylresorcinol were also investigated. These results are discussed in relation to the site of antibacterial action of hexylresorcinol.

THE influence of various factors on the antibacterial activity of phenolic compounds is a well documented subject, whereas, little data are available concerning the actual interaction of such drugs with bacteria. Some attempts have been made to relate antibacterial activity to some chemical or physico-chemical change on addition of phenol to bacteria but no real success has yet been reported. Various quantitative aspects of the interaction of hexylresorcinol with *Escherichia coli* will be described in this paper including drug binding to, and release of cellular constituents from, the organism and the consequent effects on the light scattering properties of the bacteria. Later publications will be concerned with the influence of other substances, including sodium chloride, butanol and a non-ionic surface-active agent on the interaction of hexylresorcinol with *E. coli* and evaluation of the antibacterial activity of the phenol under conditions similar to those used for drug-binding experiments. Preliminary observations have already been published[1,2].

### EXPERIMENTAL

*Hexylresorcinol.*—Commercial *p-n*-hexylresorcinol was recrystallised from light petroleum (b.p. 40–60°) as colourless needles, m.p. 69° (uncorrected), (Cox[3] gave 68–70°) and log $\epsilon$ 3·42 at $\lambda$ max 280 m$\lambda$ in distilled water. *Spectrophotometer.*—A Hilger H 700 spectrophotometer was used in conjunction with matched, fused silica cuvettes (1 cm. unless otherwise stated). *Organism.*—*Escherichia coli* (originally N.C.T.C. 5933) was maintained on nutrient agar slopes. Cultures were incubated for 18–24 hours at 37°.

*Preparation of bacterial suspensions.* The bacteria were harvested, washed once by centrifuging at 8000 *g* for 10 minutes with distilled water and finally resuspended in 0·13 M phosphate buffer (pH 7·3). The final volume of the suspension was adjusted so that on dilution 1 in

10 (on addition to drug solutions, etc.) the suspension would contain the required number of organisms. Routine standardisation was carried out nephelometrically.

*Preparation of suspensions of isolated cell walls.* Suspensions of isolated cell walls of *E. coli* were prepared following the method of Salton and Horne[4].

### *General Technique for Drug-Bacteria Contact*

A suitable volume (usually 45 ml.) of an aqueous solution of hexylresorcinol was introduced into a glass centrifuge tube contained in a water bath maintained at 25° (± 1·0°). A known volume (usually 5 ml.) of the bacterial suspension in 0·13 M phosphate buffer was added to the drug solution and the product mixed thoroughly. The final concentration of phosphate buffer was always 0·013 M. After a timed interval of 10 minutes unless otherwise stated, the bacteria were removed by centrifuging at 8000 *g* for 10 minutes and the supernatant solution further clarified by re-centrifuging before examination for the hexylresorcinol content.

Turbidity changes in suspensions of *E. coli* after the addition of hexylresorcinol were determined at 500 mμ. The drug-bacteria suspension was transferred immediately after mixing to a cuvette maintained at 25° in the spectrophotometer. The reference cuvette contained a similar suspension without hexylresorcinol. The optical density of the test suspension was measured at timed intervals after addition of the drug.

### *Analysis of Solutions derived from Drug-Bacteria Contact*

The hexylresorcinol was separated from the cell exudate by extraction with an organic solvent: 50 ml. of drug-containing solution was extracted with chloroform (reagent grade*, 4 × 50 ml portions) and a total of 20 ml. of water was used for washing. The combined solvent extracts, containing the hexylresorcinol, were evaporated to dryness under reduced pressure and the residue dissolved in water and diluted to 50·0 ml. The hexylresorcinol content of this solution was determined spectrophotometrically. The combined aqueous layers, after extraction, were boiled to remove dissolved chloroform, cooled and diluted to 50·0 ml. before spectrophotometric examination for the presence of cell exudate. All results were corrected for the slight background absorption derived from chloroform[5].

## RESULTS

### *The Rate of Interaction of Hexylresorcinol with* E. coli

Figure 1 shows the results obtained for the uptake of hexylresorcinol by and the release of cell exudate from *E. coli* with increasing time of contact. The test suspension contained 350 μg./ml. hexylresorcinol and $3 \times 10^9$ organisms/ml. The contact times ranged from 2 to 60 minutes.

* Not all commercial samples are suitable.

*The Uptake of Hexylresorcinol by* E. coli

Duplicate results obtained for the uptake of hexylresorcinol by *E. coli* ($10^9$ organisms/ml.) from solutions initially containing 20–440 $\mu$g./ml. of the drug are shown in Table I (see also curve 1 of Fig. 3). The liberation of cell exudate accompanying hexylresorcinol binding is shown in Figure 3, curve 2, as a function of the drug concentration remaining in the supernatant solution.

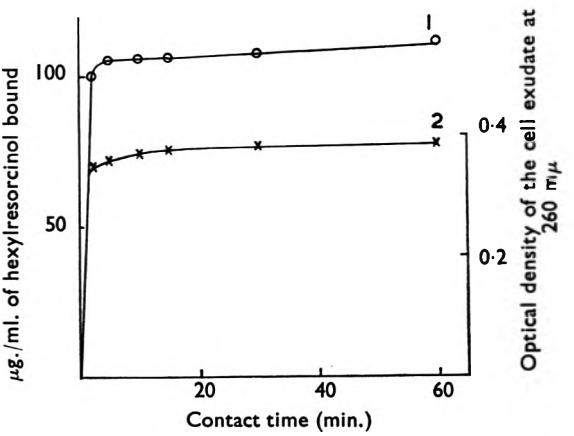


FIG. 1. Uptake of hexylresorcinol by *E. coli* ($3 \times 10^9$ organisms/ml.) and the release of cell exudate from the organisms with increasing time of contact at 25°. 1. The amount of hexylresorcinol bound (initial concentration 350 $\mu$g./ml.). 2. The amount of cell exudate released (optical density at 260 m$\mu$).

Only about 6 per cent of the drug bound by suspensions of intact bacteria was bound by isolated cell wall preparations of the same organism (initial hexylresorcinol concentration 300 $\mu$g./ml. and bacteria or isolated cell walls equivalent to $3{\cdot}3 \times 10^9$ organisms/ml.). This figure is of necessity only approximate owing to circumstances previously discussed[6].

TABLE I

THE RELATION BETWEEN THE UPTAKE OF HEXYLRESORCINOL BY *E. coli* ($10^9$ ORGANISMS/ML.) AND THE INITIAL CONCENTRATION OF HEXYLRESORCINOL

The results were obtained using a single bacterial suspension

| Initial concentration of hexylresorcinol μg./ml. | Mean uptake μg./ml. |
|---|---|
| 23·3 | 2·8 |
| 46·5 | 5·1 |
| 69·4 | 7·7 |
| 91·4 | 10·7 |
| 128·6 | 16·3 |
| 174·8 | 24·0 |
| 209·2 | 34·0 |
| 254·4 | 42·8 |
| 289·0 | 50·8 |
| 363·0 | 74·6 |
| 407·4 | 87·8 |
| 436·1 | 96·3 |

*Influence of Temperature on the Uptake of Hexylresorcinol by* E. coli

The uptake of hexylresorcinol by *E. coli* ($10^9$ organisms/ml.) from solutions initially containing 100 $\mu$g./ml. was determined at 25, 30 and 40°: the results are presented in Table II. The amount of cell exudate released under these conditions is also shown in this Table.

*Turbidity Changes in Suspensions of* E. coli *on Addition of Hexylresorcinol*

The changes in the optical density of suspensions of *E. coli* (final concentration $5 \times 10^8$ organisms/ml.) after addition of varying concentrations of hexylresorcinol are shown in Figure 2. The results shown indicate the changes at timed intervals after mixing. Stoppered cuvettes, 1 cm., thermostatically maintained at 25°, were used for these measurements. Microscopic examination of these suspensions showed that no clumping of the bacteria had occurred.

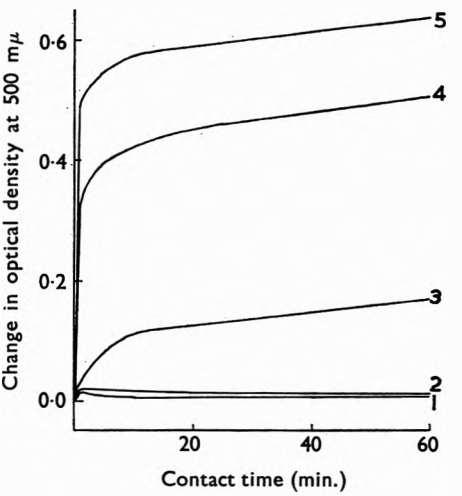


FIG. 2. Change of the turbidity of suspensions of *E. coli* ($5 \times 10^8$ organisms/ml.) at 25° after the addition of hexylresorcinol. 1. 20 $\mu$g./ml. 2. 100 $\mu$g./ml. 3. 200 $\mu$g./ml. 4. 400 $\mu$g./ml. 5. 500 $\mu$g./ml.

## DISCUSSION

*Analytical Methods*

The ultra-violet absorption curves of the solutions obtained after contact of *E. coli* with hexylresorcinol showed maxima near 280 m$\mu$; the actual position of the peak varied slightly with the initial concentration, presumably due to the relative concentrations of unchanged hexylresorcinol ($\lambda$ max 280 m$\mu$) and the cell exudate ($\lambda$ max 260 m$\mu$). Satisfactory analytical resolution of these mixtures was attained using the extraction procedure described previously[5].

*The Rate of Interaction of* E. coli *with Hexylresorcinol*

The uptake of hexylresorcinol by *E. coli* was rapid and almost complete after 5 minutes contact time (see Fig. 1); the release of cell exudate during contact showed a similar course. Ten minute contact times were, therefore, considered suitable for all subsequent work with this system.

*The Uptake of Hexylresorcinol by* E. coli

Curve 1 of Figure 3 shows that the amount of hexylresorcinol bound by *E. coli* is dependent upon the initial concentration of the phenol in solution. There was no difference between the amount of hexylresorcinol bound from aqueous solutions in the presence and absence of phosphate buffer (0·013 M and pH 7·3). The point of no further increase in the

uptake of hexylresorcinol by the bacteria on increasing the phenol concentration in the medium was not attained even with the most concentrated initial solutions (almost saturated). Although the number of bacteria in contact with the hexylresorcinol solutions could have been reduced to

TABLE II

THE EFFECT OF TEMPERATURE ON THE UPTAKE OF HEXYLRESORCINOL BY *E. coli* ($10^9$ ORGANISMS/ML.)

The initial concentration of hexylresorcinol in contact with the bacteria was 100 μg./ml. Mean results for duplicate determinations

| Temperature in °C. | Concentration in the supernatant solution μg./ml. | Amount of hexylresorcinol bound μg./ml. | Optical density of cell exudate released in the presence of the drug | Optical density of cell exudate released in the absence of the drug |
|---|---|---|---|---|
| 25 | 87·4 | 12·6 | 0·133 | 0·029 |
| 30 | 86·8 | 13·2 | 0·152 | 0·034 |
| 40 | 86·7 | 13·3 | 0·272 | 0·056 |

achieve saturation, the precision of the analytical results would have been adversely affected.

The shape of the uptake curve (Fig. 3, curve 1) indicates that the attractive forces exerted by the initially bound molecules facilitate binding of a further quantity of hexylresorcinol. This is also suggested by the changes in the electrophoretic mobilities of bacteria upon progressive addition of phenolic compounds[7]. Alternatively, the molecules bound

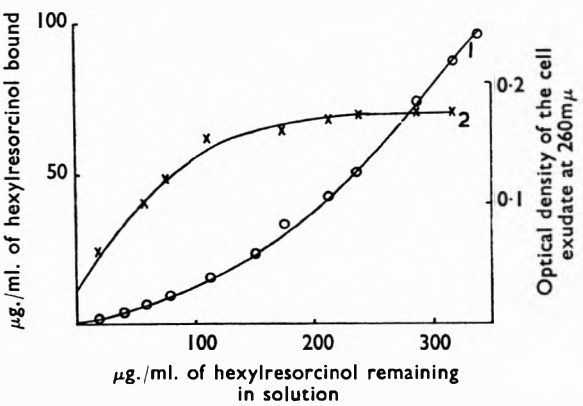


FIG. 3. Uptake of hexylresorcinol by *E. coli* and the release of cell exudate from the organisms ($10^9$ organisms/ml.). 1. The amount of hexylresorcinol bound from solutions containing 0·013 M phosphate buffer, pH 7·3 at 25°. 2. Release of cell exudate (optical density at 260 mμ) upon increasing the hexylresorcinol concentration.

initially to the cytoplasmic membrane could cause partial disorganisation of the osmotic barrier with resultant penetration by some phenolic molecules.

The amount of cell exudate liberated from *E. coli* suspensions in 10 minutes, as indicated by the optical density at 260 mμ, was influenced by the initial hexylresorcinol concentration (and also by the amount of

hexylresorcinol bound) up to 220 $\mu$g./ml. (for $10^9$ organisms/ml.). Above this concentration, a constant and limiting value was reached shown in curve 2 of Figure 3. Initially, localised damage to the cytoplasmic membrane of the bacteria is envisaged with consequent release of a limited amount of cell exudate; the components of the cell exudate may possibly be present in the "free" state or may be formed by breakdown of the cytoplasmic constituents. Progressive addition of hexylresorcinol would cause more extensive damage and the release of more cellular constituents until the drug molecules exerted their maximum effect upon the cytoplasmic membrane. Similar interdependence of the concentration of an antibacterial substance (cetyltrimethylammonium bromide, polymyxin and circulin) and the amount of cellular constituents released from bacteria has been demonstrated by other workers[8–11]; however, the amount of cell exudate liberated from the same organisms by different substances will not necessarily be the same.

The relatively small proportion (6 per cent) of hexylresorcinol bound by isolated cell walls of *E. coli* compared with the amount bound by intact cells under identical conditions provides further evidence that the cytoplasmic membrane and possibly the cytoplasm is involved in the latter. The cytoplasmic membrane has also been implicated as the site of interaction of various antibacterial substances, for example phenol[12,14], polymixin and other peptidic antibiotics[15] and some ionic surface-active agents (for a discussion see Salton[16]), with bacteria.

The uptake of hexylresorcinol by bacteria is not a reversible reaction. Therefore, although changes in the contact temperature had little effect on the amount of hexylresorcinol bound (see Table II) it does not follow that values for the heat of reaction are small and only weak binding forces are involved. Either increased breakdown of cytoplasmic constituents or changes in the permeability of the bacteria could effect the considerable increase in the amount of cell exudate liberated on raising the temperature. These results are of particular interest in relation to the temperature coefficients of bactericidal reactions associated with phenolic compounds. Nevertheless, the number of molecules exerting the biological effect may constitute only a very small fraction of those bound by the bacteria and, therefore, any correlation of the changes observed on drug binding and antibacterial activity may ultimately prove impossible.

*Turbidity Changes in Suspensions of* E. coli *on Interaction with Hexylresorcinol*

Some profound alteration in the light scattering properties of suspensions of *E. coli* (see Fig. 2) occurs after the addition of hexylresorcinol, the effect being dependent on the concentration of the drug and the time of contact. The authors have observed similar turbidity changes occur in bacterial suspensions containing many phenolic substances (for example phenol and chlorocresol) but not all (for example chloroxylenol and thymol). The optical density of bacterial suspensions between 300–600 m$\mu$ is attributed solely to the scattering of light by the organisms[17]. An increase in the optical density of a bacterial suspension

may be caused by an increase in the effective reflecting surface area of the cell or to a change in the refractive index of the cell membranes or cytoplasm or to a combination of these effects. The turbidity changes reported in the present paper will be discussed in conjunction with the biological results in Part III[18].

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# THE INTERACTION OF PHENOLIC COMPOUNDS WITH BACTERIA

## PART II. THE EFFECTS OF VARIOUS SUBSTANCES ON THE INTERACTION OF HEXYLRESORCINOL WITH *ESCHERICHIA COLI*

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The uptake of hexylresorcinol by suspensions of *E. coli* and the associated release of cellular constituents were examined using heat-killed and butanol-treated organisms. The effect of the addition of cetomacrogol to the system was evaluated in terms of the reduction in the extent of drug binding and the prevention of the hexylresorcinol-dependent light-scattering changes of *E. coli* suspensions. Sodium chloride potentiation of the latter effect and of the release of cell exudate from the bacteria on treatment with hexylresorcinol was also investigated.

IN this paper, the effects of sodium chloride and butanol on the interaction of hexylresorcinol and *Escherichia coli* are examined because of their effects upon the osmotic pressure of the drug-bacteria system. Cetomacrogol was also included since nonionic surface-active agents are employed as emulsifying and solubilising agents in pharmaceutical and cosmetic preparations. Preservation of these products is difficult since the conventional phenolic compounds (among many others) have been shown to be ineffective in the presence of nonionic substances, although the cause of this inactivation has not been established unequivocally[1,2].

The effects of sodium chloride and cetomacrogol on the antibacterial activity of solutions of hexylresorcinol against *E. coli* under analogous conditions to those herein described will be presented and discussed in Part III[3].

### EXPERIMENTAL

The experimental techniques were described in Part I[4].

*Cetomacrogol* (polyethylene glycol 1000 monocetyl ether). This material was obtained from Glovers (Chemicals) Ltd., may be represented by the formula:

$$CH_3(CH_2)_mOCH_2(CH_2OCH_2)_nCH_2OH$$

where $m$ may be 15 or 17 and $n$ may be 19 to 23. The molecular weight is approximately 1300 and the critical micelle concentration in aqueous solution is about $10^{-6}$ to $10^{-7}$ M[5].

*Preparation of suspensions of heat-killed bacteria.* Washed suspensions of *E. coli* (about $10^{10}$ organisms/ml.) in phosphate buffer were maintained at 100° for 10 minutes. The bacteria were centrifuged, washed once and resuspended in the same medium ($2 \times 10^{10}$ organisms/ml.).

*Preparation of suspensions of butanol-treated bacteria.* *n*-Butanol was added to suspensions of *E. coli* (about $10^{10}$ organisms/ml.) in phosphate

buffer to yield a final concentration of 5·0 per cent w/v (0·67 M). After 25 minutes at 20°, the bacteria were centrifuged, washed twice and resuspended in phosphate buffer ($2 \times 10^{10}$ organisms/ml.).

*Colorimetric determination of hexylresorcinol.* The method was as described previously[6]. Calibration curves were prepared for solutions containing hexylresorcinol (2–20 μg./ml.) alone and in combination with 0·001 per cent w/v cetomacrogol.

*Turbidity changes in bacterial suspensions.* The effect of sodium chloride on the turbidity changes of suspensions of *E. coli* treated with

TABLE I

THE UPTAKE OF HEXYLRESORCINOL AND THE RELEASE OF CELL EXUDATE BY HEAT-KILLED AND BUTANOL-TREATED SUSPENSIONS OF *E. coli* ($2 \times 10^9$ ORGANISMS/ML.)

The initial concentration of hexylresorcinol in contact with the bacteria was 260 μg./ml.

| | Preliminary treatment | | |
|---|---|---|---|
| | none (control) | heat-killing | butanol |
| Amount of hexylresorcinol bound in μg./ml. .. | 76·0 | 114 | 70·0 |
| Optical density at 260 mμ of the cell exudate released during preliminary treatment .. | 0·033 | 0·791 | 0·133 |
| Optical density at 260 mμ of the cell exudate released during hexylresorcinol treatment .. | 0·199 | 0·049 | 0·670 |

hexylresorcinol were determined by the method described in Part I[4], i.e., the reference cuvette contained an equivalent bacterial suspension without added drug.

Observations on suspensions of *E. coli* after the addition of either hexylresorcinol or cetomacrogol, or both, were made using water in the reference cuvette (0·2 cm. cuvettes).

*Analysis of solutions derived from drug-bacteria content.* In the absence of the surface-active agent, the extraction procedure (cf. Part I[4] was used, see discussion). The colorimetric precedure was applied only to those solutions which contained cetomacrogol.

## RESULTS

### *Influence of Various Factors on the Interaction of Hexylresorcinol with* E. coli

*Heat-killed and butanol-treated organisms.* The results obtained using heat-killed and butanol-treated suspensions of *E. coli* ($2 \times 10^9$ organisms/ml.) and an initial drug concentration of 260 μg./ml. are shown in Table I.

*Sodium chloride.* The effect of sodium chloride in various concentrations upon the uptake of hexylresorcinol by, and the release or cell exudate from, *E. coli* ($10^9$ organisms/ml.) for a solution initially containing 230 μg./ml. of the drug is indicated by the results presented in Figure 3.

*Cetomacrogol.* Figure 1, curve 1, shows the results obtained for the uptake of hexylresorcinol by *E. coli* ($3 \times 10^9$ organisms/ml.) from a solution initially containing 350 μg./ml. of hexylresorcinol and 990 μg./ml. of cetomacrogol (the solution also contained phosphate buffer,

pH 7·3, final concentration 0·013 M). The contact times ranged from 2 to 60 minutes and the molar ratio of cetomacrogol to hexylresorcinol was about 4 to 1. Curve 2 of the same Figure shows the results of a comparable experiment in which cetomacrogol was omitted (cf. Part I[4]).

The results shown in Figure 2 demonstrate the effect of increasing concentrations of cetomacrogol on the binding of hexylresorcinol by

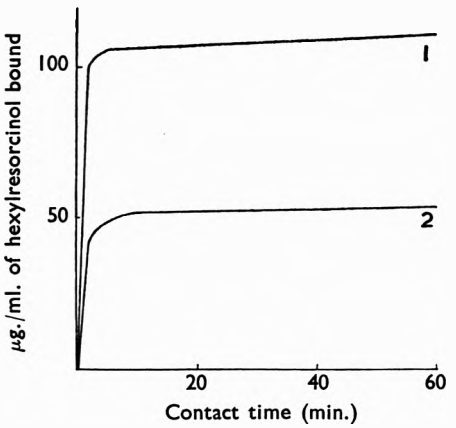


FIG. 1. The rate of uptake of hexylresorcinol by *E. coli* ($3 \times 10^9$ organism/ml.) at 25° in the presence and absence of cetomacrogol. The initial concentration of hexylresorcinol was 350 μg./ml. or $1{\cdot}8 \times 10^{-3}$ M. 1. In the absence of cetomacrogol. 2. In the presence of 1000 μg./ml or $7{\cdot}7 \times 10^{-3}$ M cetomacrogol.

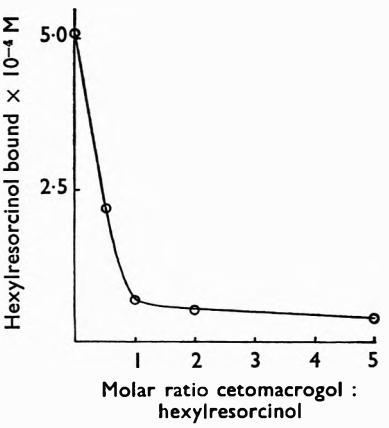


FIG. 2. Uptake of hexylresorcinol by *E. coli* ($3 \times 10^9$ organisms/ml.) at 25° in the presence of varying proportions of cetomacrogol. The initial hexylresorcinol concentration was $1{\cdot}8 \times 10^{-3}$ M.

suspensions of *E. coli* ($3 \times 10^9$) organisms/ml. The molar ratios of cetomacrogol to hexylresorcinol ranged from 0·5–5·0 to 1.

For a solution initially containing 500 μg./ml. of hexylresorcinol and cetomacrogol (molar ratio of cetomacrogol to hexylresorcinol of 0·5 : 1), the amount of the phenol bound was 24 μg./$5 \times 10^8$ organisms/ml.

TABLE II

THE TURBIDITY OF SUSPENSIONS OF *E. coli* ($10^9$ ORGANISMS/ML.) 10 MINUTES AFTER THE ADDITION OF HEXYLRESORCINOL SOLUTIONS CONTAINING VARIOUS CONCENTRATIONS OF SODIUM CHLORIDE. (0·5 CM. CUVETTES)

| Concentration of hexylresorcinol μg./ml. | Optical density at 500 mμ in presence of NaCl | | | |
|---|---|---|---|---|
| | 0·0 M | 0·05 M | 0·1 M | 0·2 M |
| 50 | 0·477 | 0·495 | 0·503 | 0·511 |
| 100 | 0·490 | 0·514 | 0·512 | 0·531 |
| 200 | 0·554 | 0·591 | 0·603 | 0·642 |
| 300 | 0·725 | 0·767 | 0·799 | 0·895 |
| 350 | 0·834 | 0·944 | 0·968 | 1·020 |
| 400 | 0·974 | 1·039 | 1·068 | 1·112 |
| 500 | 1·131 | 1·201 | 1·234 | 1·232 |

*Influence of Various Substances on the Turbidity of Suspensions of* E. coli

*Sodium chloride.* Table II shows the effect of the presence of sodium chloride on the turbidity changes of suspensions of *E. coli* observed on

addition of hexylresorcinol. The maximum percentage change in optical density was that observed for suspensions containing 350 $\mu$g./ml. of the phenol and 0·2 M sodium chloride. At each concentration of the electrolyte studied, the maximum increase in optical density of the bacterial suspensions also occurred at this concentration of the drug; higher and lower hexylresorcinol concentrations caused a smaller increase. These results were obtained for the simultaneous addition of the phenol and the salt; a greater increase in turbidity was observed when the bacterial suspension was pre-treated with sodium chloride for 10 minutes before addition of hexylresorcinol.

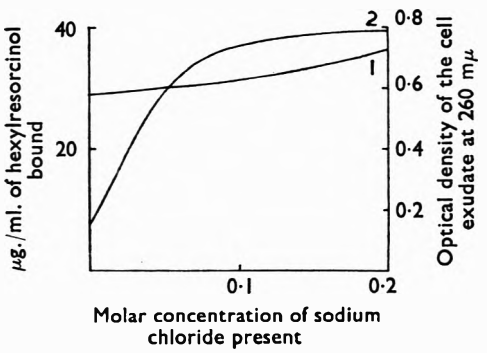


FIG. 3. The effect of sodium chloride on the interaction *E. coli* ($10^9$ organisms/ml.) with hexylresorcinol (230 $\mu$g./ml.) at 25°. 1. The amount of hexylresorcinol bound. 2. Release of cell exudate (optical density at 260 m$\mu$) upon increasing the sodium chloride concentration.

*Cetomacrogol.* The effect of cetomacrogol on the turbidity changes of suspensions of *E. coli* caused by the addition of hexylresorcinol is demonstrated by the results presented in Figure 4. The results were obtained using solutions containing cetomacrogol in addition to the phenol and a final bacterial concentration of $10^9$ organisms/ml. The optical densities of the contact suspensions at 500 m$\mu$ (in 0·2 cm. cuvettes) were measured 60 minutes after mixing. Irrespective of the hexylresorcinol concentrations, increases in the concentration of cetomacrogol above $3{\cdot}85 \times 10^{-3}$ M caused a slight decrease in the optical density of the contact suspensions, i.e., less than 0·03 optical density units under the above conditions.

## DISCUSSION

### *Analytical Methods*

The extraction procedure described in a previous paper[6] was used to separate unchanged hexylresorcinol from the solutions derived from drug-bacteria suspensions wherever possible. However, this separation was not suitable for solutions containing cetomacrogol and it was necessary to determine the phenol colorimetrically. It was impracticable to determine both the cell exudate and the unchanged hexylresorcinol in solutions containing the nonionic because of foaming.

The straight line calibration curve, obtained on plotting the optical density at 510 m$\mu$ against the hexylresorcinol concentration (up to 20 $\mu$g./ml. or $0{\cdot}1 \times 10^3$ M) in the final solution after colour development, was virtually unaffected by the presence of cell exudate and cetomacrogol (final concentration 0·001 per cent w/v or $0{\cdot}77 \times 10^{-3}$ M).

*Solutions Containing Hexylresorcinol and Cetomacrogol*

The molar ratio of cetomacrogol to hexylresorcinol in the solutions used during the present work ranged from 0·5–5·0 to 1. At lower molar ratios, a water insoluble complex separated from the solution. Observations on the probable molecular species present in solutions of hexylresorcinol and cetomacrogol and on the nature of the water insoluble complex have recently been published[1].

Since the critical micelle concentration of aqueous solutions of cetomacrogol is about $10^{-6}$ to $10^{-7}$ M, it has not been possible to examine non-micellar solutions of this material. However, the effects of other nonionic surface-active agents of *known* chain lengths at non-micellar concentrations, on the interaction of various phenols with bacteria will be reported at a later date.

*Influence of Various Factors on the Uptake of Hexylresorcinol by* E. coli

*Heat-killed bacteria.* The amount of cell exudate liberated during heat treatment of a suspension of *E. coli* is greater than during subsequent treatment with hexylresorcinol or during the interaction of a control suspension with the phenol (Table II). This increase is presumably caused by the destruction of the osmotic barrier of the bacteria, and the escape of fragments of the cytoplasmic components (cell exudate) formed by an "uncoupling reaction" of heat on the cytoplasm, since heat fixation of the cytoplasmic[7] and "protoplast"*[7,8] membranes of bacteria has been demonstrated. The term "uncoupling reaction" is used here to imply the breakdown of proteins, polypeptides and nucleic acids into their simpler components, e.g., peptides, amino acids, sugars. Thus, once the permeability of the osmotic barrier or the cytoplasmic membrane, or both, has been destroyed, the amount of cell exudate released should reflect the uncoupling efficiency of the added agent on the remaining cytoplasmic components. The small amount of cell exudate liberated during the reaction of hexylresorcinol

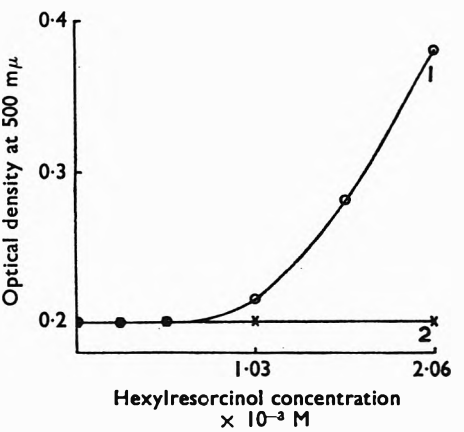


FIG. 4. The effect of cetomacrogol on the turbidity changes of suspensions of *E. coli* ($10^9$ organisms/ml.) exposed to hexylresorcinol at 25°. The readings were taken at 500 mμ in 0·2 cm. cuvettes, 60 minutes after mixing and using water in the reference cuvette. 1. In the absence of cetomacrogol. 2. With cetomacrogol ($0·77 \times 10^{-3}$ M) added to the bacterial suspension at the same time as the hexylresorcinol.

* Doubt has been expressed whether the bodies formed by growing *E. coli.* in the presence of penicillin in a hypertonic medium are protoplasts in the strict sense of the definition of that term[9].

with heat-killed organisms indicates that the limit of cytoplasmic uncoupling has been reached in the heat treatment.

*Butanol-treated bacteria.* The concentration of butanol used in these experiments (0·67 M) is in excess of that required to attain a surface pressure of 34 dynes/cm. (0·4 M). Leakage of low molecular weight constituents from bacteria, as well as haemolysis, has been shown to occur above this critical concentration of butanol; it seems reasonable to attribute the former to irreversible denaturation of the cytoplasmic membrane of the bacteria[10]. The amount of cell exudate liberated from *E. coli* by butanol alone may arise either from destruction of the osmotic barrier of the cells and the subsequent escape of naturally "free" cellular constituents, or alternatively, from destruction of the osmotic barrier and a limited uncoupling effect on the cytoplasm. Subsequent addition of hexylresorcinol may cause extensive intracellular uncoupling and release of cytoplasmic components through the previously disrupted osmotic barrier. The relatively small amount of cell exudate obtained on hexylresorcinol treatment of control suspensions of *E. coli* is explicable if the phenol is considered to penetrate the bacterial cell without grossly affecting the permeability of the osmotic barrier.

The data presented in Table II show that the amount of cell exudate released from bacteria depends on the treatment used and that not all of the "potential cell exudate" is necessarily released under one particular set of conditions (see also[11,12]).

*Sodium chloride.* Sodium chloride, *per se* (at concentration levels up to 0·2 M), has a negligible effect on the amount of cell exudate released from *E. coli* suspensions or on their turbidity. The increase in the amount of cell exudate obtained on adding hexylresorcinol solutions containing sodium chloride to suspensions of *E. coli* may be attributed to the enhanced efficiency of the phenol in the uncoupling effect on cellular constituents and liberation of these substances as cell exudate. The increased turbidity produced by hexylresorcinol in the presence of sodium chloride also indicates the enhanced activity of the phenol, since the presence of the salt has little effect on the amount of the phenol which is bound (Fig. 3). An even greater increase in optical density at 500 m$\mu$ of the bacterial suspensions on addition of hexylresorcinol observed when the organisms were pretreated with sodium chloride (0·2 M for 10 minutes) probably has significance in relation to the potentiating effect of the electrolyte on the antibacterial activity of this phenol (see Part III[3]).

The above results seem to indicate that the cell exudate liberated by low concentrations of the phenol alone is derived from a limited "uncoupling reaction" on the cytoplasm, rather than an alteration of the permeability of the osmotic barrier. This explanation accounts for the negligible turbidity changes at these concentrations of hexylresorcinol alone.

*Cetomacrogol.* The speed of uptake of hexylresorcinol was hardly affected by the presence of the nonionic substance (Fig. 1) whereas, the amount of hexylresorcinol bound was reduced in proportion to the relative molar concentration of cetomacrogol (Fig. 2). The light

scattering changes of the bacterial suspensions associated with addition of hexylresorcinol were abolished in the presence of cetomacrogol (Fig. 4) even when a sufficient concentration of the phenol was taken up to cause such changes in the absence of the nonionic substance.

Complex formation occurs in solutions containing hexylresorcinol and cetomacrogol; steric factors could, therefore, account for the observed reduction in uptake of the phenol by *E. coli* suspensions. The complexing probably prevents cell wall penetration by the phenol, since changes in the light scattering properties of bacterial suspensions upon contact with hexylresorcinol are abolished in the presence of cetomacrogol. Alternatively, it may be postulated that light scattering changes are normally caused by hexylresorcinol molecules penetrating the bacterial surface at specific sites; cetomacrogol could then block hexylresorcinol binding at these sites without interfering with binding over the remainder of the bacterial surface.

Figure 2 shows that the presence of one molecule of cetomacrogol to every two of hexylresorcinol reduces the amount of the phenol bound by approximately 56 per cent. The presence of cetomacrogol in excess of a 2:1 ratio of cetomacrogol to hexylresorcinol has little additional effect on the amount of hexylresorcinol bound by the bacteria: the amount of hexylresorcinol bound per organism under the latter conditions is less than that which would be theoretically required to form a close-packed monomolecular layer round the organism *in the absence of the nonionic substance* (cf. Part III[3]).

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# *NN*-DIMETHYLMELAMINE

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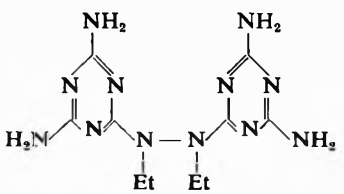
The previously unidentified product of the reaction between 2-chloro-4:6-diamino-*s*-triazine and *NNN'N'*-tetraethyldecamethylene diamine in dimethylformamide solution has been shown to be *NN*-dimethylmelamine.

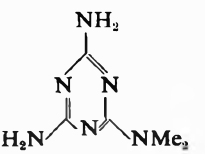
In a previous communication[1] it was recorded that prolonged boiling under reflux of a solution of 2-chloro-4:6-diamino-*s*-triazine and *NNN'N'*-tetraethyldecamethylene diamine in dimethylformamide yielded an unidentified crystalline product containing no halogen.

The analytical figures for this material corresponded reasonably well with those required for *NN'*-diethyl-*NN'*-bis (4:6-diamino-*s*-triazin-2-yl) hydrazine (I) [Found: C, 39·2; H, 6·4; N, 54·5. $C_{10}H_{18}N_{12}$ requires C, 39·2; H, 5·9; N, 54·9 per cent] and this structure appeared to be confirmed when the same product was obtained by the prolonged boiling under reflux of a mixture of 2-chloro-4:6-diamino-*s*-triazine, *NN'*-diethylhydrazine[2], potassium carbonate and dimethylformamide. Since, however, the reaction was so unexpected, and moreover the hydrogen analysis was invariably found to be somewhat high, a further explanation was sought.

Re-examination of the analysis figures showed them to correspond even more closely with those required for *NN*-dimethylmelamine (II)




(I)




(II)

[Calc. for $C_5H_{10}N_6$: C, 39·0; H, 6·55; N, 54·55 per cent]. An authentic specimen of this material was therefore prepared by treatment of 2-chloro-4:6-diamino-*s*-triazine with dimethylamine in the presence of aqueous alkali (*cf.* general method of Kaiser and others[3]) and was found to be identical with the original product.

Prolonged refluxing of a simple solution of 2-chloro-4:6-diamino-*s*-triazine in dimethylformamide also yielded the same material, although in only approximately 5 per cent yield, whereas the best yield in the original experiments was approximately 30 per cent. It appears probable therefore that the original formation of *NN*-dimethylmelamine was due to decomposition of the solvent, dimethylformamide, particularly in the presence of bases, and reaction of the liberated dimethylamine with 2-chloro-4:6-diamino-*s*-triazine.

## *NN*-DIMETHYLMELAMINE

*NN*-Dimethylmelamine resists quaternisation; thus, when a methanolic solution was left with methyl iodide at room temperature for four months, the bulk of the starting material was recovered unchanged. When a 1 per cent methanolic solution of the base was heated with either methyl iodide or decamethylene di-iodide at 110–120° under pressure for 60 hours, the only product isolated was the hydriodide of the base.

### Experimental

NN-*Dimethylmelamine:* To a slurry of 2-chloro-4:6-diamino-*s*-triazine (1·455 g., 1 mol.) in water (10 ml.) was added dimethylamine (2·7 g. 33 per cent w/w in ethanol, i.e. 100 per cent excess) together with one drop of phenolphthalein solution. The temperature was slowly raised until the mixture refluxed, and maintained here for 3 hours. Meanwhile the reaction mixture was kept just alkaline by adding 5 per cent sodium carbonate solution as required. After cooling, the reaction product was filtered, washed thoroughly with water, dried and recrystallised from ethanol. Rapid crystallisation gave colourless octahedral crystals, whilst slow crystallisation gave branched, fan-like needle aggregates of octahedra. The product (1·15 g., 75 per cent) had m.p. 306–307° (lit[4]. 307–308°), unaltered by admixture with the material originally described[1]. The *hydriodide* separated from ethanol as colourless glistening plates, m.p. 274–5° (decomp.) [Found: C, 21·5; H, 3·8; N, 29·9; I, 44·7. $C_5H_{11}N_6I$ requires C, 21·3; H, 3·9; N, 29·8; I, 45·0 per cent].

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# A STUDY OF BACTERIOLOGICAL MEDIA: THE EXAMINATION OF PEPTIDES IN BACTO-CASITONE*

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Three batches of Bacto-Casitone have been fractionated by the combined use of paper electrophoresis at pH 5·8 and paper chromatography in butanol-acetic acid-water system. Batches A, B and C showed 61, 73 and 73 subfractions respectively. Some of the subfractions have been found to be a mixture of few peptides while others consist of a single peptide by *N*-terminal amino acid analysis.

In a previous paper[1] it was found that the dinitrophenyl (DNP) peptides in Bacto-Casitone aggregated in four spots which were difficult to fractionate. This paper describes the fractionation of the peptides in three batches of casitone by the combined use of paper electrophoresis and paper chromatography. The homogeneity of each subfraction was tested by determining the *N*-terminal amino acid as DNP-derivative.

## Experimental

The apparatus for paper electrophoresis was similar to that used by Kunkell and Tiselius[2] with some modifications. The buffer vessels 19½ in. × 3 in. × 4½ in. were made of lucite, the electrodes were platinum foil 2 in. × 1 in. The electrode vessels were connected to the buffer vessels by buffer bridges so as to prevent alterations in the electrolyte composition at the electrodes from reaching the filter paper. The paper was placed between two glass plates 19⅜ in. × 18 in. of 1·1 cm. thickness which were covered lightly with Dow Corning silicone grease. To get rid of heat produced during electrophoresis the lower glass plate rested on a metal box 15½ in. × 19 in. × 1 in. which was cooled by allowing water to flow through a coil in it. A sheet of Whatman 3 MM paper 9 in. × 22¼ in. was used, a line was drawn 10¼ in. from the anode side, then 0·3 ml. of 20 per cent solution of casitone batch A was applied as a streak on this line, the paper was sprayed with buffer pH 5·8 (pyridine: acetic acid: water, 8:2:90 v/v/v) leaving about 1½ in. from either side of the sample unsprayed. Then the buffer was allowed to flow from both sides so that the sample is concentrated in a thin band. Excess of buffer was blotted and the paper was placed between the glass plates for electrophoresis. The paper was left for half an hour for equilibration then 1000 V. was applied (current 50–70 mA.) for 4 hours. The paper

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was then dried, a horizontal strip was cut from each end of the paper and the presence of the different fractions was revealed by the ninhydrin colour reaction. Using the two strips as guides vertical strips were cut from the remnant of the paper and each fraction was eluted with water. Two sheets were treated for each batch and eluates from similar fractions were pooled together. Each eluate was evaporated to dryness at room temperature in a vacuum desiccator. The residue was dissolved in 0·15 ml. of 10 per cent *iso*propanol.

Batch A gave 13 fractions, fractions F1–F6 are acidic in character, their mobilities decreases in that order, F7 is neutral, F8–F12 are basic with mobilities increasing in that order. Batches B and C gave similar results. Each fraction was subjected to electrophoresis at pH 5·8 to separate it from traces of neighbouring fractions. It was found that all fractions in addition to the main spot gave a second fraction in the neutral position designated Fx.

Each fraction was applied on Whatman No. 1 paper as 0·003 ml. spots 2 cm. apart. The papers were developed with the butanol-acetic acid-water system, then after drying two strips were cut from either end of the

TABLE I

ANALYSIS OF CASITONE

| | Number of subfractions | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Acidic fraction | | | | Neutral | | Basic fraction | | | | | |
| Batch | F3 | F4 | F5 | F6 | F7 | Fx | F8 | F8[1] | F9 | F10 | F11 | Total |
| A | 7 | 6 | 6 | 7 | 9 | 4 | 5 | 1 | 5 | 6 | 5 | 61 |
| B | 6 | 8 | 7 | 10 | 10 | 2 | 3 | – | 7 | 9 | 11 | 73 |
| C | 5 | 7 | 8 | 10 | 13 | 2 | 3 | – | 9 | 8 | 8 | 73 |

paper and the presence of the subfractions revealed by the ninhydrin colour reaction. Using the 2 strips as guides horizontal strips each representing a subfraction were cut and eluted with water. The eluate was evaporated to dryness in vacuum then dissolved in 0·1 ml. 10 per cent *iso*propanol. 0·05 ml. was completely hydrolysed with hydrochloric acid for 24 hours and after removal of hydrochloric acid, the residue was taken up in few drops of 10 per cent *iso*propanol and an aliquot was subjected to two dimensional chromatography for amino acids[3].

*Determination of* N-*terminal amino acid.* To 0·05 ml. of the subfraction was added 0·05 ml. of 2 per cent trimethylamine solution and 0·2 ml. of 5 per cent fluorodinitrobenzene (FDNB) in ethanol and the solution was shaken at room temperature for 2 hours. A few drops of trimethylamine solution and a few drops of water were then added and the excess of FDNB was extracted with ether. The aqueous layer was evaporated to dryness in a vacuum desiccator. The residue was taken up in a few drops of 6 N hydrochloric acid and hydrolysed for 8 hours at 105°. After removal of the hydrochloric acid it was subjected to two dimensional chromatography using the ethyl benzene system[4] followed by 1·5 M phosphate buffer.

## TABLE II

THE HYDROLYSATES OF ACIDIC SUBFRACTIONS*

| Subfraction | *RF* | DNP-amino acid | Asp | Glu | Orn | Lys | Arg | His | Gly | Ala | Val | Leu | Ser | Thr | Tyr | Phe | Pro | Met |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F3, 1 | 0·05–0·12 | Leu, Ph, S, Val? | 1 | 4 | – | 1 | ? | – | 1–2 | 1–2 | 1–2 | 2 | 1–2 | 1 | – | – | ? | – |
| F3, 2 | 0·08–0·15 | Ph | 1 | 4 | – | 1 | ? | – | 1–2 | 1–2 | 1–2 | 2 | 1 | trace | – | – | ? | – |
| F3, 3 | 0·08–0·16 | Ph, S, Asp, Glu, Val | 1 | 4 | – | 1 | – | – | 1–2 | 1 | 1 | 2 | 1–2 | trace | – | – | – | – |
| F3, 4 | 0·16 | Asp, Glu, Ph, Val, Thr, S, Ala? | 1–2 | 4 | – | trace | 1 | – | 1 | 1 | 1–2 | 2 | 1 | trace | – | – | 1 | 1 |
| F3, 5 | 0·21–0·39 | Ph, Val, Asp, Glu, A, S, Ala? | 1 | 4 | – | – | – | – | 1 | 2 | 2 | 2 | 1 | 1 | – | – | 1 | – |
| F3, 6 | 0·31–0·53 | Ph, Val, S | 1 | 4 | – | – | – | – | 1 | 1–2 | 1–2 | 1–2 | 1 | 1 | – | – | – | – |
| F3, 7 | 0·47–0·6 | Ph | 1 | 3 | – | – | – | – | 1 | 3 | 2 | 3 | – | – | – | – | 1 | – |
| F4, 1 | 0·044–0·11 | Ph, S | 1 | 4 | – | 1–2 | ? | – | 1 | 1 | 1 | 2 | 1 | 1 | – | – | 1 | – |
| F4, 2 | — | Ph, S, A | 1 | 3 | – | 1 | – | – | 1–2 | 1 | 1 | 1–2 | 1 | trace | – | – | 1 | – |
| F4, 3 | 0·17 | Ph, S, Asp, Glu | 1 | 3 | – | 1 | – | – | 1 | 1 | 1 | 2 | 1 | trace | – | – | 1 | – |
| F4, 4 | 0·19–0·33 | Ph, S, A, Val | 1 | 2–3 | – | trace | – | – | 1 | 1 | 2 | 2 | 1 | 1 | – | – | ? | – |
| F4, 5 | 0·48–0·62 | Ph, S, A, Val | 1 | 3 | – | – | – | – | 1 | 1 | 1–2 | 1–2 | 1 | – | – | – | 1 | – |
| F4, 6 | 0·48–0·62 | Ph, S, A, Val | 1 | 2–3 | – | trace | – | – | 1 | 1 | 1 | 1–2 | 1 | – | – | – | 1 | – |
| F5, 1 | 0·1–0·14 | Asp, Glu, S, Thr, Arg, Phe, Val, Lys, Gly? | 1 | 4 | – | 1 | – | – | 1 | 1–2 | 1–2 | 2 | 1 | 1 | – | – | 1 | – |
| F5, 2 | 0·10–0·14 | Ph, S, Thr, Val, Arg | 1 | 4 | – | 2 | 1 | – | 2 | 1–2 | 1 | 2 | 2 | 1 | – | – | 1 | – |
| F5, 3 | 0·17–0·28 | Ph, Val, Asp, Gly, S, Thr, Ala, Arg | 1 | 3 | – | 1 | 1 | – | 2 | 2 | 2 | 3 | 2 | 1 | ? | – | 1 | – |
| F5, 4 | 0·27–0·45 | Glu, Ph, Val | 1 | 2 | – | 1 | – | – | 1–2 | 2 | 2 | 3 | 1–2 | 1 | – | 1 | 1 | – |
| F5, 5 | 0·37–0·64 | Val, Ph, A | 1 | 2 | – | trace | ? | – | 1–2 | 2 | 3 | 3 | 1 | trace | – | – | 2 | 1 |
| F5, 6 | 0·48–0·68 | Val, Ph, S, Arg | 1 | 2 | – | trace | 1–2 | – | 2 | 2 | 3 | 3 | – | – | – | 1 | 1 | 1 |
| F6, 1 | 0·06–0·13 | Ph, S | 1 | 3 | – | 2 | 1 | ? | 1 | 1 | 1 | 2 | – | trace | – | – | 1 | – |
| F6, 2 | 0·09–0·23 | Ph, S, Thr? | 1 | 2 | – | 1–2 | 1 | – | 1 | 1–2 | 1 | 1–2 | trace | – | – | – | 1 | – |
| F6, 3 | 0·09–0·23 | Ph | trace | 2 | trace | 1 | 1 | – | 1 | 1 | 1 | 2 | – | – | – | – | 1 | – |
| F6, 4 | 0·24–0·54 | Ph | trace | 2 | trace | 1 | 1 | – | 1 | 2 | 1 | 1 | – | – | – | – | 1 | – |
| F6, 5 | — | Ph | 1 | 2 | – | 1 | 1 | – | 1 | 2 | 1 | 2 | 1 | – | – | – | 1 | – |
| F6, 6 | 0·4–0·56 | Ph | 1 | 2–3 | – | 1 | 1 | – | 1 | 1 | 1 | 2 | 1 | trace | – | – | 1 | – |
| F6, 7 | 0·4–0·56 | — | 1 | 3 | – | 1 | ? | – | 1 | 1 | 2–3 | 2–3 | 1 | – | – | – | 1 | – |

* In the subfractions shown the first figure indicates the number of the fraction and the second is the number of the subfraction.
Ph represents spot below and slightly to the right of Phe.
A unidentified spot slightly below DNP-alanine position.
S represents serine or ε lysine.
? doubtful.

## TABLE III

THE HYROLYSATES ON NEUTRAL SUBFRACTIONS*

| Subfraction | *RF* | DNP-amino acids | Asp | Glu | Orn | Lys | Arg | His | Gly | Ala | Val | Leu | Ser | Thr | Tyr | Phe | Pro | Met |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F7, 1 | 0·05–0·12 | Asp, Glu, S, Ph, Lys? | 1 | 3 | – | 2–3 | 1 | – | 1 | 2 | 1 | 2 | 1 | 1 | – | – | 1 | – |
| F7, 2 | 0·08–0·12 | Ph, S | 2 | 2 | – | 1 | – | – | 1 | 1 | 1 | 1 | 1 | – | – | – | 1 | – |
| F7, 3 | 0·18–0·27 | Asp, Glu, S, Gly, Ala, Val, Ph, Thr? Lys | 1 | 2 | trace | 1–2 | 1 | ? | 2 | 2 | 1 | 2 | 2–3 | 1 | – | – | 1 | – |
| F7, 4 | 0·20 | S, Gly, Thr, Ala, Ph | 1 | 2 | – | 1–2 | 1 | – | 1 | 1–2 | 1 | 2 | 1 | 1 | – | – | 1 | – |
| F7, 5 | 0·25–0·38 | Ph, S, Leu, Val, Ala, Thr, Lys | trace | 1 | – | 1 | 1 | – | 1 | 4 | 1 | 2 | 1 | 1 | – | – | 1 | – |
| F7, 6 | 0·32–0·38 | Ph, S, Glu | 1 | 2 | trace | 1–2 | 1 | – | 1 | 1–2 | 1 | 1–2 | 1 | trace | 1 | 1 | 1 | – |
| F7, 7 | 0·4 | Ph, Val, Met, Ala, S | 1 | 2 | trace | 1 | 1 | – | 1 | 2–3 | 4 | 2 | 1 | trace | 1 | – | 1–2 | 2 |
| F7, 8 | 0·43–0·59 | Ph, Leu, Val, Ala, S, Thr, Pro | 1 | 1 | – | 1 | 1 | – | 1 | 1 | 2 | 5 | – | – | – | 2 | 1 | 2–3 |
| F7, 9 | 0·47–0·56 | Ph, Leu, Val, Arg | 1 | 2 | – | 1 | 1 | – | 1 | 2 | 2 | 5 | 1 | 1 | – | 2 | 2 | 2 |
| Fx1 | 0·175 | Ph, S | trace | 1 | – | – | 1 | – | 1 | 1 | – | – | 1 | – | – | – | – | – |
| Fx2 | 0·24 | Ph, S | trace | 1 | – | – | 1 | – | 1 | 1–2 | – | – | 1 | – | – | – | – | – |
| Fx3 | 0·175 | Ph, S | trace | 1 | – | – | 1 | – | 1 | 1 | – | – | 1 | – | – | – | – | – |
| Fx4 | 0·24 | Ph, S | trace | 1 | – | – | 1 | – | 1 | 1–2 | – | – | 1 | – | – | – | – | – |

* See footnote on Table II.

## TABLE IV

THE HYDROLYSATE OF BASIC SUBFRACTIONS*

| Subfraction | *RF* | DNP-amino acid | Asp | Glu | Orn | Lys | Arg | His | Gly | Ala | Val | Leu | Ser | Thr | Tyr | Phe | Pro | Met |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F8, 1 | 0·14–0·26 | Ph, S, Val | – | 2 | – | 2 | – | – | 1 | 2 | – | 2 | – | – | – | – | – | – |
| F8, 2 | 0·15–0·3 | Ph | – | 2 | – | 2 | ? | – | 1 | 2 | 1 | 1–2 | 1 | – | – | – | – | – |
| F8, 3 | 0·15–0·35 | Ph, S | – | 2 | trace | 2 | – | – | 1 | 1–2 | 1 | 1–2 | 1 | – | – | – | – | – |
| F8, 4 | 0·18–0·35 | Ph | – | 1 | – | 1 | 1 | – | 1 | 1 | 1 | 1 | 1 | – | – | – | – | – |
| F8, 5 | 0·43–0·51 | Ph | – | 1 | – | 1 | 1 | – | 1 | 1 | 1 | 1 | 1 | – | – | – | ? | – |
| F8[i] | 0·38 | Ph, Gly | – | 1 | – | 1 | 1 | – | 1 | 1 | 2 | 1 | 1 | – | – | – | 1 | – |
| F9, 1 | 0–0·04 | Ph | 1 | 1–2 | – | 2–3 | – | – | 1 | 1–2 | 1 | 1 | 1 | 1 | – | – | – | – |
| F9, 2 | 0·07–0·12 | Ph, S | trace | 2 | 1 | 2–3 | – | – | 2 | 2–3 | 1 | 1 | 1 | – | – | – | 1 | – |
| F9, 3 | 0·17–0·25 | Ph, S, Lys | – | 2 | – | 2 | 1 | – | 1 | 2 | 2 | 1 | 1 | – | 1 | – | 1 | – |
| F9, 4 | 0·17–0·29 | Ph, S, Ala | – | 1 | – | 1 | – | – | 1 | 3 | 1 | 2 | 1 | – | – | – | – | – |
| F9, 5 | — | Ph | – | – | – | 1 | 1 | – | 1 | 2 | 1 | 2–3 | – | – | – | – | – | – |
| F10, 1 | 0–0·04 | Lys, S, Ph | – | 2 | – | 2–3 | – | – | 1 | 1–2 | 1 | 1 | 1 | – | – | – | 1 | – |
| F10, 2 | 0·06–0·11 | Lys, Ph, S | – | 2–3 | – | 4 | 1 | – | 1 | 1–2 | 1 | 3–4 | 1 | – | – | – | 1 | – |
| F10, 3 | 0·09–0·13 | Lys, Ph, S, Arg | – | 1–2 | – | 3 | 1 | – | 1 | 3 | 1 | 1 | 1 | – | – | – | 1 | – |
| F10, 4 | 0·17–0·23 | Ph, S, Glu? | – | 1 | – | 2 | – | – | 2 | 2 | 1 | 2 | 1 | – | – | – | 1 | – |
| F10, 5 | 0·17–0·29 | Ph, S, Glu? | – | 1 | – | 3 | 1 | – | 1 | 3 | 1 | 1 | 1 | – | – | – | 1 | – |
| F10, 6 | 0·27–0·39 | Ph, S, Lys | – | 1 | – | 3 | 1 | – | 1 | 2 | 2 | 2 | 1 | – | – | – | 1 | – |
| F11, 1 | 0·07–0·14 | Phy, S, Lys, Arg | 1 | 2–3 | 1 | 2–3 | 2 | – | 1 | 1–2 | 1 | 1 | 1 | – | – | – | – | – |
| F11, 2 | 0·09–0·15 | Ph, S, Lys, Arg, Thr? | – | 1–2 | – | 2–3 | 1 | – | 1 | 1 | trace | trace | 1 | 1 | – | – | – | – |
| F11, 3 | 0·19–0·24 | Ph, Arg? | 1 | 1 | 1 | 1 | 1 | – | 1 | 1–2 | 1 | 1 | – | – | – | – | – | – |
| F11, 4 | 0–2–0·33 | Ph, S, Val | – | 1 | – | 2 | 1 | – | 1 | 1 | 1 | 2 | – | – | – | – | – | – |
| F11, 5 | 0·31–0·35 | Ph, Gly? | – | – | – | 3 | – | – | 1 | 1 | 1 | 3 | 1 | – | – | – | 1 | – |

* See footnote to Table II.

## Results

Fractions F1 and F2 contain free aspartic and glutamic acids respectively. F12 and F13 showed free lysine, arginine, histidine and arginine, lysine, ornithine, respectively.

Table I gives the numbers of subfractions obtained from each fraction of the three batches of casitone. F8[1] was a slow moving spot obtained when fraction F9 was subjected to electrophoresis.

Tables II–IV show the amino acid contents in the subfractions obtained from the acidic, neutral and basic fractions respectively for batch A Arbitrary figures ranging from 1–10 indicate the relative amounts of the amino acid judged from the size and intensity of the colour. The terminal amino acid of the peptide or peptides in each subfraction is also given as the DNP-derivative.

Batches B and C were similarly analysed for the *N*-terminal and the constituent amino acids of peptides in the subfractions. Some of the subfractions showed more than one *N*-terminal amino acid while others proved to be formed of only one peptide. An unidentified spot (Ph) appeared on the chromatogram of DNP-amino acids in the *N*-terminal position which occupied a position slightly below and to the right of phenylalanine. It may be an artifact.

## Discussion

The electrophoretic patterns obtained with the three batches of casitone were similar, and demonstrated the fractionation of casitone into 11 fractions which were subjected to further separation by paper chromatography. Batches A, B and C gave 61, 73 and 73 subfractions respectively as seen in Table I.

The acidic subfractions showed a preponderance of aspartic and glutamic acid in their hydrolysates. The peptides examined had aspartic, glutamic acids, lysine, arginine and some of the neutral amino acids in the *N*-terminal position. The peptides in the basic subfractions were rich in lysine and arginine and some of them showed lysine, arginine and neutral amino acids as the *N*-terminal residue. Phosphopeptides have been separated from an enzymic hydrolysate of casein by Agren and Glomset[5]. Here phosphoserine peptides will show serine in the *N*-terminal position because DNP-phosphoserine gives DNP-serine on hydrolysis with 6N hydrochloric acid. A spot in the position of Ph has been reported previously as an artifact[6]. Some of the subfractions separated from casitone proved to be a mixture of peptides of similar mobility and $R_F$ value as appears from the *N*-terminal amino acid residue, while others are formed of one peptide.

This analysis shows the complexity of the peptide mixture of casitone as noticed from Tables II–IV.

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# BOOK REVIEW

*PHARMACOLOGY.* By J. H. Gaddum. Fifth Edition. Pp. xvi + 587 (including Index). Oxford University Press, London, 1959. 42s.

It is 6 years since the fourth edition of this popular textbook was published. Although an enormous number of new drugs have been introduced since 1953, the text has increased by only 5 per cent. Textbooks of this kind, as soon as they become too bulky, pass rapidly into the realm of reference books, so Professor Gaddum is to be congratulated on his pruning of the material.

All the important drugs of the British Pharmacopoeia 1958 are described, and now for the first time the official doses have been included. The largest new sections deal with the actions and uses of some radioactive isotopes and with the methods used in the study of the effects of drugs on the brain. At the present time, these aspects of the subject form part of the basic training of pharmacologists and it is certain that their scope will widen in future years. The subject matter has had to be concentrated but, as in previous editions, ready access to it has been secured by an efficient index. The idea of presenting so many tables illustrating the chemical relationship of closely allied substances is excellent for students and teachers alike, and this is particularly useful for the corticoids, the barbiturates, the analgesics, the local anaesthetics, the antihistamines, the phenothiazines, the acridines and the sulphonamides. It is a good plan, too, to include tables of weights and measures and a key to chemical names, for conversion factors and formulae are not always fully appreciated by biological workers.

In the first edition, which appeared in 1940, Professor Gaddum suggested that all workers connected with drugs should know something of the kind of evidence that justifies the clinical trial of new substances. In the fifth edition, it is good to find that so many of the experimental methods used before the introduction of new therapeutic measures have been retained. This also makes the book more enjoyable to read. One mistake in the text occurs on page 236 ("Most" should be "Mast"). As it is a moderately-sized book covering the general principles of pharmacology and surveying the commoner drugs, there is no hesitation in recommending it to all who are interested in medical science.

G. B. WEST.

# LETTER TO THE EDITOR

## The Chromatographic Behaviour of Aloin

SIR,—In a study involving the ability of bacteria to utilise sugars from several glucosides it became necessary to test for the presence of free sugars. Because of the low solubility of aloin in water and the highly coloured solutions produced it was necessary to use paper chromatographic procedures for this test. The liquid phase consisted of equal parts of pyridine, butanol, ethyl acetate and water. Under these conditions a deposit of aloin became reddish-violet in colour on contact with the liquid phase. Continuous irrigation of the deposit with this liquid system for 3 to 4 hours at 25° resolved the original spot into two, one yellow, the other red-violet. The yellow component had the greater mobility and advanced in a fairly solid front while the red-violet tailed. Hydrolysis of the glycoside had no effect on the chromatogram. It is assumed, therefore, that the colour is due to the anthraquinone nucleus.

Svendsen and Jensen[1] showed that a red-violet material is produced when aloin is warmed in the air; the test for sugar was positive but that for anthranol or anthraquinone was negative. Jaminet[2] uses paper chromatographic procedures to classify aloe resins according to botanical and geographical origin. All the details are not available to us but the investigator indicates that after exposure of aloin to ammonia, spots are formed at $R_F$ 0·69 (bright yellow) and 0·56 (intense sky blue). Paris and Durand[3] use a photometric determination for aloin which is based on an electrophoretic separation. It is known that aloin will form colours under certain conditions but the conditions and colours reported are not those which we have found.

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