SUMMARY

1. The method of Kekwick & Mackay (1954) for the fractionation of human plasma proteins was used successfully for the separation of the serum proteins of guinea pig, rat, rabbit, cat, dog, monkey, ox, horse and sheep.

2. By variations in pH, ionic strength, temperature and solvent concentration, three fractions consisting essentially of a mixture of α - and β -globulins (G2); γ -globulin (G3) and albumins (AP) were isolated and concentrated. From rat, rabbit, cat, dog, ox and horse serum electrophoretically pure γ -globulin could be prepared from the fraction G3. From the fraction AP electrophoretically pure albumin was prepared from guinea pig, rabbit and sheep; and 98 % pure albumin from dog and cat.

3. Tests on the fractions showed that cholesterol was largely concentrated in the fraction G2. The natural antibodies (*Salmonella* agglutinin) were concentrated in G2 in rat, ox, sheep and dog serum; and in G2 and G3 in horse, guinea pig and rabbit serum. It is suggested that the crude albumin might find a use as a vehicle for radioactive isotopes.

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Studies on Ali-esterases and Other Lipid-hydrolysing Enzymes

2. THE EFFECT OF HEPARIN INJECTION ON ALIMENTARY LIPAEMIA AND ON THE PLASMA ESTERASES*

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Although heparin does not affect the turbidity of lipaemic plasma when added *in vitro*, Hahn (1943) and Weld (1944) showed that a visible alimentary lipaemia in dogs disappears within a few minutes after the intravenous injection of small amounts of heparin. The post-heparin plasma contains a factor which is capable of clearing lipaemic plasma *in vitro* (Anderson & Fawcett, 1950); this factor has the properties of a protein and could therefore be an enzyme (Anfinsen, Boyle & Brown, 1952; Spitzer, 1952; Levy & Swank, 1954).

The clearing factor causes a decrease in the size of the large lipoprotein globules, apparently by hydrolysing the triglycerides to produce free fatty acids and glycerol (Graham *et al.* 1951; Swank & Wilmot, 1951; Anfinsen *et al.* 1952; Shore, Nichols & Freeman, 1953; Korn, 1954). It appears to be inhibited by small amounts of protamine (Brown,

1952; Spitzer, 1953) and by large amounts of sodium chloride (Brown, Boyle & Anfinsen, 1953), to be activated by heparin and to act specifically on lipoprotein (Korn, 1954). Pancreatic lipase also exhibits clearing activity *in vitro* and *in vivo* (Spitzer, 1952, 1953), but the clearing factor can be found in the post-heparin plasma of pancreatectomized dogs (Fasoli, Glassman, Magid & Foa, 1954).

It is reported that the clearing factor can be separated from plasma 'esterase' by fractional precipitation with ethanol (Brown *et al.* 1953) and that it does not hydrolyse β -naphthyl laurate (Brown, Baker & Kauffman, 1954). However, Levy & Swank (1954) found that the rate of hydrolysis of tributyrin by dog plasma increased 50–100 % within a few minutes after intravenous injection of heparin, while the esterase activity of normal dog plasma was not affected by incubation with heparin *in vitro*. On injecting small amounts of protamine after injecting the heparin, the activity

* Part 1: Myers & Mendel (1953).

of the clearing factor in the post-heparin plasma is inhibited and the esterase activity returns to normal.

However, it is known that the hydrolysis of tributyrin by normal dog plasma is due almost entirely to the presence of pseudocholinesterase (cf. Richter & Croft, 1942). In the present investigation we have found that the 50–100% increase in the rate of hydrolysis of tributyrin by the plasma after injection of heparin is due to the appearance of another enzyme capable of hydrolysing tributyrin, the activity of the pseudocholinesterase remaining unaltered. It seemed of interest, therefore, to investigate a possible relationship between this tributyrinase and the clearing factor in post-heparin plasma.

METHODS

Esterase activity was measured manometrically by the Warburg method at 37.5° and pH 7.4 in a medium containing 0.025M-NaHCO₃ and saturated with 5% CO₂ and 95% N₂ (v/v). The esterase inhibitors used were physostigmine salicylate (eserine), diethyl *p*-nitrophenyl phosphate (E600, paraoxon), diisopropyl *p*-nitrophenyl phosphate (DINP) and tri-*o*-cresyl phosphate (TOCP). The inhibitors were incubated with the esterase preparation at 37.5° for 30 min. before adding the substrate to measure the residual esterase activity (cf. Myers & Mendel, 1953).

Pseudocholinesterase activity was determined with 0.03M butyrylcholine as substrate (Cohen, Kalsbeek & Warringa, 1949) and true cholinesterase with 0.03M acetyl- β -methylcholine (Mendel, Mundell & Rudney, 1943); both enzymes are inhibited almost completely when incubated with 5×10^{-6} M eserine for 30 min. The ali-esterase activity can usually be determined with tributyrin as substrate; the ali-esterases (B-type esterases) of serum are not strongly inhibited by low concentrations of eserine but can be completely inhibited by low concentrations of E600 and related compounds (cf. Aldridge, 1953*a*). The activity of the A-esterase (E600-esterase) can be determined with phenyl acetate as substrate in the presence of 5×10^{-6} M E600 or DINP, the diisopropyl analogue of E600 (cf. Aldridge, 1953*b*; Mounter & Whittaker, 1953). Tributyrin and phenyl acetate were prepared as aqueous emulsions with 5% (w/v) gum acacia so that the final reaction mixture in the Warburg vessel contained 0.2% (v/v) ester and 0.1%

gum acacia. The lipase activity of the plasma was determined titrimetrically with an aqueous emulsion of olive oil and gum acacia as described by Cherry & Crandall (1932). In all experiments the esterase activity was calculated from the initial rate of hydrolysis after appropriate corrections had been made for the slow non-enzymic hydrolysis of the substrate in question.

To determine the activity of the clearing factor, 0.3 ml. of lipaemic dog plasma was mixed with 0.1 ml. of the plasma to be tested, and the extinction measured at 680 m μ . in a Beckman spectrophotometer (cf. Anderson & Fawcett, 1950; Spitzer, 1952; Brown *et al.* 1953; Levy & Swank, 1954). The lipaemic blood samples were drawn from a normal dog 3–4 hr. after feeding 5 g. of margarine or butter/kg. body weight; all blood samples were mixed with 0.1 vol. of 5% (w/v) sodium citrate as described by previous investigators (cf. Levy & Swank, 1954). The initial extinction of the plasma mixture was usually 0.35–0.45 and decreased rapidly in the presence of clearing factor; the initial rate of clearing, which is almost linear, was used as a measure of the clearing-factor activity. In experiments with inhibitors, the post-heparin plasma was mixed with an equal volume of water or inhibitor solution and allowed to stand for 30 min. before samples were removed to test for tributyrinase and clearing-factor activities. Under these conditions, E600 is destroyed very rapidly (Aldridge, 1953*b, c*) while DINP is relatively stable (Aldridge, 1953*c*). Control experiments in which E600 was incubated with the lipaemic dog plasma or in which an equivalent amount of *p*-nitrophenol was added to the post-heparin plasma showed no significant inhibition of the clearing factor.

Small amounts of heparin were injected intravenously into man, dog and rabbit and the post-heparin blood samples were drawn 5–7 min. later. Larger doses were injected intraperitoneally into rats, mice and guinea-pigs, and the animals were killed by exsanguination 15 min. later. In a few experiments, guinea pigs under Nembutal anaesthesia were injected with heparin in the jugular vein. In these experiments it was found that small doses of heparin injected intravenously produce approximately the same amount of clearing factor as is obtained when massive doses of heparin are administered intraperitoneally (see Table 3). Apparently the heparin is slowly absorbed from the peritoneal cavity. This fact is responsible for the large differences in the amounts of heparin which were administered intravenously and intraperitoneally to various species of animals in the experiments described below.

Table 1. *Effect of intravenous heparin injections on the esterases of dog blood*

A male dog, weighing approx. 16 kg., was injected intravenously with various amounts of heparin (135 units/mg.). Samples of blood were removed before the injection and 5–7 min. later, and mixed with 0.1 vol. of 5% (w/v) sodium citrate. The average activities of the various esterases in the normal and post-heparin citrated blood samples were determined.

Source of esterases	Substrate	Esterase activity (μ l. CO ₂ /ml. plasma or erythrocytes/30 min.) after injection of heparin (units/kg.)			
		0	20	50	100
Plasma	Tributyrin	93	166	150	212
	Butyrylcholine	1 560	1 480	1 410	—
	Acetyl- β -methylcholine	126	114	—	—
	Phenyl acetate	87 600	92 500	73 300	—
Erythrocytes	Tributyrin	313	297	320	—
	Acetyl- β -methylcholine	240	260	—	—

RESULTS

In confirmation of the results obtained by Richter & Croft (1942), it was found that the hydrolysis of tributyrin by normal dog plasma can be largely inhibited by 5×10^{-6} M eserine, a concentration which inhibits the activity of the pseudocholinesterase almost completely. Similar results were obtained with 5×10^{-6} M DINP; this compound causes complete and irreversible inhibition of the pseudocholinesterase. The small portion of the esterase activity of normal plasma towards tributyrin which cannot be inhibited by high concentrations of DINP or E 600 (see Table 2) is probably due to the large amount of A-esterase present (see Table 1).

Intravenous injection of heparin had no significant influence on the activity of the pseudocholinesterase, true cholinesterase and A-esterase of plasma nor on the true cholinesterase and al-esterase of the erythrocytes of the dog (Table 1). However, in confirmation of the results obtained by Levy & Swank (1954), it was found that the total activity towards tributyrin increases by 60–130% shortly after the injection of heparin. The increase in activity is due to the appearance of an eserine-resistant tributyrinase in the plasma (see Table 3). This enzyme is also resistant to inhibition by 5×10^{-7} and 5×10^{-6} M DINP (Tables 2 and 3), but is strongly inhibited by 5×10^{-4} M DINP or 5×10^{-6} M E 600 (Table 2).

Similar results were obtained with human plasma. The hydrolysis of tributyrin by normal human plasma is due almost entirely to the pseudocholinesterase present (Easson & Stedman, 1937; Richter & Croft, 1942; Adams & Whittaker, 1949) and can be inhibited by 5×10^{-6} M eserine or DINP (Tables 2 and 3). After injection of heparin, the plasma is found to contain another esterase which is capable of hydrolysing tributyrin and which is not inhibited by 5×10^{-6} M eserine or DINP (Table 3); the properties of this enzyme resemble those of the tributyrinase found in post-heparin dog plasma (Table 2).

The hydrolysis of tributyrin by the normal plasma of rats, rabbits, mice and guinea pigs, on the other hand, is due mainly to the presence of an al-esterase which cannot be inhibited by low concentrations of eserine (Easson & Stedman, 1937; Richter & Croft, 1942) but which can be inhibited by 5×10^{-7} or 5×10^{-6} M DINP (Table 2). The hydrolysis of tributyrin by rat serum in the presence of 5×10^{-6} M DINP followed the pattern observed with dog and human plasma; normal plasma exhibits a slight activity whereas the post-heparin plasma exhibits a high DINP-resistant tributyrinase activity (Tables 2 and 3). Analogous results were obtained with rats which had been injected

Table 2. Inhibition by E 600 and DINP of the hydrolysis of tributyrin by plasma from different species

Samples of normal and post-heparin plasma were incubated with various concentrations of E 600 or DINP at 37° for 30 min. before measuring the residual activity towards tributyrin. The amounts of heparin injected in these particular experiments were as follows: 20 units/kg. intravenously in the dog and man, 100 units/kg. intravenously in the rabbit and 2000 units/kg. intraperitoneally in the rat, guinea pig and mouse. The average esterase values obtained in other experiments are given in Table 3.

Inhibitor	Concn. of inhibitor (M)	Rate of hydrolysis of tributyrin (μ l. CO ₂ /ml. plasma/30 min.)											
		Dog		Man		Rat		Rabbit		Guinea pig		Mouse	
		Normal	Post-heparin	Normal	Post-heparin	Normal	Post-heparin	Normal	Post-heparin	Normal	Post-heparin	Normal	Post-heparin
None	—	87	165	354	472	1790	2150	1380	1375	3770	3900	4960	5240
DINP	5×10^{-4}	10	28	13	70	25	36	55	65	10	42	140	155
	5×10^{-5}	10	74	13	99	27	244	57	71	51	100	234	268
	5×10^{-6}	10	90	15	115	30	328	61	86	94	130	238	274
	5×10^{-7}	11	91	17	118	40	336	72	90	100	136	285	294
	5×10^{-8}	36	120	134	243	340	755	75	119	298	336	1652	1624
E 600	5×10^{-5}	—	23	11	11	22	24	56	63	10	—	90	—
	5×10^{-6}	—	48	—	34	24	71	—	—	46	—	230	—
	5×10^{-7}	—	61	—	54	43	320	—	—	104	—	470	—
	5×10^{-8}	—	—	—	—	410	800	—	—	635	—	2500	—

previously with TOCP in order to inhibit the ali-esterases (Mendel & Myers, 1953); in this case the plasma contains only the DINP-resistant esterases (Table 3). It might be noted that the injection of TOCP did not affect the liberation of clearing factor into the plasma after heparin injection (Table 3).

A different type of result was obtained with rabbits. The injection of heparin did not cause the liberation of an appreciable DINP-resistant tributyrinase activity into the plasma, although the post-heparin plasma exhibited a high clearing activity (Table 3).

ase thus resembles the DINP-resistant tributyrinases found in post-heparin plasma from dog, man and rat (Table 2). Nevertheless, normal plasma from the mouse and guinea pig do not exhibit clearing activity (Table 3). After injection of heparin, the plasma acquired a high clearing activity, but the activity of the DINP-resistant tributyrinases did not increase markedly (Table 3).

The results fail to support the suggestion of Levy & Swank (1954) that the tributyrinase in post-heparin dog plasma might be concerned with its clearing activity. Further experiments were

Table 3. *Effect of heparin injections on the clearing activity and on the hydrolysis of tributyrin by plasma from different species*

Post-heparin blood samples were usually drawn 6 min. after intravenous injection or 15 min. after intraperitoneal injection of a heparin preparation containing 135 units/mg. The clearing activity is expressed as the decrease in the extinction of lipaemic dog plasma at 680 m μ ., calculated from the initial rate of clearing at room temperature after mixing 0.3 ml. of citrated lipaemic dog plasma with 0.1 ml. of the citrated plasma containing the clearing factor. The rate of hydrolysis of tributyrin was measured in the usual way at 37.5° after previous incubation of the citrated plasma in bicarbonate buffer with or without inhibitor for 30 min. The 'TOCP-rats' were male rats weighing 200–250 g., which had been injected intraperitoneally with 1 ml. TOCP/kg. one day previously, this dose being the maximum tolerated by the rats.

Species	No. of measurements	Heparin injection		Rate of hydrolysis of tributyrin by the plasma in the presence of the following inhibitors (μ l. CO ₂ /ml. plasma/30 min.)			Clearing activity of the plasma ($E_{680 \text{ m}\mu}$ /100 min.)
		Dose (units/kg.)	Route of injection	No inhibitor	5×10^{-6} M DINP	5×10^{-6} M eserine	
Dog	13	0	Intravenous	93	8	15	—
	11	20		166	80	85	12.8
	3	50		150	—	63	—
	1	100		212	—	107	—
Man	5	0	Intravenous	404	16	16	—
	5	20		529	132	130	4.0
Rat	8	0	Intraperitoneal	1223	21	—	0.07
	7	500		1676	343	—	9.5
	2	1000		1615	403	—	—
	8	2000		1335	407	—	21.5
TOCP-rat	2	0	Intraperitoneal	20	20	—	—
	10	500		261	260	—	9.2
	2	2000		298	295	—	—
Rabbit	5	0	Intravenous	1129	57	—	0.02
	2	20		1210	64	—	6.6
	3	100		1020	80	—	9.5
	1	1000		1090	70	—	—
Guinea pig	3	0	Intravenous	1659	71	—	0.09
	3	50		1692	146	—	11.5
Guinea pig	5	0	Intraperitoneal	3488	133	—	—
	5	500		3230	165	—	—
	6	2000		3754	192	—	14.2
Mouse	3	0	Intraperitoneal	4630	228	—	0.0
	3	2000		4330	249	—	13.9

The results obtained with guinea pigs and mice were complicated by the fact that the normal plasma contains an ali-esterase which is inhibited by 5×10^{-6} M DINP, and a small amount of another esterase which is not inhibited by 5×10^{-6} M DINP but which is inhibited by E 600 or by high concentrations of DINP. This DINP-resistant tributyrin-

carried out in order to substantiate this conclusion. Post-heparin plasma from rat and dog was heated at 48–50° for various periods. Both the DINP-resistant tributyrinase and the clearing factor were partially inactivated; however, the clearing factor was more sensitive to destruction than the tributyrinase (Table 4). Post-heparin plasma was also

incubated with various concentrations of E 600 before testing the enzyme activities. Again both enzymes were inhibited but in this case the tributyrinase is more sensitive than the clearing factor (Table 5). From these experiments it can be concluded that the clearing factor and the DINP-resistant tributyrinase are different enzymes.

Table 4. *Inactivation of the enzymes in post-heparin plasma by exposure to high temperatures*

Citrated plasma, obtained from rats 15 min. after intra-peritoneal injection of 2000 units heparin/kg. or from a dog 6 min. after intravenous injection of 20 units heparin/kg., was heated in a water bath at 48° or 50° for various periods of time. The activity of the clearing factor in the control and heated samples of plasma was determined in the usual way with lipaemic dog plasma as substrate. The activity towards tributyrin was determined manometrically in the presence of 5×10^{-6} M DINP.

Species	Temp. (°)	Time of heating (min.)	Percentage activity remaining	
			Clearing factor	DINP- resistant tributy- rinase
Rat	48	10	45	71
	48	30	17	44
	48	45	9	32
	50	20	8	32
Dog	50	30	11	58

However, it is interesting to observe that the clearing factor is fairly sensitive to inhibition by E 600 and almost completely resistant to DINP (Table 5). In this respect, this enzyme resembles the lipase of pancreas (cf. Aldridge, 1954). However, the relative activities of these enzymes on lipaemic dog plasma and on olive oil differ considerably. When pancreatic lipase was liberated into the blood stream of rats by the subcutaneous injection of acetyl- β -methylcholine plus eserine (Popper & Necheles, 1943), a marked increase in the rate of

hydrolysis of olive oil by the plasma was observed, together with a slight increase in the DINP-resistant tributyrinase activity and the production of a small clearing activity (Table 6). Injection of heparin into rats, on the other hand, produces a large clearing activity and a smaller increase in the rate of hydrolysis of olive oil, together with the liberation of large amounts of the DINP-resistant tributyrinase into the plasma (Table 6).

Table 5. *Inhibition of the enzymes in post-heparin plasma by incubation with E 600 and DINP*

Citrated post-heparin plasma was incubated with an equal volume of water or inhibitor solution for at least 30 min. at room temperature. Subsequently samples were removed to determine the activities of the clearing factor and DINP-resistant tributyrinase in the usual manner.

Species	Inhibitor	Concentration of inhibitor in incubation mixture (M)	Percentage activity remaining	
			Clearing factor	DINP- resistant tributy- rinase
Dog	DINP	3×10^{-3}	83	25
		3×10^{-3}	9	9
		3×10^{-4}	48	20
		1×10^{-4}	73	49
Rat	E 600	3×10^{-3}	9	5
		3×10^{-4}	18	8
		3×10^{-5}	89	27
		3×10^{-3}	4	6
Man	E 600	3×10^{-4}	18	9
		3×10^{-5}	39	9

DISCUSSION

The fact that heparin, incubated with normal blood *in vitro*, does not produce clearing activity suggests that the clearing factor must be liberated from the tissues after injection of heparin into the animal. Extracts of normal heart and lung do indeed exhibit clearing-factor activity (Anfinson *et al.* 1952; Korn, 1954). The activity of these extracts is

Table 6. *Comparison of the effects of injections of heparin and of acetyl- β -methylcholine plus eserine on the enzymes of rat plasma*

Male rats weighing approx. 300 g. were injected twice at a 15 min. interval with 0.3 mg. acetyl- β -methylcholine/kg. plus 0.3 mg. eserine/kg. subcutaneously; the rats were killed and citrated blood collected 60 min. after the first injection. Other rats were injected with 2000 units heparin/kg. intraperitoneally and the citrated blood collected 15 min. later. The activities of the clearing factor and the DINP-resistant tributyrinase in the plasma were determined in the usual manner. The hydrolysis of olive oil was measured titrimetrically by the method of Cherry & Crandall (1932).

Substance injected	Activity of the plasma enzymes		
	Clearing factor ($-\Delta E_{480 \text{ m}\mu}/$ 100 min.)	DINP-resistant tributy- rinase (μ moles acid/ml. plasma/60 min.)	Olive oil hydrolysis (μ moles acid/ml. plasma/60 min.)
None	0.07	1.8	0.29
Heparin	10.1	38.7	1.05
Acetyl- β -methylcholine + eserine	1.1	4.1	5.29

increased in the presence of small amounts of heparin but the heparin is not essential (Korn, 1954); the clearing factor is, in fact, inhibited by larger amounts of heparin (Brown *et al.* 1953). Moreover, there does not seem to be any correlation between the heparin content and the clearing activity of the blood during shock (Levy & Swank, 1953). In other words, the primary factor involved in the production of clearing activity *in vivo* is not the activation of the clearing factor by heparin but rather the liberation of this enzyme from one or more tissues (cf. Weld, 1944; Swank & Levy, 1952; Anfinson *et al.* 1952; Levy & Swank, 1954).

The results obtained in the present investigation indicate that heparin is also capable of liberating a DINP-resistant tributyrinase from tissues. The origin of this esterase is still uncertain, although preliminary experiments have shown that the heart and lung of the normal rat contain a DINP-resistant tributyrinase together with a DINP-sensitive ali-esterase. Further experiments are required in order to establish the mechanism by which heparin and other heparin-like substances liberate clearing factor and the DINP-resistant tributyrinase from the tissues. The results obtained in a recent investigation by Seifter & Baeder (1954) suggest that the liberation of these enzymes might be connected with the effect of hyaluronidase on the ground substance of the tissues.

SUMMARY

1. The effect of heparin injections on alimentary lipaemia and on the plasma esterases has been investigated in six species of animals. In all cases the post-heparin plasma contained a factor capable of clearing lipaemic plasma. The injection of heparin in man, dog and rat liberates a tributyrinase into the plasma which is not inhibited *in vitro* by low concentrations of eserine or diisopropyl *p*-nitrophenyl phosphate (DINP). The DINP-resistant tributyrinase activity of rabbit, mouse and guinea pig plasma does not increase markedly after the injection of heparin.

2. Both the clearing factor and the DINP-resistant tributyrinase in post-heparin plasma are inactivated by heating at 48–50° or by incubation with diethyl *p*-nitrophenyl phosphate (E 600). However, the tributyrinase is less sensitive than the clearing factor to destruction by heating and more sensitive to inhibition by E 600.

3. It is concluded that the clearing factor and the DINP-resistant tributyrinase are different en-

zymes, both of which may be liberated from the tissues into the blood stream by the injection of heparin.

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