enzyme activity when two or more enzymes of similar substrate specificities are present. More accurate estimates of the respective amounts of A and B await the development of methods for the quantitative separation of the two enzymes, or alternatively the establishment of conditions for reducing the activity of one of the enzymes to zero without affecting the activity of the other.

Some evidence has been presented (Dodgson *et al.* 1954) which suggested that the 'insoluble' enzyme of rat liver was not concerned with the synthesis of arylsulphates, but these observations were not extended to enzymes A and B. It seems likely that the three enzymes, which are so widely distributed in various animal tissues, may well have some important general function rather than a more specific function (e.g. detoxication) which is confined to relatively few organs.

SUMMARY

1. Human liver and a number of other tissues have been shown to possess three different arylsulphatases.

2. Two of these enzymes were readily solubilized and were comparable to enzymes A and B of rat liver. The other enzyme could not be obtained in

soluble form and had properties which were very similar to those of the microsomal arylsulphatase of rat liver.

3. The three enzymes have been separated from each other and their characteristics investigated.

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Studies on the Acetylation of Human Fibrinogen

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The effects of substitution and other modifications on the biological and physical properties of fibrinogen have been widely examined in recent years. Jaques (1943) investigated the chemical changes on iodination and showed that the aromatic residues are involved in the reaction. Laki & Mihalyi (1949) also studied the effect of iodination, and found that under the conditions of their experiments fibrinogen became incoagulable by thrombin. Physicochemical studies on the polymerization of iodinated fibrinogen by Laki & Steiner (1952) demonstrated the formation of intermediate polymers differing from those formed by untreated fibrinogen, and also a change in the pH optima of the various stages of association. The action of tyrosinase on the components of the clotting system was studied by Sizer & Wagley (1951) and Krugelis & Sizer (1954); a progressive increase in the clotting time of fibrinogen occurred with the period of enzyme action. Oxidation by gaseous oxygen increases the

clotting time of plasma (Palos, 1949). Formaldehyde and potassium permanganate have occasionally been used to stop the thrombin-fibrinogen reaction, but the properties of the modified protein have not been investigated.

In this investigation, human fibrinogen has been acetylated under exceptionally mild conditions with *NS*-diacetylthioethanolamine, which reacts only with free amino groups (Baddiley, Kekwick & Thain, 1952), and the properties of the modified protein, its reaction with thrombin and the behaviour of mixtures of the acetylated and untreated fibrinogen in the thrombin-fibrinogen reaction have been examined.

MATERIALS AND METHODS

Fibrinogen. This protein, prepared by the method of Kekwick, Mackay, Nance & Record (1955), had clottable N values between 97 and 98%, and was essentially free of plasminogen and plasmin.

Thrombin. Human thrombin was prepared by the method of Lyttleton (1954), with the ether-fractionation technique. In the later stages of this investigation a more purified thrombin prepared by a modified method was used (Kekwick & Mackay, unpublished work); the activity of this material was 200 u./mg. of protein N.

Acetylation. The protein to be acetylated was dissolved in borate buffer, the pH of which was varied between 8.5 and 9.6 as required. A slight excess (calculated from the number of free amino groups) of *NS*-diacetylthioethanolamine was added, and a shallow layer of the mixture was shaken gently under oxygen at 18° for 24 hr. (Baddiley *et al.* 1952). After the reaction was complete all solutions were dialysed to remove reaction products and excess of reagents. The extent of acetylation was determined by measuring the number of residual free amino groups by the van Slyke manometric method. Acetyl content was expressed as the percentage of total free amino groups acetylated.

Nitrogen. This was determined by the micro-Kjeldahl method, with a Markham (1942) still; a copper sulphate-potassium sulphate catalyst digestion mixture was employed. The quantities estimated were 0.2–0.5 mg. of N.

Electrophoresis. Electrophoretic analyses were carried out in the Tiselius apparatus at 0°, the diagonal schlieren optical system with monochromatic light, λ 546 m μ ., being used. Solutions were dialysed against phosphate buffer, pH 8 (*I*, 0.2), protein concentrations being between 1.0 and 1.5 g./100 ml. Mobilities were determined relative to the mobility of the unmodified protein under the same experimental conditions. A modified Durrum (1950) method was employed for electrophoresis on paper.

Sedimentation. Ultracentrifugal examinations were made in the Svedberg oil-turbine machine at 54 000 rev./min. (240 000 *g*) with the diagonal schlieren optical system (Philpot, 1938). Solutions were made up either in phosphate buffer, pH 8 (*I*, 0.2), +NaCl (*I*, 0.15) or in citrate saline (NaCl, 0.88 g./100 ml.; trisodium citrate, 0.37 g./100 ml.).

Measurement of clotting time. These tests were carried out in thin-walled 2 × $\frac{3}{8}$ in. tubes immersed in a glass-sided thermostat tank at 37°; solutions were given 10–15 min. to reach temperature equilibrium before any additions were made. Thrombin was added instantaneously by forcing a known volume into the test sample of fibrinogen solution by means of a capillary pipette attached with pressure tubing to a tuberculin syringe; the stop-watch was started at the same moment. Thrombin solutions were made from reconstituted freeze-dried material and diluted with veronal buffer, and the activity of these solutions was checked at 20 min. intervals during the course of an experiment. The veronal buffer, pH 7.3 (*I*, 0.15), contained 215 ml. of 0.2*N*-HCl, 14.43 g. of NaCl and 11.74 g. of sodium diethyl barbiturate in 2 l.

Chromatography. Chromatographic analysis was carried out by the ascending technique on Whatman no. 4 paper with *n*-butanol–acetic acid–water (4:1:5, v/v) as the solvent system. The chromatograms were developed with ninhydrin.

RESULTS

Acetylation experiments were carried out at various pH values to establish the degree of substitution occurring under different conditions. There is considerable variation in substitution, even over the short pH range studied (Table 1).

The reaction was allowed to proceed for 24 hr., assuming equilibrium to be reached in that period. In a longer period there is a slightly higher substitution (Table 1, no. 3). The unmodified fibrinogen contained 0.063 mg. of amino N/mg. of total N (240 free NH₂ groups/340 000 g.); acetylation of all the available free amino groups was not attempted.

Table 1. *Effect of pH on the degree of acetylation of fibrinogen, and the coagulation time of the products obtained*

No.	pH	Acetyl (%)	Clotting time (sec.)
1	—	—	30.9
2	8.5	5.1	32.0
3*	8.5	9.6	38.0
4	9.2	32.2	106.2
5	9.6	42.9	No clot in 12 hr.

All solutions were dialysed against veronal buffer, pH 7.3 (*I*, 0.15), for the clotting tests.

* This sample had a reaction time of 48 hr. with the acetylating reagent; the other samples had a standard period of 24 hr.

The acetylated fibrinogens were considerably more stable than native fibrinogen in solution, especially at 4°; solutions stored at this temperature for 2 months showed no signs of precipitation.

On electrophoresis at pH 8 in phosphate buffer, *I* = 0.2 (Fig. 1), these samples had only one

Fig. 1. Sedimentation diagrams and electrophoresis patterns of acetylated human fibrinogen. (a) Electrophoresis pattern after 50 mA hr. of a 0.5% (w/v) solution in phosphate buffer, pH 8 (*I*, 0.2); (b) sedimentation diagram after 20 min. at 240 000 *g* of a 0.4% (w/v) solution in citrate-saline; (c) sedimentation diagram after 20 min. at 240 000 *g* of a 0.4% (w/v) solution in citrate-saline after preparative ultracentrifuging. Arrows show direction of movement.

component; the mobility relative to untreated fibrinogen increased with the acetyl content (Table 2). Sample no. 5 (42.9% acetyl) when subjected to prolonged electrophoresis at pH 8 still had only one component, the electrophoresis diagram being similar to that of native fibrinogen under these conditions (Kekwick *et al.* 1955).

Table 2. *Effect of acetylation on the electrophoretic mobility of fibrinogen*

Sample	Mobility (mm./mA/hr.)
Control	0.188
Ac/8.5	0.208
Ac/9.6	0.257

Protein concentration 0.66 g./100 ml., by refraction in phosphate buffer, pH 8 (I, 0.2).

In the ultracentrifuge the samples of acetylated fibrinogen displayed a major component having the same sedimentation coefficient ($S_{20, w} \approx 7$) as native fibrinogen. A heavy component ($S_{20, w} \approx 18$), similar to that frequently found in small amounts in native fibrinogen, was present to the extent of 5–10% of the total protein; it was possible to remove most of this in a preparative ultracentrifuge (Spinco Model L). There were no noticeable differences in the ultracentrifuge diagrams obtained with solutions in phosphate–NaCl buffer or citrate–saline.

The effect of acetylation on the clotting properties of fibrinogen is shown in Fig. 2. There is a pronounced increase in clotting time with even quite low degrees of substitution; on interpolation for 25% of acetyl the clotting time is doubled, and when 40% or more of the free amino groups are acetylated no coagulation takes place. One sample of 42.9% acetyl content was incubated with thrombin for over 36 hr. without any clot forming.

In an attempt to elucidate the mechanism of this loss of coagulability, experiments were undertaken to find the effect of adding acetylfibrinogen (Table 1, sample 5) on the normal thrombin–fibrinogen reaction. In the first series of tests fibrinogen and acetylfibrinogen were mixed and the thrombin was added to the mixture. The variation of clotting time at constant thrombin concentration with the quantity of acetylfibrinogen added is shown in Fig. 3. It is evident that acetylfibrinogen is an inhibitor of the thrombin–fibrinogen reaction, and that this inhibition varies in a linear manner with concentration over the range studied. Control tests with the acetylating reagent alone showed no effect on the clotting time, even after incubating the reagent with fibrinogen at 37°, pH 7.3, for 15 min.

When thrombin and acetylfibrinogen are incubated together and native fibrinogen is added to samples at intervals, a more marked inhibitory effect occurs, increasing with time of incubation until a constant level is reached after a period depending on the concentration of acetylfibrinogen

Fig. 2. Effect of acetyl content on clotting time. Fibrinogen concentration 0.1 g./100 ml., thrombin 0.5 u./ml., final volume 1 ml. (Clotting time of untreated fibrinogen, 30.9 sec.)

Fig. 3. Inhibition of clotting by acetylfibrinogen. Concentration of native fibrinogen 1 mg./ml., thrombin 1.5 u./ml., final volume 1 ml.

(Fig. 4). The experiment in Table 3 demonstrates the partial reversibility of this effect. A mixture of thrombin and acetylfibrinogen was incubated for 30 min. and a sample diluted. On addition of the diluted sample to fibrinogen a shorter clotting time was obtained than with an equivalent amount of the undiluted mixture. The effect of dilution appears to be instantaneous, since no further decrease in clotting time takes place on incubating the diluted sample and testing it at intervals.

Attempts to demonstrate the nature of the inhibition, by using the clotting time for determining the reaction rates, were inconclusive. It is difficult to analyse the results by the method of Lineweaver & Burk (1934), because clotting times

are difficult to determine accurately at low fibrinogen concentrations. The results obtained by plotting clotting time in place of the more usual $1/v$ are shown on Fig. 5a. Better results were obtained with the method of Augustinsson & Nachmansohn (1949), also shown on Fig. 5b, the ratio of clotting times being used instead of the velocity ratio.

A reaction still appears to take place when the acetylfibrinogen is incubated with thrombin (Fig. 6); the original picture of the acetylated fibrinogen, showing only a trace of heavy component after high-speed centrifuging (Fig. 1c) is replaced by that of a definite multicomponent system, the proportion of the components varying with the time of incubation. The thrombin used in these experiments was free from plasmin and plasminogen, and the change in properties must therefore be due to the formation of some of the soluble intermediate polymers in the clotting reaction. From the ultracentrifuge experiments it was impossible to obtain evidence for the formation of any smaller breakdown products.

Peptide material is liberated by thrombin from acetylfibrinogen as with untreated fibrinogen

Fig. 4. Incubation of acetylfibrinogen with thrombin and effect on the clotting time. Fibrinogen concentration, 0.1 g./100 ml., thrombin 0.4 u./ml., final volume 1 ml. ○, Thrombin incubated with acetylfibrinogen; ●, control thrombin incubated with citrate-saline.

Table 3. *Reversibility of inhibition by acetylated fibrinogen on dilution*

Solution (a): 1 ml. of thrombin (2.5 u./ml.) + 1 ml. of acetylated fibrinogen (sample 5).

(i) 0.6 ml. of fibrinogen + 0.3 ml. of citrate saline + 0.1 ml. of soln. (a). Clotting time 47 sec.

(ii) 1 ml. of soln. (a) + 3 ml. of citrate saline incubated and tested at intervals

Fibrinogen (0.6 ml.) + 0.4 ml. of incubation mixture

Incubation time (min.)	0	2.5	5	7.5	10	30
Clotting time (sec.)	40.8	41	40.6	41.2	41.2	41.8

Fig. 5. Nature of the inhibition by acetylfibrinogen. (a) ●, No inhibitor; △, inhibitor 1/12; ○, inhibitor 1/6. s = concn. of fibrinogen (g./100 ml.). (b) ○, 0.9 u. thrombin/ml.; △, 1.2 u./ml.; ●, 1.8 u./ml. Final volume of all tests, 1 ml.

(Lorand, 1951, 1952; Bettelheim & Bailey, 1952). Thrombin was allowed to react with comparable quantities of acetylated and native fibrinogen dissolved in 0.05M ammonium carbonate. Trichloroacetic acid (to 10% w/v) was added and the protein-free supernatant solution obtained was extracted with ether to remove the trichloroacetic acid. The extracted solutions were then evaporated to dryness under vacuum, a procedure which also

Fig. 6. Sedimentation diagrams after the action of thrombin on acetylfibrinogen, after 30 min. at 240 000 g of 0.4% (w/v) solutions in citrate-saline. (a) Incubated with thrombin (0.5 u./ml.) for 60 min.; (b) incubated with thrombin (0.5 u./ml.) for 240 min.

volatilized most of the salt. The residue obtained was examined by electrophoresis on paper at pH 4.2 (acetate buffer) and chromatographically on paper in *n*-butanol-acetic acid-water as solvent. Two peptides were detected from both native and acetylated fibrinogen on developing with ninhydrin.

DISCUSSION

The results indicate quite clearly that the substitution of acetyl groups into the free amino groups has a considerable effect on some of the properties of human fibrinogen. The solubility and homogeneity of the acetylated protein were not substantially different from those of untreated fibrinogen, but solutions in the cold were much more stable. Electrophoretic analysis in all cases revealed only a single component, with the expected change in mobility due to the alteration in the number of ionized groups.

The heavier component of acetylfibrinogen is almost certainly an artifact produced by the conditions of reaction on this very labile protein. Samples of highly purified fibrinogen have a marked tendency to polymerize spontaneously in solution on storage, especially in the cold. There is also the possibility of intermolecular cross-linking, intramolecular -S-S- bonds being reduced by the free thiol derived from the acetylating reagent and the -SH groups liberated afterwards reoxidizing to form intermolecular -S-S- bonds giving rise to a polymer (Baddiley *et al.* 1952). Although the acetylation experiments were carried out under oxygen to avoid these complications by oxidizing

the deacetylated reagent, polymer formation still occurred. The two component mixtures normally obtained and the material isolated by high-speed centrifuging did not differ noticeably in their clotting properties.

The decrease in the rate of clot formation by thrombin with increasing substitution, which reduces the number of positively charged groups, agrees with the view that in the polymerization stage of the clotting reaction (Ferry, Katz & Tinoco, 1954) molecules are initially oriented and possibly later held together by electrostatic forces of attraction. Moreover, since the alterations due to acetylation affect only the *N*-terminal α -amino groups and the ϵ -amino groups of lysine the results suggest that these groups may play a major role in the final polymerization process. A very similar variation in coagulation properties with the extent of chemical modification was observed by Sizer & Wagley (1951) after tyrosinase had been allowed to act on fibrinogen for varying periods. Laki & Mihalyi (1949) state that iodinated fibrinogen is no longer clottable by thrombin; later physico-chemical studies by Laki & Steiner (1952) show that intermediate polymers different from those formed from native fibrinogen are produced, and that the pH optima of the polymerization reaction are changed.

The effect of acetylfibrinogen on the reaction between native fibrinogen and thrombin can be explained as a competitive inhibition of the enzyme in the first stage of the clotting process. This would be in agreement with the work of Sherry & Troll (1954), who demonstrated that a synthetic ester (toluene-*p*-sulphonylarginine methyl ester) was a competitive inhibitor in the reaction of thrombin with fibrinogen. The results with acetylfibrinogen indicate that the reversible reactions between enzyme and substrate and between enzyme and inhibitor proceed at different rates, and that the equilibrium in the inhibitor reaction is attained more slowly as in the thrombin-acetylfibrinogen incubation experiments. The slow reversible reaction between thrombin and acetylfibrinogen would then effectively reduce the available thrombin and slow down the clotting reaction. The extent of the inhibition appears to depend on the absolute concentration of enzyme and inhibitor in the mixture, as was shown by the effect of dilution. In contrast to the reaction of thrombin with the synthetic ester of Sherry & Troll (1954), acetylfibrinogen does not lose its inhibitory activity during the course of the reaction; it is not removed from the system as the reaction proceeds and remains capable of combining with thrombin, after reacting with thrombin.

It is of great interest to note that the modified protein still reacts with thrombin. The release of

peptide material ('fibrinopeptide') from acetyl-fibrinogen can be demonstrated; the ultracentrifuge data show definitely that some of the heavier intermediate polymers are formed in the reaction and that only the 'gel' stage of clotting is completely inhibited. This suggests that the site of attachment of thrombin to fibrinogen is on the bulk part of the molecule and not on the peptide portion split off by thrombin. The strong adsorption of thrombin on fibrin leads to the same conclusion.

SUMMARY

1. Fibrinogen was acetylated under mild conditions at varying pH, giving materials of varying degrees of substitution very similar in physico-chemical properties to the native protein.

2. The clotting properties of the protein are modified by the degree of substitution. The material is no longer clottable by thrombin when the number of free amino groups substituted exceeds 35 %.

3. Acetylated fibrinogen inhibits the reaction between thrombin and fibrinogen. This inhibition appears to be competitive.

4. Although the acetylated fibrinogen does not form a clot when acted upon by thrombin, a peptide is split off and soluble polymers are formed.

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The Effects of Anticholinesterases on Oxidative Phosphorylation in Brain, and their Relation to Potassium Loss and Nerve Degeneration

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A number of reports (Rothenberg, 1950, for the squid axon; Greig, Faulkner & Mayberry, 1953, for the mammalian erythrocyte; Kirschner, 1953, for frog skin) indicating the possible importance of cholinesterase in relation to cell-membrane permeability led to an investigation into the effects of cholinesterase inhibitors on potassium loss from brain slices (Strickland & Thompson, 1955). From this work it was concluded that cholinesterase was not directly concerned with the prevention of the loss of measurable amounts of potassium from slices of chicken brain; thus in each case the cholinesterase inhibitors DFP (diisopropyl phos-

phorofluoridate), eserine and Nu 1250 (the synthetic prostigmine analogue, *N*-*p*-chlorophenyl-*N*-methyl carbamate of *m*-hydroxyphenyltrimethylammonium bromide) caused potassium loss only when a concentration several times greater than that necessary for complete inhibition of cholinesterase activity was reached. Some evidence suggested that these inhibitors caused potassium loss because of their action on the energy-producing reactions of the cell.

In addition to this work on potassium loss, Barnes & Denz (1953) and Cavanagh (1954) have shown that there is a marked similarity between the degenerative changes induced in peripheral nerves of the hen by anticholinesterases such as

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