978. *The Condensation of Long-chain Fatty Acids with Polysaccharides and Proteins.*

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The esterification of D-glucose, D-glucosamine, bacterial dextran, and the somatic polysaccharide from *Mycobacterium tuberculosis* with stearic acid, has been investigated and the methods adapted to the production of esters of biologically active, branched-chain fatty acids with polysaccharides. Fatty acids have been condensed with proteins by means of the fatty azide, and also by reaction of the fatty acid with *p*-phenylenediamine, followed by diazotisation of the resulting amine and its coupling with protein.

The work of Anderson (*Chem. Reviews*, 1941, **29**, 225) and Polgar *et al.* (*Biochem. J.*, 1948, **42**, 206; *Nature*, 1950, **166**, 693) has shown the great importance of lipids in the constitution of *Mycobacterium tuberculosis* and other organisms of the acid-fast group. In the lipids of *Myco. tuberculosis* there were found branched-chain fatty acids, some of which appeared to be involved in the tubercle-forming property of the organism (Sabin, *Physiol. Reviews*, 1932, **12**, 141). David, Polgar, and Robinson (*J.*, 1949, 1541) and Polgar, Robinson and Seijo (*J.*, 1949, 1545) have synthesised various branched-chain fatty acids and have shown that some of them, particularly 3 : 12 : 15-trimethyldocosanoic acid * and 2 : 12 : 15-trimethyldocosanoic acid, were able to cause granuloma formation on injection into animals.

The present work was undertaken in order to determine whether fatty acids are capable of acting as specific hapten groups in serological reactions, and to ascertain whether a granuloma-forming fatty acid will form antigens capable of conferring a protective action against the granuloma-forming action of the acid itself. To this end, methods of synthesising fatty acid derivatives of polysaccharides and proteins have been examined.

The preparation of pentastearoyl α-D-glucose by the action of stearoyl chloride on D-glucose in the presence of pyridine has been reported by Hess and Messmer (*Ber.*, 1921, **54**, 499). This has been successfully repeated, and the method applied to the stearoylation of D-glucosamine hydrochloride, pentastearoyl D-glucosamine being formed. The method

* Geneva nomenclature (CO_2H = 1). This is not the nomenclature used in *J.*, 1949, 1541, 1545.

was, however, unsuitable for stearoylation of polysaccharides: either there was no reaction or a mixture of degraded products was formed.

The method used by Stacey and Swift (unpublished results: cf. Clarke, Malm, and Stinchfield, B.P. 287,880/1927) for esterification of bacterial dextran with stearic acid, namely, the condensation of the free acid with the polysaccharide in the presence of chloroacetic anhydride and a trace of sodium perchlorate at 80°, proved more successful. Conditions are now reported for the maximum yield of stearoyl dextran with the maximum utilisation of fatty acid (Table 1, p. 5018). The latter condition was particularly important when working with rare, branched-chain fatty acids. The maximum utilisation in the stearoylation of dextran by this method was 59%. The esters formed had a fatty acid content varying from 50 to 80% according to the conditions employed and were soluble in non-polar solvents. It did not appear possible to obtain by this method appreciable quantities of a water-soluble stearoyl dextran. The somatic polysaccharide of *Myco. tuberculosis* (Haworth, Kent, and Stacey, *J.*, 1948, 1211) was similarly esterified to give a derivative containing 22% of stearic acid and resembling stearoyl dextran in solubility.

This method also proved effective for the esterification of dextran and tubercle polysaccharide with the synthetic branched-chain acids, 3 : 12 : 15-trimethyldocosanoic and 2 : 12 : 15-trimethyldocosanoic acid. In these reactions two products were usually isolated, a solid which was insoluble in all the solvents available and a syrup which was soluble in non-polar solvents.

All these fatty acid derivatives of the carbohydrates were highly resistant to hydrolysis with dilute aqueous or alcoholic alkali, probably owing to their extreme insolubility. Hydrolysis was accomplished, however, by prolonged boiling with aqueous 30% sodium hydroxide or by boiling with alcoholic potassium hydroxide with benzene as mutual solvent. The former method was the more convenient for the estimation of fatty acid content.

Condensation of fatty acids with proteins was accomplished by two methods. The first was an application of that due to Clutton, Harington, and Mead (*Biochem. J.*, 1937, **31**, 764). Palmitoazide, prepared by Curtius's method (*J. pr. Chem.*, 1901, **64**, 419), readily condensed with gelatin under mildly alkaline conditions. The product, which was isolated as a water-soluble granular white powder, gave on hydrolysis 7·2% of benzene-soluble material. Much of the fatty material was only weakly bound inasmuch as it could be removed from the protein with boiling benzene. The extracted material, which contained nitrogen, appeared to be heterogeneous and was insoluble in most solvents, but was sparingly soluble in benzene. The exact nature of the gelatin–palmitoyl complex was not known, therefore, and it may have been an adsorption complex of gelatin with a reaction product of palmitoazide. It was noted, however, that palmitic acid itself was unable to form a complex with gelatin under the same conditions. Palmitoazide also condensed with γ-globulin at pH 8 in aqueous dioxan to produce a water-soluble derivative which contained 0·8% of fatty acid. Electrophoretic analysis of the material (kindly carried out in this Department by Dr. G. A. Gilbert) showed that a new component had been formed. Control experiments showed that this was not due to the action of the solvents. This method of condensing fatty acids with proteins could not be successfully applied to the branched-chain fatty acids, however, mainly because their azides could not be characterised.

The second method used for coupling fatty acids with proteins was an application of the classical diazo-reaction (Goebel, *Nature*, 1939, **143**, 77). Stearic acid readily reacted with *p*-phenylenediamine to form *N*-stearoyl-*p*-phenylenediamine (Sulzberger, D.R.-P. 193,451/1908; *Chem. Zent.*, 1908, **79**, I, 1011). This material was diazotised and coupled in aqueous acetone or aqueous dioxan at an alkaline pH with casein, serum mucoid, γ-globulin, and ovalbumin. The products were pink, water-soluble compounds containing 0·5—1·3% of stearic acid.

3 : 12 : 15-Trimethyldocosanoic acid also reacted with *p*-phenylenediamine but the product could not be obtained pure. The crude derivative, upon diazotisation and coupling with γ-globulin, gave a pink protein which contained 6·6% of the fatty acid. No lipid material was extractable from these complexes by neutral organic solvents,

indicating that the fatty acids were firmly bound. The serological investigation of these substances is being carried out by Dr. Ungar of Glaxo Laboratories Ltd. and will be reported in a separate communication.

Experimental

Pentastearoyl D-*Glucosamine.*—To a suspension of D-glucosamine hydrochloride (1·2 g.) in dry chloroform (30 ml.) and dry pyridine (10 ml.) at −15°, stearoyl chloride (10 g.) in dry chloroform (10 ml.) was slowly added. The mixture was allowed to warm to room temperature, then boiled for 8 hours, cooled, and poured into water (500 ml.). The resulting emulsion was extracted with chloroform. The chloroform layer was washed with water (twice), dilute hydrochloric acid (twice), and again with water (twice), dried ($MgSO_4$), and evaporated to dryness. The resulting syrup was repeatedly extracted with boiling methanol, and the insoluble residue repeatedly crystallised from light petroleum (b. p. 60—80°)–ethanol (2 : 1) and finally from chloroform–methanol (2 : 1), giving *pentastearoyl* D-*glucosamine* (4·5 g.) as a waxy crystalline mass, m. p. 71—72%, $[\alpha]_D^{25}$ +16° (*c*, 2·0 in $CHCl_3$) (Found: C, 76·0; H, 12·0; N, 0·74. $C_{96}H_{183}O_{10}N$ requires C, 76·3; H, 12·2; N, 0·93%).

Stearoylation of Bacterial Dextran.—Mixtures of bacterial dextran (1 g.) [isolated from *Betacoccus arabinosaceous*, Birmingham strain (Stacey and Swift, *J.*, 1948, 1555)], sodium perchlorate (0·0025 g.), and stearic acid were heated with chloroacetic anhydride at 80° for various times (see Table 1). The mixture was extracted with warm methanol and the residue extracted further with light petroleum (b. p. 60—80°). Addition of ethanol to the light petroleum extract gave a white, flocculent precipitate, which was dried to a white powder (light petroleum-soluble fraction 1). The residue from the light petroleum extraction was extracted with water, which removed unchanged dextran, and in some instances a small residue (2), soluble in aqueous acetone, remained. The methanol extract, which contained mainly stearic acid and chloroacetic anhydride, was poured into water, and the solid filtered off, washed with water, and dried *in vacuo* (P_2O_5). The material was extracted with light petroleum to remove unchanged stearic acid, and the residue gave a fraction (3) which was soluble in ethanol. The remaining material (2) was soluble in aqueous acetone.

Table 1.

Chloroacetic anhydride (g.)	10	10	2·5	10	1	1	10
Stearic acid (g.)	8	2	2	1	1	0·1	0·1
Heating at 80—90° (hr.)	4	4	4	3	15	15	3
Light petroleum-soluble fraction (1) (g.)	3·2	2·0	0·95	0·80	—	—	0·04
Aqueous acetone-soluble fraction (2) (g.)	—	—	—	Trace	0·07	0·23	Trace
Ethanol-soluble fraction (3) (g.)	—	—	—	Trace	Trace	—	0·16
Stearic acid in (1) (%)	75	59	84	48	—	—	59
Stearic acid in (2) (%)	—	—	—	—	0·12	2·2	—
Stearic acid in (3) (%)	—	—	—	—	—	—	4·6
Utilisation of stearic acid (%)	30	59	40	39	—	—	24

Stearoylation of the Somatic Polysaccharide of Myco. tuberculosis (*Human Strain*).—Stearic acid (2 g.), somatic tubercle polysaccharide (1 g.) ($[\alpha]_D$ +85°; Haworth, Kent, and Stacey, *loc. cit.*), sodium perchlorate (0·0025 g.), and chloroacetic anhydride (10 g.) were heated at 80° for 4 hours. The mixture was extracted with warm methanol, and the resulting residue extracted further with light petroleum. Addition of ethanol to the light petroleum solution precipitated the stearoylated polysaccharide. The material was purified by reprecipitation from light petroleum and was isolated as a slightly coloured powder (1 g.), soluble in light petroleum, chloroform, or benzene and insoluble in ethanol, methanol, or water (Found: Stearic acid, 22%).

Esterification of Dextran and Tubercle Polysaccharide with Biologically Active, Branched-chain Fatty Acids.—The experimental conditions were similar to those described above. In most instances two products were obtained. One was a waxy solid (A), insoluble in all the solvents available. The other was a viscous syrup (B), soluble in light petroleum, chloroform, or benzene, but insoluble in ethanol, methanol, or water. The results are summarised in Table 2.

Reaction of Palmitoazide with Gelatin—A solution of palmitoazide (from palmitoylhydrazine hydrochloride, 0·6 g.; Curtius, *loc. cit.*) in dioxan (10 ml.) was added, with shaking, to an ice-cold solution of gelatin (1·8 g.) in 50% aqueous ethanol, adjusted to pH 9 with dilute sodium hydroxide. The mixture was kept at this pH by the dropwise, simultaneous addition of N-sodium hydroxide. After 20 hours at 0° the solution was adjusted to pH 5 with 10% acetic acid, and the resulting mixture centrifuged at 3000 r.p.m. for 15 minutes. The supernatant liquid and unchanged palmitoazide, which collected at the surface of the liquid, were removed

by decantation. The residual solid was washed with 50% aqueous ethanol (twice) and dried by washing it with ethanol followed by ether. The complex was obtained as a white powder (1·35 g.) (Found: N, 13·3%), readily soluble in warm water. After hydrolysis with boiling

TABLE 2. *Experiments carried out with 200 mg. of polysaccharide.*

	Polysaccharide			
	Bacterial dextran		*Myco. tuberculosis* polysaccharide (somatic)	
Fatty acid	Insol. product (A)	Sol. product (B)	Insol. product (A)	Sol. product (B)
3 : 12 : 15-Trimethyldocosanoic acid	200 Mg.; fatty acid, 43%	Nil	150 Mg.; fatty acid, 51%	100 Mg.; fatty acid, 64%
2 : 12 : 15-Trimethyldocosanoic acid	200 Mg.; fatty acid, 50%	80 Mg.; fatty acid, 56%	26 Mg.; fatty acid, 29%	128 Mg.; fatty acid, 35%

5N-hydrochloric acid, 7·2% of benzene-soluble material was extracted. When gelatin itself was hydrolysed in a similar manner no material soluble in benzene was obtained.

Palmitoyl-γ-globulin.—A solution of palmitoazide (from 0·7 g. of palmitoylhydrazine hydrochloride) in dioxan (10 ml.) was added, with steady mechanical stirring, to a solution of human γ-globulin (supplied by courtesy of Dr. J. T. Edsall) (1 g.) in 0·2M-borate buffer (pH 8·5; 60 ml.) kept at 0°. After 18 hours at 0°, the solution was dialysed against tap water for 24 hours and unchanged palmitoazide and denatured protein were filtered off. To the filtrate (75 ml.), solid ammonium sulphate was added (30 g.), the resulting precipitate removed at the centrifuge, washed with 0·6-saturated ammonium sulphate solution (twice), and dissolved in water, and the resulting solution dialysed against repeated changes of tap water at 0° for 3 days. The solid which separated was dissolved by the addition of 0·2M-acetate buffer (pH 4; 1 ml.), and the resulting clear solution dried from the frozen state. *Palmityl-γ-globulin* was obtained as a white, flocculent, water-soluble solid (0·75 g.) (Found: N, 13·1; Ash, 5; palmitic acid, 0·83%).

N-*Stearoyl*-p-*phenylenediamine* (cf. Sulzberger, *loc. cit.*).—*p*-Phenylenediamine (1 g.) (purified by sublimation in carbon dioxide) and stearic acid (2 g.) were ground together in a mortar and the resulting mixture heated at 200° for 15 mins. The resulting solid, crystallised three times from ethanol, formed white needles of *N*-stearoyl-*p*-phenylenediamine (2·5 g.), m. p 118—119° (Found: C, 76·4; H, 11·2; N, 7·3. Calc. for $C_{24}H_{42}ON_2$: C, 76·9; H, 11·3; N, 7·5%). It is essential that the reaction and all subsequent operations be carried out in an oxygen-free atmosphere.

Diazotisation of N-*Stearoyl*-p-*phenylenediamine.*—A solution of *N*-stearoyl-*p*-phenylenediamine (0·1 g.) in chloroform (10 ml.), acetic acid (10 ml.), and 10N-hydrochloric acid (0·1 ml.) was treated with sodium nitrite (50 mg.) at room temperature for 5 mins. The solution was filtered and ether (50 ml.) added to the filtrate. The precipitated diazonium salt was washed repeatedly with ether, dried quickly by suction, and immediately dissolved in aqueous dioxan or aqueous acetone for coupling with proteins.

p-*Stearamidophenylazocasein.*—A filtered solution of casein (2 g.) in 0·2N-sodium hydroxide (75 ml.) was mixed with acetone (50 ml.). A solution of *p*-stearamidobenzenediazonium chloride (50 mg.) in aqueous acetone (1 : 5) (15 ml.) was added and the resulting deep red solution set aside for 10 mins. at room temperature. The solution was adjusted to pH 5 by addition of 10% acetic acid, and the resulting precipitate collected at the centrifuge and exhaustively extracted with absolute ethanol at room temperature. Upon drying with ether p-*stearamidophenylazocasein* (0·5 g.) was obtained as a pink solid, soluble in phosphate buffer of pH 8 (Found: N, 15·2; Stearic acid, 0·83%).

p-*Stearamidophenylazoserum-mucoid.*—Serum mucoid (0·5 g.) was dissolved in ice-cold distilled water (30 ml.). Acetone (20 ml.), cooled to −15°, was added with stirring and the solution cooled to −15°. A solution of *p*-stearamidobenzenediazonium chloride (50 mg.) in aqueous acetone (1 : 5; 50 ml.) was added slowly. After 10 mins. ice-cold 0·2M-phosphate buffer (pH 8; 30 ml.) was added and the solution kept at 0° for 4 hours. After dialysis against repeated changes of tap water for 18 hours, the protein was precipitated with ice-cold ethanol (3 vols.), and the precipitate separated at the centrifuge and dried with ethanol and ether. The *product* was obtained as a red solid (0·35 g.), easily soluble in water (Found: N, 12·9; Stearic acid, 0·99%).

p-*Stearamidophenylazo-γ-globulin.*—A solution of human γ-globulin (1 g.) in distilled water (60 ml.) was cooled to 0° and dioxan (30 ml.) added slowly. A solution of *p*-stearamidobenzenediazonium chloride (50 mg.) in aqueous dioxan (1 : 5; 10 ml.) was added and the solution kept

for 10 mins. Then 0·2M-phosphate buffer (pH 8; 30 ml.) was added at 0°. After 15 hours at 0° the liquid was dialysed against repeated changes of tap water for 48 hours. The precipitate which formed was centrifuged off and suspended in water, and 0·2M-phosphate buffer of pH 8 added until most of the material was dissolved. Insoluble particles were centrifuged off, and the solution was dried from the frozen state to give a pink *product* (0·85 g.) [Found: N, 13·6 (corrected for ash); Ash, 32; stearic acid, 1·27%].

p-*Stearamidophenylazo-ovalbumin*.—Ovalbumin (2 g.) was coupled with *p*-stearamidobenzenediazonium chloride as described above. The *product* (1·2 g.) contained N, 13·2; stearic acid, 0·47%; and inorganic material, 7·8%.

Reaction of 3 : 12 : 15-*Trimethyldocosanoic Acid with* p-*Phenylenediamine*.—3 : 12 : 15-Trimethyldocosanoic acid (0·5 g.) was heated with *p*-phenylenediamine (0·18 g.) at 200° in carbon dioxide for 30 mins. The cooled solution was mixed with benzene (50 ml.), and the solution extracted five times with warm water. The benzene solution was dried ($MgSO_4$), the solvent removed *in vacuo*, and the resulting syrup dissolved in methanol (3 ml.). After 2 hours at 0° the product had separated as a waxy solid. This was impure but could not be purified further. Upon diazotisation and coupling with β-naphthol it gave a deep red azo-compound. The material was used for the next stage of the reaction.

3 : 12 : 15-*Trimethyldocosanamidophenylazo-γ-globulin*.—The product obtained by the diazotisation of *N*-3 : 12 : 15-trimethyldocosanoyl-*p*-phenylenediamine (50 mg.) was coupled with γ-globulin (0·5 g.) as described above. The product (0·27 g.) was isolated as a pink flocculent solid [Found: N, 13·8% (corrected for ash)] which contained inorganic material, 24%, and fatty acid, 6·6%.

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