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EXPLANATION OF PLATE

Representative sections from the central portions of the pituitaries of rats used in the experiment shown in Fig. 1. (Masson Trichrome $\times 950$ green filter.) The sections show the appearance of the pituitary of the thiouracil-treated animal after (a) no further treatment, (b) L-thyroxine (1 $\mu\text{g./day}$) and (d) L-triiodothyronine (0.64 $\mu\text{g./day}$) compared with the gland of the normal animal (c). The appearance of enlarged basophils (pale cells) and decreased number of darker appearing acidophils resulting from thiouracil treatment is partially prevented by 1 $\mu\text{g. L-thyroxine}$ (b) and completely prevented by 0.64 $\mu\text{g. L-}$

triiodothyronine (d). Representative sections of the kidneys (upper) and hearts (lower) of the animals whose growth curves are given in Fig. 5 (haematoxylin and eosin $\times 3$ green filter). (e) Sham-operated controls; (f) thyroidectomized controls; (g) thyroidectomized + 1 $\mu\text{g. L-triiodothyronine/day}$; (h) thyroidectomized + 20 $\mu\text{g. triiodothyronine/day}$. The sections have been taken from approximately the same level in all cases. The animals receiving the large dose (h) show a relative increase in the size of the kidney cortex and the ventricles of the heart.

Studies on the Determination of the Sequence of Amino-Acids in Peptides and Proteins

1. THE PREPARATION, PROPERTIES AND CHROMATOGRAPHIC ADSORPTION OF THE AZOBENZENE-*p*-SULPHONYL DERIVATIVES OF VARIOUS AMINO-ACIDS

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Almost simultaneously with the conception of the polypeptide structure of proteins, attempts were made to devise methods for the determination of the amino-acids situated at that end of the polypeptide chain which bears the free α -amino group. Fischer & Abderhalden (1907) were the first to introduce such a method of end-group determination, and clearly to state their aims. By reaction of the free amino group with naphthalene-2-sulphonyl chloride and subsequent hydrolysis of the peptide bonds, they obtained the amino-acid bearing the end group in form of the *N*-naphthalene-2-sulphonyl derivative. However, further work showed that the method was inapplicable in certain cases, and the yields were often very poor. Later, Gurin & Clarke (1934) used benzenesulphonyl chloride as a 'marker' in an investigation on the nature of the free amino groups in gelatin.

In these methods the *N*-substituted ('marked') amino-acids were isolated from the hydrolysate by methods depending ultimately on differences in solubility. The difficulties were considerable and losses occurred during the isolation.

Sanger (1945), in a brilliant investigation of the free amino groups of insulin, used 1-fluoro-2:4-dinitrobenzene as 'marker'. The 2:4-dinitrophenyl-amino acids were isolated from the hydrolysate by partition chromatography on silica columns.

We resolved to investigate the possibility of the application of adsorption-chromatographic methods to the problem of the separation of 'marked' amino-acids, and to use as 'markers' other derivatives more appropriate for the particular problems we proposed to investigate.

Karrer, Keller & Szőnyi (1943) applied to the resolution of amino-acid mixtures the method first introduced for the separation of sugars by one of us (Reich, 1939) using the coloured *N-p*-phenylazobenzoyl derivatives. As the *N*-acyl derivatives prepared by reaction of amino-acids with carboxylic acid chlorides are very unstable to acid hydrolysis, we prepared the present series of derivatives with sulphonyl chlorides to investigate their value for end-group determinations.

Part of this work has been communicated to the Biochemical Society (Reich, 1948).

EXPERIMENTAL

Esterification of amino-acids. The amino-acids were esterified by the classical method of Curtius & Goebel (1888). A suspension of the amino-acids in dry methanol was saturated with dry HCl, the solution containing the methyl ester hydrochloride was evaporated to dryness *in vacuo* and the residue was crystallized usually from acetone, or sometimes from methanol. With L-lysine and L-phenylalanine the esterification mixture was poured in to a large amount of dry ether. Table 1 gives data on the methyl ester hydrochlorides not previously reported.

Preparation of azobenzene-*p*-sulphonyl chloride. Azobenzene-*p*-sulphonyl chloride (sulphazoyl chloride) ($p\text{-C}_6\text{H}_4\text{.N=N.C}_6\text{H}_4\text{.SO}_2\text{Cl}$) was described by Chrzasczczewska & Dobrowolski (1937) and Pearl (1945), who gave the m.p. 124–125° (corr.). Janowsky (1881) described it as a compound with m.p. 82°, which he obtained by the chlorination of azobenzene-*p*-sulphonic acid prepared by Griess (1870). We followed the conditions set out by these workers, but obtained only the compound with the higher m.p. 122–123° (124–125° corr.). We prepared this compound by chlorosulphonation of azobenzene with chlorosulphonic acid (yield, 40%), or by chlorination of azobenzene-*p*-sulphonic acid with PCl_5 (overall yield 30%). The best method of preparation was as follows. Chlorosulphonic acid (225 g.) was stirred mechanically, and azobenzene (75 g. dry, finely powdered) was added during 5 min. The temperature was raised to 95°, and maintained between 95 and 100° for 1 hr. After cooling, the solution was poured slowly into 3 l. of cracked ice, with vigorous mechanical stirring, and the stirring was continued for 30 min. The solution was extracted with 3–4 l. of benzene, the benzene solution shaken with 2% K_2CO_3 solution (250 ml. to every 1.5 l. of benzene solution), washed several times with water, and evaporated *in vacuo* to dryness. The residue twice recrystallized from benzene-light petroleum, b.p. 60–80° (10:90), was a deep red crystalline product, m.p. 124–125° (corr.); yield, 40%.

N-Sulphazoylation in pyridine

The *N*-sulphazoylamino acid methyl esters can be obtained by reaction of the methyl ester hydrochlorides of amino-acids dissolved in pyridine with sulphazoyl chloride at 40°.

To a solution of methyl ester hydrochloride (2 g.) in dry pyridine (50 ml.) is added a 10–15% excess of sulphazoyl chloride. The solution is left for 48 hr. in a glass-stoppered flask at 40°; water (0.5 ml.) is then added, and the solution is shaken vigorously and left overnight at room temperature. The product is poured with stirring into aqueous Na_2CO_3 (5%, 500 ml.) and left for 1 hr. The orange solid which precipitates is collected, washed free from pyridine and inorganic salts, and recrystallized (generally from light petroleum, b.p. 80–100°). Table 2 gives the data on some *N*-sulphazoylamino acid methyl esters prepared by this method.

α -Di-*N*-sulphazoyl-L-lysine methyl ester. This derivative was prepared in a similar way. After pouring into Na_2CO_3 a very fine precipitate formed which was extracted with ether; the washed ether was evaporated *in vacuo*. The residual oil solidified after drying for 3 days *in vacuo* over H_2SO_4 . The solid was dissolved in dry CHCl_3 , and precipitated by the addition of light petroleum, b.p. 40–60°. A final recrystallization from ethanol-light petroleum (1:3)

Table 1. Data on methyl ester hydrochlorides of amino-acids not previously reported

(All rotations for *D* line at 16–20°; $l=1$ dm.; and solvent, for methyl ester hydrochlorides, ethanol, except where shown otherwise.)

Amino-acid	Yield (%)	M.p. (corrected) (°)	$[\alpha]_D^{20}$	Methyl ester hydrochloride									
				Found					Required				
				C	H	N	Cl	C	H	N	Cl		
DL-Leucine	74	138–139	—	46.2	8.8	8.0	—	—	—	—	—	—	—
D-Leucine	86	147–148	$[\alpha]_D^{16}, -21.6 \pm 4^\circ$ (c, 0.46)	46.6	8.5	8.2	—	—	—	—	—	—	—
DL-Isoleucine	60	136–137	—	46.2	8.6	7.8	—	—	—	—	—	—	—
D-Isoleucine	80	148–149	$[\alpha]_D^{17}, -25.2 \pm 2.1^\circ$ (c, 0.91)	—	—	—	—	—	—	—	—	—	—
D-Isoleucine	80	147–148	$[\alpha]_D^{17}, +24.6 \pm 3^\circ$ (c, 0.69)	46.8	8.8	8.3	—	46.3	8.8	7.7	—	—	—
DL-Norleucine	78	120–121	—	46.2	8.8	7.5	—	—	—	—	—	—	—
L-Norleucine	70	126–127	$[\alpha]_D^{17}, +21.1 \pm 2.6^\circ$ (c, 0.77)	46.2	8.7	7.8	—	—	—	—	—	—	—
L-Phenylalanine	73	160–161	$[\alpha]_D^{19}, +38.4 \pm 1.8^\circ$ (c, 1.1)	55.5	6.5	6.7	16.2	55.7	6.5	6.5	16.5	—	—
L-Lysine	90	203–204	$[\alpha]_D^{20}, +15.9 \pm 0.8^\circ$ in water (c, 1.29; $l, 2$ dm.)	36.1	7.6	11.9	30.3	36.0	7.7	12.0	30.5	—	—

Table 2. Data on the methyl esters of *N*-sulphazoyl amino acids

(All rotations are for 6438 Å. in chloroform.)

Methyl ester	Yield (%)	M.p. (°)	[α] ²⁰	<i>N</i> -Sulphazoyl derivative									
				Found					Analysis				
				C	H	N	S	C	H	N	S	Required	
Glycine	95	148-149	—	53.7	4.7	12.6	—	54.0	4.5	12.6	—	C ₁₂ H ₁₅ O ₄ N ₃ S	
D-L-Alanine	95	127-128	—	55.2	5.1	11.9	9.1						
	60	154-155	[α] _D ¹⁸ , +34.1 ± 1.0° (<i>c</i> , 0.98; <i>l</i> , 2 dm.)	54.9	4.9	12.5	9.1						
	73	152-153	[α] _D ¹⁹ , -34.5 ± 2.0° (<i>c</i> , 0.99; <i>l</i> , 1 dm.)	55.5	5.0	12.1	—	55.3	4.9	12.1	9.3	C ₁₂ H ₁₇ O ₄ N ₃ S	
D-L-Leucine	98	121-122	—	58.7	5.8	10.6	7.8						
	94	139-140	[α] _D ²⁰ , +50.2 ± 1.0° (<i>c</i> , 1.1; <i>l</i> , 2 dm.)	58.7	6.0	10.3	7.8						
	90	138-139	[α] _D ¹⁶ , -50.0 ± 1.8° (<i>c</i> , 1.17; <i>l</i> , 1 dm.)	58.3	6.0	10.7	8.3						
D-L-Isoleucine	95	119-120	—	58.6	5.7	—	7.9						
	98	138-139	[α] _D ²⁰ , -50.4 ± 1.1° (<i>c</i> , 0.88; <i>l</i> , 2 dm.)	58.2	5.9	11.1	8.3	58.4	5.9	10.7	8.2	C ₁₂ H ₁₅ O ₄ N ₃ S	
	96	138-139	[α] _D ¹⁸ , +50.4 ± 1.1° (<i>c</i> , 1.1; <i>l</i> , 2 dm.)	58.4	5.6	11.0	8.2						
D-L-Norleucine	98	107-108	—	58.3	5.7	9.8	7.9						
	92	129-130	[α] _D ²⁰ , +47.0 ± 1.3° (<i>c</i> , 1.2; <i>l</i> , 2 dm.)	58.7	5.9	10.7	8.0						
D-L-Phenylalanine	90	142-143	—	62.0	4.9	10.0	8.0	62.3	5.0	10.0	7.57	C ₂₂ H ₂₁ O ₄ N ₃ S	
	96	148-149	[α] _D ²⁰ , +26.2 ± 0.8° (<i>c</i> , 1.26; <i>l</i> , 2 dm.)	62.3	5.1	10.2	7.9						

gave crystals, m.p. 104–105° (corr.); yield, 52%; $[\alpha]_{D}^{19}$ +53.1 ± 0.9° in CHCl_3 (c, 1.1; l, 2 dm.). (Found: C, 57.3; H, 4.9; N, 12.7. $\text{C}_{31}\text{H}_{33}\text{O}_5\text{N}_4\text{S}_2$ requires C, 57.3; H, 5.0; N, 12.9%.)

N-Sulphazoylation in aqueous acetone containing NaHCO_3

A solution of an amino-acid in water containing an excess of NaHCO_3 reacts with sulphazoyl chloride dissolved in an equal volume of acetone; by shaking the mixture for 2 hr. at room temperature, or by maintaining it at 40° for 2 hr. with occasional shaking, quantitative acylation of the amino group occurs. As an example, the preparation of *N*-sulphazoylglycine and *N*-sulphazoylalanine proceeded in the following way.

Glycine (0.5 g.) was dissolved in water (60 ml.) containing NaHCO_3 (1.5 g.). A solution of sulphazoyl chloride (2.0 g.) in acetone (60 ml.) was added, and the mixture was shaken for 2 hr. at room temperature. The acetone was evaporated off *in vacuo* and unchanged sulphazoyl chloride was removed by extraction with CHCl_3 . On acidification with HCl , *N*-sulphazoylglycine was precipitated. Yield, 2.1 g. (100%); m.p. 195–196°. Recrystallized from ethanol-water (30:70) it gave 2.0 g.; m.p. 197–198°. (Found: C, 52.4; H, 4.1; N, 13.0; S, 9.8. $\text{C}_{14}\text{H}_{13}\text{O}_4\text{N}_3\text{S}$ requires C, 52.6; H, 4.1; N, 12.9; S, 10.0%.)

A similar preparation using DL-alanine gave *N*-sulphazoyl-DL-alanine in 97% yield; m.p. 186–187° (189–190°, corr.). (Found: C, 53.7; H, 4.6; N, 13.0. $\text{C}_{15}\text{H}_{15}\text{O}_4\text{N}_3\text{S}$ requires C, 54.0; H, 4.6; N, 12.6%.)

Esterification of N-sulphazoylamino acids with diazomethane

Solutions of *N*-sulphazoylamino acids in dry dioxan, on treatment with a solution of diazomethane in ether, give quantitative yields of the methyl esters. The following is an example.

N-Sulphazoyl-DL-alanine (1.6 g.) was dissolved in dioxan (50 ml.) and mixed with a solution of diazomethane (1 g.) in ether (40 ml.). The solution was left for 2 hr. at room temperature, and was then evaporated *in vacuo* to approximately 30 ml., and poured into water (400 ml.); the orange solid which precipitated was filtered off; the mother liquors, being very pale yellow, were discarded. Yield, 1.7 g.; m.p. 124–126°. Recrystallized from ethanol-light petroleum (5:95), it gave 1.6 g. of orange needles, m.p. 127–128°.

The substance was identical with that obtained by esterification of *N*-sulphazoyl-DL-alanine by the methanol-HCl method, and also with the substance obtained by *N*-sulphazoylation of the DL-alanine methyl ester hydrochloride in pyridine. There was no depression of mixed melting points.

ϵ -Sulphazoyl-L-lysine methyl ester

L-Lysine monohydrochloride (4 g.) in water (250 ml.) was treated with an excess of Ag_2O , AgCl was removed by filtration and the hot filtrate was treated with excess basic CuCO_3 ; on filtration a deep blue solution of the chelate complex was obtained (300 ml., including washings from the precipitate). By the formation of this copper complex the α -amino group was protected from acylation. To the complex was added NaHCO_3 (7.0 g.) and a solution of sulphazoyl chloride (8.4 g.) in acetone (400 ml.); the mixture

was shaken for 2 hr. at room temperature. The precipitated brownish yellow copper complex was thoroughly washed with water and acetone, and dried. It was suspended in 2*N*-HCl (400 ml.), cooled to 0° and saturated with H_2S . The precipitate was extracted with 200 ml. of boiling ethanol-6*N*-HCl (1:1). On cooling, 3.8 g. of an orange solid, m.p. 228–229° (decomp.), were obtained; by concentrating the mother liquors, a further 3.2 g., m.p. 227–228° (decomp.) were obtained. Total yield 7.0 g. (74.5%). This was recrystallized twice from boiling 3*N*-HCl, giving 5.0 g. of ϵ -sulphazoyl-L-lysine hydrochloride, m.p. 229–230° (decomp.). (Found: C, 49.0; H, 5.3; N, 13.0; S, 7.6; Cl, 8.3. $\text{C}_{18}\text{H}_{23}\text{O}_4\text{N}_4\text{SCl}$ requires C, 50.6; H, 5.4; N, 13.1; S, 7.5; Cl, 8.3%.)

For esterification the substance was suspended in dry methanol and the mixture was saturated with HCl. The solution was evaporated to dryness *in vacuo* (<35°), and the residue dissolved in methanol. Pyridine was added until the solution was alkaline to Congo red. It was then poured into excess 5% NaHCO_3 , and extracted with CHCl_3 . The CHCl_3 extract was washed with water, dried, and purified chromatographically on alumina columns (the alumina having been treated with 10% (v/v) acetic acid in methanol for 9 hr. at 40° and dried in air). The columns were developed with 0.5% (v/v) methanol in benzene; the impurities remained strongly adsorbed at the top of the column, and the required compound formed a sharply defined band containing over 90% of the coloured compound. The band was eluted with acetone-methanol (2:1), and the solution evaporated *in vacuo*. The residue, recrystallized from hot benzene, gave orange needles of ϵ -sulphazoyl-L-lysine methyl ester, m.p. 124–125°; yield, 90%; $[\alpha]_{D}^{19}$ +15.8 ± 1.1° in CHCl_3 (c, 0.92; l, 2 dm.). (Found: C, 56.2; H, 5.9; S, 7.8. $\text{C}_{18}\text{H}_{24}\text{O}_4\text{N}_4\text{S}$ requires C, 56.4; H, 6.0; S, 7.9%.)

Chromatography

N-Sulphazoylamino acid methyl esters can be selectively adsorbed on alumina columns from their solutions in certain organic solvents. The alumina (Hopkins & Williams, pure aluminium oxide) was treated for 9 hr. with 10% acetic acid in methanol (v/v), then thoroughly washed with methanol, and dried in air. It was found possible to separate on such columns the methyl esters of the *N*-sulphazoyl derivatives of glycine, alanine, leucine, disulphazoyllysine and ϵ -sulphazoyllysine. Other combinations of these compounds with derivatives of phenylalanine, valine, isoleucine or norleucine were also separated. As an example, the separation of a mixture containing the methyl esters of the *N*-sulphazoyl derivatives of glycine, L-phenylalanine, ϵ -sulphazoyl-L-lysine, and α -disulphazoyl-L-lysine is described. A slurry of alumina in dry benzene was gradually packed into a column 22 × 400 mm. (under N_2 pressure of 130 mm. Hg) until the column was 280 mm. long. The rate of flow was then 150 ml./hr. under atmospheric pressure. The mixture of the sulphazoyl derivatives dissolved in CHCl_3 (20 ml.) was added to the column, which was then developed first with 400 ml. of benzene and then with 160 ml. of methanol-benzene (0.2:99.8). The band containing the sulphazoyl-L-phenylalanine methyl ester was in the bottom of the column and had partly passed into the effluent. The other three compounds formed clearly separated bands in the following order from the top of the columns. ϵ -sulphazoyl-L-lysine methyl ester, α -disulphazoyl-L-lysine methyl ester, sulphazoylglycine methyl ester. The bands

were eluted with acetone. The recoveries were as follows: (SA=sulphazoyl, ME=methyl ester).

Band	Quantities added	Quantities recovered
A	46 mg. ϵ -SA-L-lysine-ME, m.p. 124–125°	45.6 mg., m.p. 124–125°
B	54.2 mg. α -di-SA-L-lysine-ME, m.p. 104–105°	53.8 mg., m.p. 104–105°
C	51.6 mg. SA-glycine-ME, m.p. 148–149°	51.0 mg., m.p. 148–149°
D	50.6 mg. SA-L-phenylalanine-ME, m.p. 148–149°	50.2 mg., m.p. 147–148°

However, with alumina prepared in this way, a mixture of the derivatives of phenylalanine and alanine gave a single band; it was also not possible to separate a mixture of valine, isoleucine, norleucine, and leucine.

Although alumina prepared in various ways, and SiO₂ (British Drug Houses, dried at 230°) were used in an attempt to separate α -sulphazoyl-L-lysine methyl ester and ϵ -sulphazoyl-L-lysine methyl ester, no successful separation of these compounds was obtained.

Using alumina of more strongly adsorbing qualities (either untreated alumina dried at 230° or alumina which had been treated with methanol and then dried at 230°), a chemically pure compound, sulphazoyl-DL-leucine methyl ester (prepared from DL-leucine obtained synthetically from 4-methylpentanoic acid (*isocaproic acid*)), was separated into two bands, each of a melting point identical with the compound originally added to the column, each optically inactive and with no apparent difference in their dipole moments.

To an alumina column 22 × 400 mm. (slurry of alumina (120 g.) with benzene (250 ml.) and methanol (0.4%, v/v)) was added a solution of *N*-sulphazoyl-DL-leucine methyl ester (0.5640 g.) in CHCl₃ (60 ml.). The column was developed first with CHCl₃ (60 ml.), and then with 0.4% methanol in benzene (130 ml.). Two separate bands were obtained, which when eluted and recovered gave 0.2550 and 0.2575 g. respectively. Both samples had m.p. 121–122°.

These two separate bands were repeatedly chromatographed until chromatographically homogeneous; they were optically inactive, and their dipole moments were found to be identical within the limits of experimental error. (We thank Dr F. R. Goss for the measurements. He also kindly calculated the possible differences in dipole moments of the *cis* and *trans* isomers, and concluded that a difference of about 2 Debye units is to be expected.) Hence this seems to rule out the possibility that these two compounds were *cis-trans* isomers.

Also, mixtures of the derivatives of DL-leucine and DL-norleucine, DL-isoleucine and DL-norleucine, L-norleucine and D-leucine were separated into two approximately equal bands, neither band being pure, but apparently an equimolecular mixture of the two compounds originally added to the columns; this was shown by the optical rotation of mixtures of optically active compounds, and by the melting points.

An example of the chromatographic separation of such a mixture is given below. A column was prepared in a tube (22 × 400 mm.) with a slurry of alumina (treated with methanol and dried overnight at 230°) in 0.5% methanol in benzene (v/v). The length of the column was 300 mm. and the rate of flow 100 ml./hr. A solution in CHCl₃ (40 ml.) of

the methyl esters of sulphazoyl-L-norleucine (234.4 mg.; [α]_D²⁰₄₃₈, +47.0°; m.p., 129–129.5°) and sulphazoyl-D-leucine (234.5 mg.; [α]_D¹⁶₄₃₈, –50.0°; m.p., 138–139°) was added to the column followed by CHCl₃ (40 ml.). After development with a solution of 0.5% methanol in benzene (110 ml.) the appearance of the column was as follows, 40 mm. practically white, 16 mm. deep orange (A), 60 mm. very pale orange, 16 mm. deep orange (B), remainder white. Recovered by elution with acetone: band A, 221.5 mg.; m.p. 115–116°; [α]_D²⁰₄₃₈, –1.5°; band B, 219.0 mg.; m.p. 115–116°; [α]_D²⁰₄₃₈, –1.6°.

On rechromatography, each band gave two separate bands, one containing about 8–9 times the quantity of material in the other. These bands were chromatographically homogeneous, of negligible rotation, and m.p. 115–116°.

Stability of sulphazoylamino acids to acid hydrolysis

The sulphazoylamino acids (70–100 mg.) were kept at 100° in evacuated, glass-stoppered flasks with 6*N*-HCl (70–100 ml.) for varying periods. The solutions were then evaporated to dryness *in vacuo*. In the case of sulphazoyl-glycine and sulphazoyl-DL-phenylalanine, the residue was dissolved in dioxan, diazomethane in ether was added, and after standing for 2 hr. the red solutions were evaporated to dryness *in vacuo*.

Fig. 1. Stability of some *N*-sulphazoylamino acids to acid hydrolysis. The substances were maintained for varying periods of time in 6*N*-HCl at 100° in evacuated flasks, then recovered chromatographically, and the recovered substances estimated by weight. (SA=sulphazoyl.)

The residue was extracted with CHCl₃, and the sulphazoylamino acid methyl ester was purified on alumina columns. The columns were developed with 10% light petroleum, b.p. 60–80°, in benzene; some impurity was washed completely out of the column, the remainder was very strongly adsorbed at the top of the column, whereas the required compound occupied the centre position. It was eluted with acetone and the purity was determined by means of the melting point and mixed melting point with an authentic specimen.

ϵ -Sulphazoyl-L-lysine was methylated in methanol solution with dry HCl; the deep red solution thus obtained was evaporated *in vacuo* (<35°), taken up in methanol, neutralized with pyridine to Congo red, and poured into excess 5% (w/v) NaHCO₃; the mixture was extracted with CHCl₃, and the extract was dried and purified chromato-

graphically. As the esterification by methanol-HCl is not quantitative (yields of the order of 90% being obtained), it was necessary to apply a correction to give the stability of the compound to the conditions of hydrolysis. The data obtained are shown in Fig. 1.

DISCUSSION

The methyl esters of *N*-sulphazoyl derivatives of various amino-acids, have been prepared in two different ways. First, by reaction of azobenzene-*p*-sulphonyl chloride (sulphazoyl chloride) with methyl esters of amino-acids in pyridine, and secondly, by reaction of sulphazoyl chloride with amino-acids in water—NaHCO₃—acetone solution, and subsequent methylation of the *N*-sulphazoyl-amino acids with diazomethane.

Only the latter procedure could be used for the determination of the amino-acids situated at those ends of polypeptide chains which bear the unsubstituted α -amino groups. Under the conditions described in the paper, the reaction proceeds smoothly and quantitatively.

However, the stability of the *N*-sulphazoyl-amino acids under conditions required for the hydrolysis of peptide bonds is not very great; especially in the case of the glycine derivative, a considerable proportion of it is destroyed under hydrolytic conditions. This is an important factor curtailing the use of these derivatives for the end-group determination in peptides and proteins.

The investigation concerning the chromatographic separation of mixtures of *N*-sulphazoyl-amino acid methyl esters has demonstrated the importance of assessing the homogeneity of chromatographic bands by physico-chemical means independent of chromatographic characterizations. Mixtures of the derivatives of DL-leucine with DL-norleucine, of DL-isoleucine with DL-norleucine, and of L-norleucine with D-leucine were separated into two bands, neither band being pure but apparently an equimolecular mixture of the two compounds added to the column (as shown by melting points and the optical rotation of the active compounds).

Even more unexpected is the fact that a chemically pure substance, *N*-sulphazoyl-DL-leucine methyl ester (prepared from DL-leucine obtained synthetically from isocaproic acid), was separated into two bands; the melting point of both was

identical with the compound originally added to the column, each compound was optically inactive, and there was no apparent difference in their dipole moments.

The *N*-sulphazoyl derivatives crystallize well and have sharp melting points. They are orange to deep red substances, and the chromatographic bands are easily visible even in very small amounts.

SUMMARY

1. A method of preparation of azobenzene-*p*-sulphonyl chloride (sulphazoyl chloride) is described.
2. Some methyl esters of amino-acids which have not been previously described in the literature have been prepared by methylation in methanolic hydrogen chloride.
3. *N*-Azobenzene-*p*-sulphonyl derivatives (sulphazoyl derivatives) of various amino-acids were prepared by reaction of sulphazoyl chloride with amino-acids in aqueous acetone containing sodium bicarbonate.
4. These sulphazoylamino acids were methylated either with methanolic hydrogen chloride or with diazomethane in dioxan yielding *N*-sulphazoylamino acid methyl esters.
5. An alternative method of preparation of *N*-sulphazoylamino acid methyl esters by reaction of sulphazoyl chloride with amino-acid methyl ester hydrochlorides in pyridine solution is reported.
6. The chromatographic resolution of mixtures of *N*-sulphazoylamino acid methyl esters has been investigated, and examples are described.
7. The necessity of assessment of the chemical homogeneity of various fractions obtained chromatographically is emphasized. Examples are described which demonstrate that chromatographically homogeneous fractions may be mixtures of chemically distinct compounds.
8. An example is noted of a compound which as far as could be ascertained was chemically pure and homogeneous, but which could be chromatographically separated into two homogeneous fractions.
9. The applicability of *N*-sulphazoylation of amino-acids to the end-group determination in peptides is discussed.

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