case considered is $A \rightleftharpoons nB$, but the treatment could be easily extended. It leads to a differential equation for transport of material across a plane, due to the combined effects of differential migration velocities and diffusion.

2. Although the differential equation cannot be directly integrated, numerical integration can be performed. This has been done, using the various quantities appropriate to human haemoglobin at pH 5.06, available from the experimental results of the preceding paper; only the velocity constant of the dissociation has to be guessed.

3. The agreement between the calculated and observed spreading of the boundary is satisfactory. In particular it is shown that the shape of the boundary and the variations of its rate of spreading with time are remarkably insensitive to the heterogeneity caused by a fairly rapidly established dissociation equilibrium.

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Clearing Factor and Lipase

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Since the work of Hahn (1943), it has been repeatedly shown that alimentary lipaemia disappears after the intravenous injection of heparin. It is also a well-established fact that this is not a direct effect of heparin, but that the administration of heparin gives rise to the appearance in the blood of another substance, which has been called 'clearing factor' (Anderson & Fawcett, 1950). Since then the work of other groups has brought to light many chemical and biological features of this interesting substance. One of the unsettled problems is the relationship between clearing factor and lipase. Clearing factor clears lipaemia *in vitro*, which heparin is unable to do, and during the clearing process fatty acids are liberated. This suggested that clearing factor might be a kind of lipase or an activator of this enzyme.

However, we and several other groups of investigators have failed to show an increase of the lipase activity in the plasma of rats and dogs after heparin administration, although there are investigators who hold the opinion that the lipase activity is enhanced. A cause of these divergent results might be that several types of fat-splitting enzymes (lipases) occur in the plasma of various species of animals, and that the activity of different enzymes may be measured depending on the substrate used and other conditions of the experiment.

Mendel, Myers, Uyldert, Ruys & Bruyn (1953) introduced the term 'ali-esterases' for enzymes which hydrolyse only esters of lower fatty acids (like tributyrin) but not esters of palmitic, oleic and other higher fatty acids. These ali-esterases are inhibited by sodium *p*-aminophenylarsonate (atoxyl), tri-*o*-cresyl phosphate (TOCP) and taurocholate. 'True' lipase degrades both esters of lower and higher fatty acids, is inhibited by quinine but not by atoxyl and TOCP, and is even activated by taurocholate. In this paper we employ the terms 'ali-esterase' and 'lipase' for these two separate enzymes and 'tributyrynases', when both are discussed. In human beings both types of tributyrinases occur in the plasma. Ali-esterases are produced in the liver, lipase in the pancreas (Srinivasan & Patwardhan, 1952). It will be shown that in other species of animals either one or other type occurs almost exclusively in the plasma.

The aim of this investigation was to ascertain whether ali-esterases or lipase are essential for the formation and/or action of clearing factor. For this purpose we studied first the influence of well-known tributyrinase inhibitors on the clearing process *in vitro*. Later rats were freed from ali-esterase activity by pre-treatment with TOCP, whereas in dogs the lipase activity of the plasma was reduced by pancreatectomy. Heparin was then injected

into both types of animals under conditions which in normal individuals had proved to give rise to the appearance of clearing factor in their plasma.

MATERIAL AND METHODS

Clearing activity. As a substrate we used lipaemic plasma from normal mongrel dogs. The animals were given a fat meal, and 3 hr. later, when lipaemia had developed, they were bled from the carotid artery. The blood was collected in 3.8% (w/v) sodium citrate; 4 vol. of blood were run into 1 vol. of citrate solution. The blood was centrifuged and the plasma was stored in a frozen state in small bottles; under these conditions there was no deterioration for a period of at least 3 months.

For the production of clearing factor 10 anticoagulant units of heparin were injected intravenously into rats. Exactly 10 min. later the animals were bled from the abdominal aorta and 4 ml. blood added to 1 ml. 3.8% sodium citrate solution. In many instances pooled plasma from several rats was used. The dogs received an intravenous injection of 125 units/kg. and a blood sample was taken from a leg vein 30 min. after the injection.

Since the clearing-factor activity of blood decreases rapidly on standing at room temperature, it was centrifuged immediately and the plasma used without delay. The activity was determined by adding 0.5 ml. of the clearing factor-containing plasma to 1.5 ml. of lipaemic dog plasma (or in one case to dog lipoproteins suspended in rat plasma) and measuring the optical density at 650 μ . before and after 1 hr. incubation at 37°, using either an Evelyn colorimeter with tubes of 7 mm. diameter, or a Lumetron colorimeter (model 402E). In a series of forty-nine duplicate determinations with clearing activities (decreases of optical density) ranging from 0.000 to 0.300 a standard deviation of ± 0.006 was found for the differences between duplicates.

Determination of fatty acids. This was performed in the plasma mixtures by a method described by van de Kamer (1948) for the estimations in faeces, as modified by van der Vies (Appendix) for use in blood plasma. The determinations were made on 2 ml. of citrate plasma.

Lipase activity. This was determined by a modification of the method of Srinivasan & Patwardhan (1952).

Plasma (0.5 ml.) was added to 10 ml. of substrate solution (3 ml. tributyrin + 100 ml. 0.25% (w/v) solution of sodium diethylbarbiturate). The mixture was shaken for 3 hr. at 37° and finally poured into 60 ml. of 96% (w/v) ethanol. After standing overnight, the proteins were filtered off and two 25 ml. samples titrated with standard alkali solution (0.050N-NaOH), using thymol blue as

indicator. For each plasma, a blank was run to which the plasma was added at the end of the incubation period. The lipase activities in the tables are expressed in ml. 0.050N-NaOH used for titration. In a series of thirty-two determinations a standard deviation of ± 0.047 was found for the difference between duplicates.

For the experiments with rats, animals of about 200 g. were used irrespective of sex. In order to obtain ali-esterase-free animals, one subcutaneous injection of 0.1 ml. TOCP was given on the day before the experiment. Concentrations of inhibitors added *in vitro* are indicated in the tables.

The plasma was incubated with the inhibitor at 37° for 30 min. before the determination of the clearing activity. This was necessary because the inhibition of tributyrinase by some inhibitors, e.g. atoxyl develops slowly.

For the study of true lipase, male and female mongrel dogs of 5–16 kg. were used. After pancreatectomy the animals were injected twice daily with sufficient insulin to maintain them in a moderately hyperglycaemic but otherwise good condition.

RESULTS

The differences between ali-esterase and lipase as mentioned in our introductory remarks are clearly demonstrated in Table 1, which shows that rat plasma contains practically nothing but ali-esterase (little activation by taurocholate after inhibition of ali-esterase activity by TOCP), whereas dog plasma contains practically nothing but lipase (significant activation by taurocholate and inhibition by TOCP).

It is important to stress the point that this statement in its full significance holds only for the conditions of our own experiments. We found that slight alterations of the experimental conditions, for example a larger amount of plasma used for the tributyrinase determinations, would have led us to the conclusion that only relatively less lipase was present instead of practically nothing but ali-esterase.

This same limitation holds for our conclusions on the quinine inhibition of the tributyrinases, which is mentioned below, and possibly for other points. Consequently, our experiments on the possible relationship between ali-esterase and clearing factor were performed in rats, whereas the relation between lipase and clearing factor was studied in dogs.

Table 1. *Lipase and ali-esterase activity of rat and dog plasma*

The enzyme activity is expressed in ml. 0.05N-NaOH required for neutralizing the acid liberated from 0.3 ml. tributyrin after incubation with 0.5 ml. plasma for 3 hr. at 37°.

Addition	Rat plasma		Dog plasma	
	Without taurocholate	Taurocholate (0.2%)	Without taurocholate	Taurocholate (0.2%)
None	0.60	0.23	0.08	0.38
TOCP (sat.)	0.10	0.16	0.07	0.36

Table 2. Absence of increased tributyrinase activity in clearing-factor-containing rat and dog plasma

Activity measured as in Table 1.

	Rat plasma		Dog plasma			
	Normal	Clearing factor	Without taurocholate		Taurocholate (0.2%)	
			Normal	Clearing factor	Normal	Clearing factor
	0.85	0.95	0.05	0.04	0.10	0.10
	0.97	1.03	0.07	0.08	0.26	0.25
	1.21	1.22	0.10	0.10	0.22	0.17
			0.14	0.16	0.35	0.33
			0.13	0.16	0.37	0.37
Av.	1.01	1.07	0.09	0.11	0.26	0.24

Table 3. Effects of lipase inhibitors on the clearing factor

0.1 ml. solution, containing 4 mg. of inhibitor or 0.1 ml. water in the blanks was incubated for 30 min. at 37° with 0.5 ml. of 'clearing-active' rat plasma. Then 1.5 ml. of lipaemic dog plasma was added and the clearing estimated, using the Lumetron colorimeter with 0.5 cm. cuvettes. For these experiments three pools of clearing-active plasma were used. The differences, compiled in the last column, are statistically significant ($P < 0.001$). Figures in the table are optical densities.

Inhibitor	No. of experiments	Blanks, previously incubated with water	Previously incubated with inhibitor	Difference (inhibition)
Atoxyl	6	$0.106 \pm 0.009^*$	0.037 ± 0.003	0.069 ± 0.010
Sodium taurocholate	6	0.106 ± 0.009	0.002 ± 0.004	0.104 ± 0.010
Quinine hydrochloride	3	0.150 ± 0.003	0.077 ± 0.002	0.073 ± 0.004

* Standard error of mean.

Fig. 1. Relationship between clearing (plain columns) and release of fatty acids (black columns) in: *a*, normal plasma; *b*, normal plasma + atoxyl; *c*, plasma containing clearing factor; and *d*, plasma containing both clearing factor and atoxyl.

Fig. 2. The effect of various concentrations of atoxyl on the clearing in plasma. Solutions (0.1 ml.) containing varying amounts of atoxyl were incubated 30 min. with 0.5 ml. of clearing-active plasma at 37°. Then water (0.5 ml.) and lipaemic plasma (1.5 ml.) were added, and the clearing estimated in duplicate at each concentration.

Table 2 shows that in neither rat nor dog plasma is the tributyrin-splitting capacity enhanced by the presence of clearing factor, even after activation of the dog lipase by taurocholate. This means that if clearing factor should prove to be a kind of lipase it would be of a different type to ali-esterase and pancreatic lipase, as it does not hydrolyse tributyrin. This experiment also excludes the possibility that clearing factor is an activator of a tributyrinase. It does not exclude the possibility that one of

the tributyrinases could be essential for either the formation or the action of clearing factor.

Tables 3 and 6 and Figs. 1 and 2 show that clearing factor and the tributyrinases behave similarly in some respects but differ in others.

The clearing *in vitro* is inhibited by the ali-esterase inhibitors atoxyl and sodium taurocholate

and by the lipase inhibitor quinine. Not only the clearing, but also the liberation of fatty acids is inhibited by atoxyl to about the same extent.

Table 4 records an experiment in which sufficient atoxyl was added to rat plasma to obtain a decrease of ali-esterase activity from 0.46 to 0.37, and a decrease of the clearing from 0.179 to 0.129. The addition of 0.5 ml. ali-esterase-containing rat plasma which augmented the enzyme activity to values of 0.75 and 0.56 respectively did not improve the clearing. This shows that atoxyl inhibits the action of clearing factor regardless of the ali-esterase activity. Apparently the action of clearing factor is also independent of the true lipase activity (which is present in the mixture because of the addition of lipase-containing lipaemic dog plasma) as atoxyl does not inhibit this type of lipase, although it inhibits clearing factor.

After thus studying the relationship between the action of clearing factor and the tributyrinases, we

It is clear from Table 5 that, although in the three pooled plasma mixtures (each from three rats) the ali-esterase activity after TOCP treatment was exceedingly low and true lipase was also absent, the clearing was unaltered whereas the fatty acid production was only slightly lower. Apparently the production of clearing factor is entirely independent of the ali-esterase content of the plasma, and the action is independent both of the ali-esterase and the true lipase activity.

For the study of the relationship between production of clearing factor and true lipase, five dogs were pancreatectomized and maintained with insulin. Plasma lipase fell in all instances within a few days after operation, but surprisingly (with the exception of one dog) rose again to normal levels within a period of about 2 weeks. In these dogs the production of clearing factor after the injection of heparin also proved to be temporarily impaired. (We do not know the cause of the restoration; it

Table 4. *Effect of plasma ali-esterase on an inhibited clearing system of rat plasma*

The components indicated in the second column were incubated at 37° for 30 min. Then the components mentioned in the third column were added. Each mixture was prepared in triplicate and clearings estimated by following the drop in extinction at 650 m μ . Afterwards the triplicate mixtures were pooled and tributyrinase activity was determined in duplicate.

Mixture no.	Components incubated together	Components added after incubation	Clearing obtained in triplicate experiment ($-\Delta E_{650\text{m}\mu}$)			Mean	Tributyrinase activities (ml. 0.05 N alkali)	
			0.180	0.175	0.182		0.46	0.46
1	0.1 ml. water + 0.5 ml. clearing-active rat plasma	0.5 ml. water + 1.5 ml. lipaemic dog plasma				0.179		
2	0.1 ml. water + 0.5 ml. clearing-active rat plasma	0.5 ml. normal rat plasma + 1.5 ml. lipaemic dog plasma	0.160	0.158	0.154	0.157	0.77	0.73
3	0.1 ml. atoxyl (2 mg.) + 0.5 ml. clearing-active rat plasma	0.5 ml. water + 1.5 ml. lipaemic dog plasma	0.131	0.131	0.124	0.129	0.37	0.37
4	0.1 ml. atoxyl (2 mg.) + 0.5 ml. clearing-active rat plasma	0.5 ml. normal rat plasma + 1.5 ml. lipaemic dog plasma	0.124	0.131	0.123	0.126	0.56	0.56

passed to the possible connexion between the production of clearing factor and the tributyrinases. For this purpose rats were injected subcutaneously with 0.1 ml. TOCP and bled the next day. This treatment reduced the ali-esterase activity of the plasma almost to zero level. Nevertheless, the animals still produced clearing factor after injection of heparin. The results of a final experiment are presented in Table 5. Here all ali-esterase activity was excluded by TOCP treatment of the rats and the lipase activity by isolation of the lipoproteins of the dog plasma (by leaving the lipaemic dog plasma in the cold room and suspending the slowly separating lipoproteins in plasma of TOCP-treated rats).

Table 5. *Clearing activity in the absence of ali-esterase*

Plasma from rats treated with tri-*o*-cresyl phosphate compared with plasma from untreated rats. Ali-esterase activity measured as in Table 1; clearing activity as decrease in optical density in 1 hr. at 37°; fatty acids expressed as ml. N-NaOH. Other details as in text.

	Ali-esterase activity	Clearing activity	Increase in fatty acids
Untreated control	0.51	0.172	3.11
	0.59	0.157	2.96
	0.65	0.152	2.97
TOCP treated	0.03	0.152	2.63
	0.01	0.163	2.65
	0.01	0.164	2.56

might mean a compensatory increased production of intestinal and gastric lipase.)

It was a distinct advantage of the temporary character of the fall of the lipase activity and clearing-factor production that it enabled us to demonstrate the satisfactory correlation which exists between the two. This is shown in Fig. 3, in which, because of the difference of the levels in the various animals, all data are expressed in standard measures (standard deviation units, i.e. for each series of observations the differences between the individual values and the average were divided by the standard deviation).

Fig. 3. The correlation between clearing-factor production and plasma lipase activity in depancreatized dogs.

The striking accumulation of points in the left lower and the right upper quadrant immediately shows the apparently good correlation. This was confirmed by the statistical calculation which showed that the correlation coefficient $r=0.647$ (for 43 points) with a value for t (for 41 degrees of freedom) of 5.47 and $P<0.001$.

Apparently in these depancreatized dogs a definite relationship exists between the lipase activity of the plasma and the ability to produce clearing factor.

DISCUSSION

The *in vitro* studies presented in this paper show that the clearing process is inhibited by a number of substances which are well-known tributyrinase inhibitors.

We showed that at least one of these (atoxyl) inhibits clearing factor directly, independently of

the activity of the two tributyrinases. This sensitivity to tributyrinase inhibitors and the release of fatty acids during clearing, which is also inhibited by atoxyl, indicates that clearing factor resembles the tributyrinases in some respects. However, clearing factor is not identical with one of the tributyrinases as is demonstrated by the fact that its behaviour towards the various inhibitors is different (Table 6).

Table 6. *Effect of various inhibitors on clearing factor, ali-esterase and lipase*

- = Inhibition; + = activation; 0 = no effect.

Inhibitor	Clearing factor	Ali-esterase	Lipase
Atoxyl	-	-	0
Taurocholate	-	-	+
TOCP	0	-	0
Quinine	-	0	+

Moreover, clearing factor does not split tributyrin, nor does it enhance the tributyrin-splitting capacity of rat and dog plasma (Table 2). Finally, clearing factor can clear lipaemic plasma in the almost complete absence of both ali-esterase and lipase (Table 5). These findings show that the action of clearing factor seems to be in every respect independent of ali-esterase and lipase.

Since clearing factor itself has some properties in common with both tributyrinases it might be considered to be a similar though not identical enzyme, for example a lipoproteinase as suggested by Korn (1954).

With regard to the production of clearing factor the situation is different. Although it could be shown in rats that the administration of heparin gives rise to a normal production of clearing factor in the absence of ali-esterase (Table 5), the production of clearing factor in dogs seems to be correlated with the lipase activity of the plasma (Fig. 3). It may seem somewhat peculiar that the rat produces clearing factor without lipase in its plasma, but it should be considered that clearing factor need not necessarily be produced in the circulating blood and that the pancreas of the rat contains lipase. The correlation between lipase activity and clearing-factor production may prove to be of considerable interest as it seems to be consistent with some findings of other investigators.

Neches, Plotke & Meyer (1942) showed that the pancreas secretes less lipase in old people than in young ones, and Meyer, Sorter & Neches (1942) showed that the lipase activity of the plasma in old age is significantly lowered. Becker, Meyer & Neches (1949) also counted the chylomicra in the serum of young and old people after a fat meal and

found that in old subjects the chylomicron curves are much higher and fall more slowly than in young people.

It is of great interest that in the same paper they mention how after the oral administration of lipase this 'old-type curve' was transformed into a 'young-type curve'. With regard to clearing factor Utz, Mann & Barker (1953) reported that the production of clearing factor after a standard dose of heparin in atherosclerotic patients is subnormal. It should be noted that these studies are not strictly comparable because of the use of different methods and different subjects (pancreatectomized dogs, normal old people and atherosclerotics). However, keeping this in mind we would like to suggest the following tentative hypothesis:

During ageing the production of lipase by the pancreas decreases causing a lowering of the plasma level of lipase. Consequently, the ability to produce clearing factor diminishes and the normal chain of reactions of the lipoproteins (Gofman, Lindgren, Jones, Lyon & Strisower, 1951) is disturbed and fat is removed from the blood at a much slower rate, which eventually may promote the development of atherosclerosis. In this respect it is of interest that Stamler & Katz (1951) could show that, in chickens on a cholesterol-oil diet, pancreatectomy led to a much higher cholesterol level of the blood plasma and to much more severe atherosclerotic lesions.

The previously mentioned result of Becker *et al.* (1949) suggests the possibility of restoring the normal sequence of events not only by heparin (Graham *et al.* 1951; Jones *et al.* 1951), but also by oral administration of lipase. To aim at the prevention of atherosclerotic changes seems to be more hopeful than trying to cure already advanced lesions of the wall of the blood vessels and for the administration of a prophylactic drug the oral route would probably be the only practicable way. From this angle lipase looks more attractive than heparin. A clinical study will have to prove the correctness of these ideas.

SUMMARY

1. *In vitro* studies on the action of clearing factor prove that neither ali-esterase nor lipase are

necessary for its action. Clearing factor acts even in the almost complete absence of both enzymes.

2. The behaviour of clearing factor towards various inhibitors of ali-esterase and lipase, together with the liberation of fatty acids during clearing, indicates that clearing factor might be a new type of enzyme (a lipoproteinase) which possesses some characteristics of both ali-esterase and lipase without being identical with either of them.

3. *In vivo* studies on the production of clearing factor showed that it can be produced in the absence of ali-esterases, but that in pancreatectomized dogs a good correlation existed between the lipase activity of the plasma and the production of clearing factor after injection of heparin.

4. The possible implications of these findings are discussed in relation to the problem of atherosclerosis.

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