A Search for Specific Chemical Methods for Fission of Peptide Bonds

2. EXPERIMENTS INVOLVING REDUCTION OF C-TERMINAL RESIDUES*

By J. C. CRAWHALL AND D. F. ELLIOTT

National Institute for Medical Research, Mill Hill, London, N.W. 7

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In the first paper of this series (Elliott, 1952) it was shown that *N*, *O*-acyl migration could be used to bring about cleavage of the silk fibroin molecule at some of the serine residues, but that other peptide bonds involving serine remained resistant to the reagent. It seemed possible that this resistance may have been due to steric hindrance by bulky side chains in the neighbourhood of serine residues. In view of the very high content of glycine and alanine in silk fibroin it could be assumed that they are linked frequently to serine and that it is these bonds which undergo the acyl migration reaction, whereas bonds involving serine and amino acids with larger side chains remained unaffected. In order to test this hypothesis a quantitative *C*-terminal end-group method of wide scope was needed. No such method existed when this work commenced, but reduction of the *C*-terminal residues with a metallo-hydride seemed promising. In addition it was hoped that reduction of the carboxyl groups of peptides would form the starting point for a stepwise degradation method as outlined below.

Fromageot, Jutisz, Meyer & Pénasse (1950) had already shown that lithium aluminium hydride reduced the free carboxyl groups of proteins to primary alcohol groups and that amino alcohols were obtained on subsequent hydrolysis, but the results obtained on insulin were far from quantitative. Chibnall & Rees (1951, 1952) had used lithium borohydride to determine the distribution of amide groups in insulin. Our initial experiments consisted in the reduction of a few simple peptide derivatives by lithium aluminium hydride and lithium borohydride, in connexion with the stepwise degradation method. Attempts were then made to develop a reliable end-group method for large peptides and proteins.

RESULTS

Reduction of simple peptides and related compounds by mixed metallo-hydrides

In some of these experiments benzoylated glycol peptides were used; these were readily prepared by the condensation of 2-phenyloxazalone with amino

acids in the presence of aqueous alkali, and were converted into their methyl esters by the action of diazomethane. At first, attempts were made to use lithium aluminium hydride for reduction on account of its comparatively low cost. In most cases no attempt was made to isolate the main products as crystalline substances, but instead the crude material was hydrolysed and submitted to paper chromatography. It was hoped that all products would be detected by this method and not merely those present in largest amount. Reduction at the boiling point of the solvent, tetrahydrofuran (THF), even for a short while, was not satisfactory. Under these conditions and after acid hydrolysis, benzoylglycylalanine methyl ester yielded a considerable amount of *N*-benzylglycine in addition to glycine and alaninol. There was also present a fourth ninhydrin-positive substance which may have been a secondary amine derived from the reduction of the glycylalanine linkage. When methyl hippurate was treated with lithium aluminium hydride in boiling THF 2-benzylamino-ethanol was produced in 65% yield. The spot due to *N*-benzylglycine persisted in all chromatograms obtained from reductions of benzoylglycyl peptide esters carried out at room temperature or at 0° for several hours. Benzoylglycylalanine methyl ester was not reduced by lithium aluminium hydride at -50°. Since this work was completed, Jutisz, Meyer & Pénasse (1954) have carried out similar work with lithium aluminium hydride and hippuric acid or its methyl ester. They observed both reduction of the benzamido group to a benzylamino group and reductive cleavage of the C—N linkage of the amide group. Their experiments also showed that 60–90% reduction of the end groups of dipeptides resulted when they were treated for 2 hr. at 0° with lithium aluminium hydride, but traces of the second amino acid appeared as an amino alcohol even in these reductions. In view of these results it seems likely that some peptide bonds in proteins would be attacked by lithium aluminium hydride.

Reductions with lithium borohydride gave much more satisfactory results. The end group of benzoylglycylalanine methyl ester was reduced completely with no side reactions when treated with

* Part 1: Elliott, D. F. (1952).

lithium borohydride for 18 hr. at 0°. It was also found that *N*-benzoyl ethanolamine and *N*-benzoylglycylethanolamine were unaffected by the reagent under the same conditions. When reduced at the boiling point of THF by lithium borohydride, however, benzoylglycylalanine methyl ester gave rise to a new spot on the chromatogram which had an R_f value identical with that of ethanolamine. No attempt was made to identify this substance, but experiments carried out later leave little doubt that it was ethanolamine; reductive cleavage of the peptide bond was not envisaged at that time. These preliminary experiments indicated that lithium borohydride was a satisfactory reducing agent for simple peptides, but that further study of the side reactions occurring under vigorous conditions might be necessary. This problem is discussed below. Recently, Grassmann, Hörmann & Endres (1953) have described the reduction of peptides by lithium borohydride at the boiling point of THF.

Attempts to devise a stepwise degradation of peptides

A number of attempts were made to develop a stepwise degradation based on the acyl migration reaction used in the degradation of silk fibroin (Elliott, 1952). The following scheme of reactions was envisaged:

This would have constituted a valuable degradative procedure for the peptides containing *N*-terminal serine or threonine resulting from the fission of proteins by the acyl migration method. A great

deal of time was devoted to studying the use of hydrogen chloride in anhydrous methanol and nitromethane, which could be readily removed at the end of the reaction, to bring about the *N, O* rearrangement. Although many different conditions of time and temperature and various concentrations of hydrogen chloride in methanol were examined, the rearrangement was never quantitative and was generally accompanied by side reactions. It was also found that free ethanolamine was produced by the action of methanolic hydrogen chloride on *N*-benzoylglycylethanolamine; this probably occurred by alcoholysis of the *O*-peptidyl linkage. Attempts to force the latter reaction to completion by using more vigorous conditions resulted in partial cleavage of the benzoyl group. These experiments are summarized in Table 1. Desnuelle & Bonjour (1951) showed that a saturated solution of hydrogen chloride in methanol caused cleavage of peptide bonds in addition to those involving serine and threonine in proteins when the reaction time was prolonged.

It was found that after 20 hr. at room temperature in nitromethane-hydrogen chloride the theoretical quantity of amino N was formed from *N*-benzoylglycylethanolamine. The reagent itself was unstable and gave rise to a crystalline ppt. after about 3 days. It was shown, however, that after 20 hr. there was insufficient of this substance present to affect the amino N determinations. Paper chromatography revealed, however, that an appreciable quantity of free ethanolamine was present in the product. The mechanism by which this substance is produced under anhydrous conditions is not clear.

The product obtained by lithium borohydride reduction in the cold of benzoylglycylalanine methyl ester gave similar results to the ethanolamine derivative on rearrangement by the two methods described above.

Cold concentrated sulphuric acid, which was found to be a satisfactory reagent in the protein

Table 1. *Products obtained (indicated by ×) on treatment of N-benzoylglycylethanolamine with methanolic hydrogen chloride under various conditions*

Additional, unidentified substances were obtained in experiments carried out at 100°.

Normality of HCl soln. used	Mol. prop. HCl used	Temp. of reaction (°)	Time (min.)	Ethanolamine	Glycine methyl ester	O-Benzoylglycyl-ethanolamine
3	5	100	5	×	×	×
1	5	100	20	×	×	×
0.3	5	100	90	×	×	×
3	1	100	10	×	Trace	×
3	1	100	90	×	×	×
1	1	100	20	×	×	×
3	1	20	1020	×	—	×
3	5	20	1020	×	—	×
1	5	20	1020	×	—	×

field, was unsuitable for small peptides, which do not lend themselves to straightforward isolation from this reagent. Orthophosphoric acid was unsatisfactory for the same reason and glacial acetic acid at 100° did not cause rearrangement. Fuming sulphuric acid in nitromethane caused no production of amino N from benzoylglycylethanolamine.

The formation of free amino alcohols during rearrangement gave rise to difficulties in the acetylation stage. When acetylation of the rearranged peptide was carried out at pH 5 according to the procedure described by Elliott (1952) the amino group of the *O*-peptidyl form was blocked, but that of the free amino alcohol was unattacked. Since the quantity of amino alcohol present was generally about 20 % of the total end group, it did not seem justifiable to ignore this amount. It would then have been necessary either to carry out a second acetylation at a higher pH or to devise a technique for the isolation of the amino alcohol. In either event the process would have become rather involved and unsuitable for small-scale operation. This work was therefore discontinued.

Isolation of amino alcohols from mixtures with amino acids and inorganic salts

The isolation of pure amino alcohols from the mixtures obtained after metallo-hydride reduction and hydrolysis of peptides and proteins is not easy, partly on account of the presence of inorganic salts formed from the metallo-hydrides. In many cases electrolytic desalting was necessary before isolation procedures could be carried out (Chibnall & Rees, 1953; Fromageot & Jutisz, 1953). There appears to be no solvent, except ether, in which amino acids and inorganic residues from the metallo-hydrides are insoluble and the amino alcohols soluble; even in this solvent, ethanolamine and the aminodiols are practically insoluble. These amino alcohols were more easily extracted by repeated shaking of a hydrolysate, made alkaline by potassium carbonate, with 1 % ethereal anisaldehyde. This was presumably due to the formation of Schiff bases which were more soluble in the organic solvent. The amino alcohols were recovered in the pure state from the ethereal extracts by evaporation and steam distillation of the acidified residue to remove the anisaldehyde.

Some attempts were then made to develop chromatographic procedures for the separation and identification of amino alcohols. Dr A. T. James of this Institute kindly carried out gas-liquid chromatography, but found that, whereas the simpler amino alcohols were readily separated from each other, the aminodiols gave rise to trailing peaks in all the systems used. Liquid-liquid chromatography, using 'Hyflo Super-Cel' as support, aqueous potassium hydroxide as the stationary

phase and various organic solvents as the moving phase was also investigated but the degree of resolution was rather poor.

The conversion of the amino alcohols into their 2:4-dinitrophenyl (DNP) derivatives was next investigated (cf. James & Synge, 1951; Waldkötter, 1938); it seemed likely that these substances would be readily soluble in ether and therefore easily isolated from hydrolysates. They were formed by means of 1-fluoro-2:4-dinitrobenzene under various conditions, but it was found that serinol gave rise to a small amount of a yellow substance in addition to the *N*-substituted derivative. This was probably the *ON*-bis-dinitrophenyl or the *OO'*-*N*-tris-dinitrophenyl derivative. The reaction of the hydroxyl group of ethanol with fluorodinitrobenzene has been observed by Zahn & Wurtz (1952). The formation of a by-product in the preparation of DNP-amino alcohols was very undesirable; the yellow compound from serinol, for instance, had the same *R* value as *N*-DNP-threoninol in one of the solvent systems used for chromatography.

Eisen, Belman & Carsten (1953) have shown that sodium 2:4-dinitrobenzenesulphonate slowly reacts with protein amino groups at pH 10-11 to give DNP derivatives. This reaction, which occurs by elimination of a molecule of sodium sulphite, is analogous to the formation of amino-substituted quinolines by heating 2- and 4-quinoline sulphonc acids with bases (Walker, 1947). It seemed possible that sodium 2:4-dinitrobenzenesulphonate (DNBS) would react only with the amino groups of amino alcohols. An apparent disadvantage of the reagent was the high pH needed for the reaction, but it was soon evident that DNP-amino alcohols have a higher stability to alkali than DNP-amino acids. A number of different reaction conditions were examined with alaninol as test substance, a quantitative yield of the DNP derivative being obtained when it was heated at 95° for 20 hr. with DNBS in the presence of borax solution. These conditions were also suitable for the isolation of amino alcohols from hydrolysates; serinol, ethanolamine and alaninol were recovered almost quantitatively in this manner after being mixed with 40 parts of silk-fibroin hydrolysate. There appeared to be no substitution of the hydroxyl groups under these conditions; even tyrosinol gave only the *N*-substituted derivative.

Chromatography of DNP-amino alcohols. Since this work was commenced the isolation and chromatography of DNP-amino alcohols has been described by several workers (Grassmann, Hörmann & Endres, 1954; Jatzkewitz & Tam, 1954; Luck & Wilcox, 1953; Jutisz, Privat de Garilhe, Suquet & Fromageot, 1954). The choice of solvents for chromatography of DNP-amino alcohols is rather limited on account of their high solubility in

[illegible]

small portion was treated with 15% (w/v) methanolic hydrogen chloride for 30 min. at room temperature, when the formyl groups were removed without peptide bond fission (Elliott, 1953). The *N*-terminal residues were estimated by amino *N* determinations and the *N*-terminal serine residues by the periodate technique of Rees (1946). The figure obtained for the total *N*-terminal residues represented also the maximum possible value for the *C*-terminal residues, that for *N*-terminal serine being the minimum value.

residue of lysozyme is leucine (Thompson, 1952; Harris, 1952; Ohno, 1953). When a portion of the lysozyme, after reduction by lithium borohydride, was treated with fluorodinitrobenzene under the usual conditions of Sanger (1945) and the product hydrolysed, a number of different DNP-amino acids were produced. Thus there appeared to be no particular specificity in this reductive breakdown.

Some attempts were made to devise conditions whereby lithium borohydride could be used in aqueous media in which the protein would be

Table 4. *Yields of amino alcohols produced by LiBH₄ reductions under various conditions of the formyl peptide mixture obtained by degradation of silk fibroin*

Results expressed in terms of m-equiv./g. of peptide.

Pretreatment of peptide mixture	Time (hr.)	Temp. (°)	Yield of amino alcohol			
			Ethanolamine	Alaninol	Serinol	Total
Esterification	72	20	0.32	0.09	0.02	0.43
Esterification	12	70-75	0.50	0.215	0.025	0.74
Esterification	18	70-75	0.77	0.40	0.065	1.235
None	18	70-75	0.195	0.16	0.04	0.395

Total *N*-terminal residues, 0.93; *N*-terminal serine residues, 0.84.

The formylpeptides were esterified by diazomethane before reduction. At first, attempts were made to bring about reduction of the esterified peptide mixture with lithium borohydride in THF at room temperature, but even after 3 days only about 50% reduction had occurred. It was clear that these poor results were due to the insolubility of the peptide mixture in the solvent and the effect of raising the temperature was therefore examined. When the reaction was carried out at 70-75° for 12 hr., the yield of amino alcohols approached the theoretical quantity, but in an 18 hr. reduction the yield was far greater than theoretical. When a similar reduction was carried out on the peptide mixture without previous diazomethane treatment the same amino alcohols (ethanolamine, alaninol and serinol) were obtained in about one-third of the quantity obtained from the esterified peptides. That this result was not entirely due to reduction of the free carboxyl groups was demonstrated by a similar treatment of hippuric acid, whereby only a 12% yield of ethanolamine was obtained. These results are summarized in Table 4, where the values obtained for the total *N*-terminal residues and the *N*-terminal serine residues are also given.

It was then found that lysozyme, which had been recrystallized four times, gave rise to ethanolamine and alaninol as well as several other amino alcohols, when treated with lithium borohydride in THF at 70-75° (see Table 5). There is no doubt that, with the possible exception of leucinol, these arose by fission of the peptide chain. It has been shown by two independent methods that the *C*-terminal

Table 5. *Amino alcohols obtained from lysozyme on treatment with LiBH₄ in THF at 70-75° for 18 hr.*

Results expressed as moles/mole of enzyme (14700 g.)

Amino alcohol	Yield
Ethanolamine	1.23
Alaninol	2.02
Serinol	0.48
Leucinol or isoleucinol	0.74
Valinol	0.25
Phenylalaninol	0.17

soluble. The reagent decomposed slowly when added to water, but it was found possible to bring about partial reduction of methyl hippurate in aqueous methanolic solution by addition of an excess of reagent in several portions over a long period of time. The effects of various catalysts were also investigated. In none of these experiments could the yield of ethanolamine be raised above about 40%. Unfortunately no DNP-leucinol was produced from esterified lysozyme after reduction in aqueous solution.

EXPERIMENTAL

Synthesis of model compounds

Melting points are uncorrected.

N-Benzoyl ethanolamine. Prepared by the method of Knorr & Rössler (1903).

N-Benzoylglycyl ethanolamine. 2-Phenylloxazalone (5.3 g.) was added in small portions during 10 min. to ethanolamine (2 g.), dissolved in a mixture of water (10 ml.) and ethanol (20 ml.), with vigorous shaking. A clear soln. was obtained

and after it had been kept for 1.5 hr. at room temp. it was evaporated to dryness under reduced pressure. The residual oil of *N*-benzoylglycyllethanolamine was crystallized from a mixture of ethanol and benzene and had m.p. 126°. (Found: C, 59.6; H, 6.2; N, 12.6. $C_{11}H_{14}O_2N_2$ requires C, 59.5; H, 6.3; N, 12.6%.) The yield was 4.45 g. (61%).

DL-Benzoylglycylalanine. 2-Phenyloxazolone (1.6 g.) was added in small portions during 10 min. to a vigorously shaken soln. of *DL*-alanine (0.89 g.) in *N*-NaOH (10 ml.) and ethanol (10 ml.). Ethanol was removed by evaporation at reduced pressure and the soln. acidified with conc. HCl. After the soln. had stood for 45 min. the ppt. was filtered off, washed with water and crystallized from boiling water. The yield was 1.97 g. (79%) and the m.p. 200° (Curtius & Lambotte (1904), report 202° for this compound). (Found: N, 10.9. Calc. for $C_{12}H_{14}O_4N_2$: N, 11.2%.)

DL-Benzoylglycylphenylalanine. This was prepared in a similar manner to the alanine derivative in 43% yield and had m.p. 202° after crystallization from hot water (Curtius & Müller (1904), report m.p. 172° for this compound). (Found: N, 8.7. Calc. for $C_{18}H_{18}O_4N_2$: N, 8.6%.)

L-Benzoylglycylproline. 2-Phenyloxazolone (0.8 g.) was added in small portions during 5 min. to a vigorously shaken soln. of *L*-proline (0.57 g.) in *N*-NaOH (5 ml.) and ethanol (5 ml.). The clear soln. was allowed to stand for 1 day. The ethanol was then removed under reduced pressure and the aqueous soln. acidified with cold conc. HCl (Congo red). No ppt. formed. The soln. was evaporated to dryness under reduced pressure and the residue extracted with ethanol. *L-Benzoylglycylproline* (0.53 g., 39%), m.p. 205°, separated from the ethanolic extract on addition of ether. It was recrystallized from ethanol and had m.p. 207°, $[\alpha]_D^{20} + 8.75 \pm 0.5^\circ$ in 0.042*N*-NaOH (c, 0.575). (Found: C, 60.4; H, 6.0; N, 9.6. $C_{14}H_{16}O_4N_2$ requires C, 60.9; H, 5.8; N, 10.2%.)

N-Benzylglycine. This was synthesized by the method of Baker, Ollis & Poole (1949).

Reduction of peptides and their esters by LiAlH₄ and LiBH₄

Purification of THF. The solvent was allowed to stand 24 hr. over NaOH flakes and was then distilled over fresh NaOH flakes. The distillate was refluxed 6 hr. with Na wire and distilled a second time. Last traces of water were removed by refluxing 1 hr. with a small quantity of LiAlH₄ and distilling into a carefully dried receiver. It was stored in the dark over Na wire to prevent formation of peroxides.

Preparation of solutions of LiAlH₄. The reagent was obtained in the form of solid masses of irregular size from which a portion (2.5 g.) was broken, rapidly ground and added to THF (50 ml.) in a flask closed with a CaCl₂ tube. After refluxing for 1 hr. the soln. was rapidly filtered through dry kieselguhr and stored in a stoppered bottle kept in a sealed box containing CaCl₂ as a desiccant. The LiAlH₄ content of the soln. was roughly estimated by the vol. of H₂ evolved on decomposition by 10% aqueous dioxan in a Warburg flask connected to a gas burette. In general the LiAlH₄ content of the solid reagent was about 80%. A soln. prepared in this way was therefore approximately molar.

Chromatography of the reduction products. At the end of a reduction the mixture was cooled in ice and unchanged reagent decomposed by addition of a small excess of water.

The soln. was refluxed 10 min., cooled again and the ppt. removed by centrifuging. The soln. was evaporated to dryness and a portion hydrolysed with conc. HCl at 100° for 18 hr. The hydrolysate was submitted to paper chromatography, using the butanol-acetic acid-water mixture of Fromageot *et al.* (1950) and the sheet sprayed with ninhydrin. A hydrolysate of the starting material was used as a control.

Esterification of the peptides was carried out by addition of excess of ethereal diazomethane to a suspension in a 1:1 (by vol.) mixture of ether and methanol. When effervescence had ceased, the excess of reagent was decomposed with a few drops of acetic acid and the solvent evaporated.

Reduction of DL-benzoylglycylalanine methyl ester by LiAlH₄. (a) *At the b.p. of THF*. The ester (0.88 g.) dissolved in THF (5 ml.) was added dropwise during 20 min. to a boiling *m* soln. of LiAlH₄ in THF (10 ml.). The soln. turned a deep orange colour. After heating for 1.5 hr. a portion of the product was hydrolysed and examined chromatographically. Glycine (*R_F* 0.05), alaninol (*R_F* 0.24) and *N*-benzylglycine (*R_F* 0.40) were present in addition to an unidentified substance, *R_F* 0.58. Another portion of the reduction product was hydrolysed with 5*N*-H₂SO₄ at 100° for 18 hr. The liberated benzoic acid was extracted with ether and estimated by titration. Only about 20% of the theoretical amount of benzoic acid was present.

(b) *At 0°*. Reduction was carried out as above, except that 5 mol. prop. of the reagent were used and the reaction mixture was cooled in ice. After remaining at 0° for 18 hr. the product was examined as before. *N*-Benzylglycine was present in addition to glycine and alaninol and was still present in the product when the reaction time was decreased to 2 hr.

(c) *At -50°*. As in (a) except that the LiAlH₄ soln. was cooled to -50° during the addition of the ester and the reaction mixture then maintained for 4 hr. at -50°. No alaninol could be detected in the hydrolysed product.

N-Benzylglycine was also obtained when benzoylglycylalanine was reduced by LiAlH₄ at room temp. for 18 hr.

Reduction of methyl hippurate at the b.p. of THF. The ester was reduced exactly as for benzoylglycylalanine methyl ester and the product treated with ethereal picric acid. *N*-Benzylaminoethanol picrate was produced in 65% yield and had m.p. 136° (Gabriel & Stelzner (1896), recorded m.p. 135-136° for this compound). (Found: C, 48.0; H, 4.5; N, 14.7. Calc. for $C_{15}H_{16}O_8N_4$: C, 47.3; H, 4.2; N, 14.7%.)

When *DL*-benzoylglycylphenylalanine methyl ester was reduced by LiAlH₄ at 0° for 3 hr. *N*-benzylglycine was found in the hydrolysate of the product, but was absent from a similar reduction of *L*-benzoylglycylproline.

Reduction of DL-benzoylglycylalanine methyl ester by LiBH₄. The sample of LiBH₄ used in these experiments was almost pure and it was assumed that the wt. of LiBH₄ in soln. was equal to the amount weighed out. It dissolved rather slowly in THF at room temp. and left a small amount of white residue.

(a) *At 0°*. A solution of 1 *m*-mole of the ester in 5 ml. THF was cooled to -50° and to it was added a solution of LiBH₄ (5 mol. prop., 110 mg.) in THF (15 ml.) also cooled to -50°. The mixture was then maintained at 0° for 18 hr. At the end of this time the soln. was cooled to -50° and water (0.1 ml.) added. The soln. was then refluxed 2 min., cooled and filtered. The filtrate was evaporated to dryness

and hydrolysed in the usual way. A chromatogram showed only glycine and alaninol. It was also shown that *N*-benzoyl ethanolamine and *N*-benzoylglycylethanolamine were unaffected by the reagent under the same conditions.

(b) *At the b.p. of THF.* The ester and reagent were mixed as in (a) and then refluxed 4 hr. The chromatogram showed the presence of glycine and alaninol and in addition a substance, R_F 0.15. Ethanolamine also had R_F 0.15 in the same solvent mixture.

Acyl migration experiments on reduced peptides

Amino N was estimated in the volumetric apparatus of Van Slyke (1911). After addition of the reagents and the sample to the reaction vessel the mixture was shaken for 1 min. at room temp. (18–22°) and reaction allowed to proceed for 1 hr. without shaking. After further shaking for 1 min. the volume of nitrogen evolved was measured in the usual way. This procedure gave quantitative results with cyclohexylamine, alaninol, ethanolamine and *O*-benzoyl ethanolamine hydrochloride.

The presence of the *O*-peptidyl form of a reduced peptide was demonstrated by application of duplicate samples from a rearrangement to a sheet of chromatography paper and exposing one sample to NH_3 vapour for a few seconds. The sheet was then developed in the solvent of Fromageot *et al.* (1950) and sprayed with ninhydrin. The spot due to the rearranged peptide was absent in the sample treated with ammonia, whereas amines formed by side reactions were unaffected. Glycine methyl ester gave a yellow spot with ninhydrin.

The experiments with *N*-benzoylglycylethanolamine, recorded in Table 1, were carried out on 50 mg. of the substance in sealed glass tubes. At the end of the reaction the tubes were opened and the methanol and HCl removed in a vacuum desiccator over NaOH and H_2SO_4 . In the experiments carried out at 100° minor amounts of ninhydrin-reactive substances were present in addition to those recorded; these were not identified. An amino N determination on the product obtained by treating *N*-benzoylglycylethanolamine with 30 mol. prop. of methanolic HCl showed that only 60% of the theoretical amount was present. Rearrangements with HCl in nitromethane were carried out by suspending the substance in the dry solvent and passing in dry HCl until the soln. was saturated. The clear soln. which was obtained was allowed to stand for at least 20 hr. at room temp. The product was isolated in the same manner as described for rearrangements in methanol-HCl.

Synthesis of DNP-amino alcohols

Amino alcohols. DL-Alaninol, DL-valinol, DL-leucinol, DL-isoleucinol, DL-phenylalaninol and DL-glutamidiol were prepared from the amino acid esters by the method of Karrer, Portmann & Suter (1948) and were purified as their oxalates, with the exception of glutamidiol which was used as a crude oil. The serinol oxalate used was a gift from Dr H. R. V. Arnstein. DL-Tyrosinol hydrochloride and its *O*-methyl derivative were synthesized by the method of Berlinguet (1954).

DL-Threoninol was prepared as follows. DL-Benzoyl-threonine was esterified with ethereal diazomethane and the ester crystallized from ether. DL-*N*-Benzoylthreonine methyl ester had m.p. 85–86°. (Found: C, 61.3; H, 6.4. $\text{C}_{15}\text{H}_{19}\text{O}_4\text{N}$ requires C, 60.8; H, 6.4%.) The ester (1 g.) was added to a soln. of LiBH_4 (0.2 g.) in THF (20 ml.). The

mixture effervesced slightly and became warm. It was allowed to stand 2 days and the excess reagent decomposed by addition of water and a few drops of conc. HCl. The solvent was removed under reduced pressure and the residue hydrolysed with 6*N*-HCl (10 ml.) at 105° for 16 hr. The hydrolysate was cooled, filtered from benzoic acid and the filtrate evaporated to dryness. The crude hydrochloride was used for the preparation of the DNP derivative as described later.

DL-Dibenzoyllysine, prepared by the method of Fischer & Weigert (1902), was dissolved in 50% (v/v) aqueous methanol and treated with excess of diazomethane in ether. The crude ester (0.01 mole) was reduced by refluxing 4 hr. with LiBH_4 (100 mg.) in THF (20 ml.). The crude DL-lysine hydrochloride was obtained as in the case of threonine.

DL-Benzoylaspartic acid (Fischer, 1899) was esterified with ethereal diazomethane and the product crystallized from ether. DL-Benzoylaspartic acid dimethyl ester had m.p. 83.5°. (Found: N, 5.65. $\text{C}_{13}\text{H}_{15}\text{O}_6\text{N}$ requires N, 5.3%.) The ester was reduced as in the case of DL-threoninol and the hydrochloride of DL-aspartidiol isolated in the usual way.

Preparation of DNBS (cf. Fierz, 1920). 1-Chloro-2,4-dinitrobenzene (20.2 g.), powdered $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ (25 g.), ethanol (65 ml.) and water (15 ml.) were mixed and stirred under reflux on the steam bath for about 8 hr. The orange solution was filtered hot and cooled at 0° for several hr. The product was filtered, washed with an ethanol-water mixture (13:3, by vol.) and purified by crystallization from water, using 2 ml. per g. of solid. The yield was 10 g.

Reaction of DNBS with amino alcohols. For preparative purposes it was convenient to use aq. Na_2CO_3 as the reaction medium. A short reaction period sufficed to give yields of 80–85%.

The amino alcohol (free base, hydrochloride, or oxalate, 0.5 m-mole) was mixed with DNBS (300 mg., 100% excess), $\text{N-Na}_2\text{CO}_3$ (2.5 ml.) and water (2.5 ml.). The mixture was heated in a sealed tube at 100° for 1 hr. with occasional shaking until the solids had dissolved. The DNP derivative frequently separated as an oil from the soln. during the reaction. At the end of the reaction, the mixture was diluted with an equal volume of water and the DNP-amino alcohol extracted with chloroform and then crystallized from the appropriate solvent. The following products were obtained (solvent for crystallization in parenthesis):

N-DNP-Ethanolamine (benzene), m.p. 89°. It gave no depression in m.p. with a specimen prepared in a different manner by Dr A. T. James.

N-DNP-DL-Alaninol (benzene–light petroleum, b.p. 60–80°), m.p. 94–95°. (Found: N, 17.2. Calc. for $\text{C}_9\text{H}_{11}\text{O}_5\text{N}_3$: N, 17.4%.) (Cf. Grassmann *et al.* 1953; Luck & Wilcox, 1953; Jatzkewitz & Tam, 1954.)

N-DNP-DL-Valinol was an oil which slowly solidified and had m.p. 67–71° in the crude state. It could not be recrystallized but behaved as a single substance in solvent systems nos. 3, 4 and 5 (Table 2). (Cf. Grassmann *et al.* 1953; Jatzkewitz & Tam, 1954.)

N-DNP-DL-Isoleucinol was an oil, but behaved as a single substance in solvent systems nos. 4 and 5.

N-DNP-DL-Leucinol (light petroleum), m.p. 83–84°. (Found: C, 51.2; H, 6.1; N, 14.6. $\text{C}_{12}\text{H}_{17}\text{O}_5\text{N}_3$ requires C, 50.85; H, 6.05; N, 14.8%.) Described as an oil by Grassmann *et al.* (1953).

N-DNP-DL-Phenylalaninol (benzene-cyclohexane), m.p. 122–123°. (Found: C, 57.1; H, 4.9. Calc. for $C_{15}H_{11}O_3N_3$: C, 56.8; H, 4.8%.) (Cf. Grassmann *et al.* 1953; Jatzkewitz & Tam, 1954.)

N-DNP-DL-Serinol (chloroform), m.p. 133°. (Found: C, 42.4; H, 4.3; N, 16.1. Calc. for $C_9H_{11}O_3N_3$: C, 42.0; H, 4.3; N, 16.35%.) (Cf. Jatzkewitz & Tam, 1954; Waldkötter, 1938.)

N-DNP-DL-Threoninol (benzene containing a few drops of ethanol), m.p. 126–127°. (Found: C, 44.7; H, 4.7. $C_{10}H_{13}O_3N_3$ requires C, 44.3; H, 4.8%.)

N-DNP-DL-Glutamidiol and *N*-DNP-DL-prolinol. The crude glutamidiol used contained prolinol as pointed out by Karrer *et al.* (1948). The crude product from the reaction with DNBS was subjected to column chromatography using solvent system no. 2. Two main bands were obtained: *R*, 1.25 and *R*, 0.13. The latter was assumed to be DNP-glutamidiol by comparison with other DNP-aminodiols. The band *R*, 1.25 was purified further in system no. 3 to give a substance *R*, 1.04. Comparison with other DNP-amino alcohols in systems nos. 3, 4 and 5 indicated that this was probably DNP-prolinol. Neither of these DNP derivatives could be crystallized.

The following DNP derivatives were prepared, using borax solution as reaction medium, by the technique described later:

N-DNP-DL-Aspartidiol. This substance was obtained as an oil. It was not easily distinguished from DNP-glutamidiol in any of the solvent systems given in Table 2.

N-DNP- and *N,N'*-bis-DNP-DL-lysinal. Two products were obtained from the reaction and had *R*, 0.21 and *R*, 0.9 in system no. 3. These were assumed to be the DNP- and bis-DNP derivatives respectively. Neither was obtained crystalline.

N-DNP-DL-Tyrosinol (chloroform containing a few drops of ethanol), m.p. 186–187°. (Found: N, 12.6. $C_{15}H_{15}O_3N_3$ requires N, 12.6%.)

N-DNP-*O*-Methyl-DL-tyrosinol (benzene-light petroleum, b.p. 40–60°), m.p. 138°. (Found: N, 12.1. $C_{16}H_{17}O_3N_3$ requires N, 12.1%.)

Preparation and use of the columns

Hyflo Super-Cel (Johns-Manville Co., London) was made into a thin cream with 6*N*-HCl and the mixture allowed to stand overnight. It was then filtered and treated twice more with 6*N*-HCl in the same way. The solid was finally washed free of chloride and dried at 100°. The dried solid was then ignited at 600° for 8 hr. The columns were glass tubes about 35 cm. long and internal diameter 12 mm. The Hyflo Super-Cel (6 g.) was mixed with the stationary phase (3 g.) and poured, in the form of a slurry with the moving phase, into the tube. The column was packed with a steel plunger as described by Howard & Martin (1950). The solvent systems described in Table 2 gave very stable columns which could be used many times. When not in use they were preserved in stoppered glass tubes long enough for the column to be completely enclosed together with sufficient moving phase to cover the solid support both inside and outside the column.

The columns were used under slight positive pressure (3–5 cm. Hg) and were allowed to become just dry before application of the sample in 1 ml. or less of the moving phase. This was then followed by two 0.2 ml. portions of moving phase before filling the upper part of the tube.

When dealing with an unknown mixture it was convenient to use first solvent system no. 1 in which all but the aminodiols derivatives ran very fast. Identification of the constituents of the fast-moving material was carried out in systems nos. 3, 4 or 5. The separation of DNP-alaninol and dinitroaniline proved to be difficult. System no. 6 was developed for this purpose. A feature of interest is the great difference in *R* value between DNP-serinol and other DNP-aminodiols in system no. 7. This may be due to interaction between the OH groups of glycerol and the serinol derivative.

The solvent was removed from the eluates by evaporation on the water pump at 30–40°. The residue was dissolved in chloroform and its light absorption measured in the Hilger Uvispek spectrophotometer at 350 μ . A standard curve was constructed for ethanolamine and alaninol; other DNP derivatives were estimated by reference to the alaninol curve, due allowance being made for molecular weight; it was assumed that the error involved would be small. In some cases an appreciable residue of glycol remained after removal of the mobile phase from the eluate. This did not interfere with the absorption measurement but if it was necessary to apply the residue to another column the glycol was removed by heating at 50°/0.01 mm. Hg for a few minutes.

Isolation of amino alcohols from hydrolysates

The technique used for the preparation of DNP-amino alcohols, although satisfactory when the amino alcohols were fairly pure, gave much lower yields in the presence of a protein hydrolysate. DNP-Ethanolamine was recovered in only 54% yield when a mixture of one part of ethanolamine and 40 parts by weight of a silk hydrolysate was heated with DNBS and *N*- Na_2CO_3 solution at 100° for 1 hr. In view of the high yields of DNP-derivatives obtained from pure amino alcohols the low yield was considered to be due to decomposition of DNP-ethanolamine rather than to incomplete reaction. Amino alcohols were found to react rather slowly with DNBS in the presence of Na_2CO_3 at 30 and 40°; when NaOH was used in place of Na_2CO_3 extensive destruction of the DNP derivative took place. It was then found that a 95% yield of DNP-alaninol was obtained by heating alaninol with excess of DNBS in the presence of borax solution at 95° for 17–20 hr. This method was also effective when amino acids were present.

Silk fibroin (20 mg.) was hydrolysed in the usual way and the hydrolysate evaporated to remove excess HCl. The residue was dissolved in water (1 ml.) and 0.5 mg. each of the oxalates of ethanolamine, alaninol and serinol were then added. To this solution was then added *N*-NaOH until the pH was 9.5–10.0 (indicator paper). The mixture was washed into a tube containing borax (1 g.) and DNBS (200 mg.); the volume of liquid was then about 5 ml. The tube was sealed and heated at 95° for 20 hr., with occasional shaking at first until the borax had dissolved. At the end of the reaction water (10 ml.) was then added and the DNP-amino alcohols were extracted with ether. The ether extract was evaporated to dryness and applied to a Hyflo Super-Cel column using solvent system no. 1, which separated DNP-serinol from the DNP derivatives of ethanolamine, alaninol and ammonia, which formed a single fast-moving band. This mixture was then applied to a column using system no. 3 in which DNP-ethanolamine was separated from dinitroaniline and DNP-alaninol. The

last two substances were separated in system no. 6. The yields of DNP-serinol, DNP-ethanolamine and DNP-alaninol were 80, 89 and 85% respectively.

It was found convenient to use the apparatus shown in Fig. 1 for the ether extraction of DNP-amino alcohols after the DNBS reaction. The walls of the washing limb C, and of the extraction limb B were thickened close to the lower side-arms so as to provide narrow orifices through which ether was forced by hydrostatic pressure forming fine jets and creating considerable turbulence of the liquid. After

Fig. 1. Diagram of extraction apparatus.

proceeding for a short distance the liquid streams broke up into fine droplets which travelled to the surface of the aqueous layer before coalescing. At the commencement of an extraction the drain-cocks attached to B and C were closed and sealed with water. Ether was poured into the reservoir A and allowed to flow into limb B until the orifice was just covered with ether. Tap E was then closed and the solution to be extracted washed into limb B, the final volume being not more than 15 ml. The thistle funnel D was then fixed in position and tap E opened. As soon as the ether extract from B had reached the orifice in limb C, about 12 ml. of water were quickly added to this limb and D was half-filled with fresh ether. The progress of the extraction was controlled by tap E, on which no lubricant other than the solvent was used. Extraction of DNP-amino alcohols was complete after 100 ml. of ether had been passed through the apparatus; this required about 10 min. In the case of mixtures containing a large quantity of dinitroaniline, which has a much higher water solubility than most DNP-amino alcohols, the aqueous layer in limb C was coloured deep yellow and the washed ether layer remained pale yellow even after this volume of ether had

been passed through. Control experiments showed, however, that the extraction could safely be interrupted at this point.

Action of LiBH_4 on the formyl peptides obtained from silk fibroin

The peptide mixture (100 mg.) (Elliott, 1953) was dissolved in water (10 ml.) and methanol (20 ml.) added to the soln. Ethereal diazomethane soln. was then added in small portions with shaking until sufficient was present in excess to maintain a yellow colour for at least 2 min. After standing for 0.5 hr. the excess of reagent was destroyed with a drop of acetic acid and the solution evaporated under reduced pressure until the ether and methanol had been removed. The aqueous residue was then dried in the frozen state. The above operations and the reduction described below were carried out in a thick-walled cylindrical flask of approx. 6 cm. diam. and 8 cm. length, excluding the neck. To the residue was then added dry THF (25 ml.), LiBH_4 (25 mg.) and a number of small glass beads (occupying a volume of about 10 ml.). In the reaction at room temperature the flask was mounted in a horizontal position and slowly rotated, but in the experiments at 75° the flask was mounted in an upright position in an oil bath, and agitation of the contents was obtained by means of a stout glass stirrer. At the end of the reduction excess of reagent was decomposed by addition of a few drops of 3N-HCl and the solvent removed under reduced pressure. A sticky residue remained which was removed from the flask by boiling for a few min. with several portions of 6N-HCl and decanting from the glass beads. The acid extracts were evaporated to small bulk and then heated in a sealed tube at 100° for 16 hr. The hydrolysate was treated with DNBS (1 g.) in the presence of borax (2.5 g.), exactly as described for the silk fibroin hydrolysate.

The reduction of the unesterified peptide mixture was carried out in a similar fashion.

Action of LiBH_4 on lysozyme

The reduction was carried out exactly as for the peptides from silk fibroin. The lysozyme was prepared from egg-white by the method of Fevold & Alderton (1949) and was recrystallized four times.

To identify the N-terminal residues liberated during the reduction a separate experiment was carried out and the excess of LiBH_4 decomposed with a few drops of glacial acetic acid. The solvent was removed under reduced pressure and the residue dried in a vacuum desiccator over KOH. The residue was treated with 10% (w/v) NaHCO_3 (10 ml.), ethanol (10 ml.) and fluorodinitrobenzene (0.1 ml.), and the mixture vigorously stirred for 4 hr. Excess of 6N-HCl was then added and the soln. evaporated almost to dryness. The sludge of DNP-peptides was hydrolysed by refluxing 16 hr. with 6N-HCl and the DNP-amino acids were isolated in the usual way. Dinitrophenol was removed from the DNP-amino acid mixture by the method of Isherwood & Cruickshank (1952). Chromatography of the mixture was carried out on paper buffered at pH 6, using *tert.*-amyl alcohol as solvent (Blackburn & Lowther, 1951). Six spots of about equal intensity were obtained. These had R_f values identical with those of the DNP derivatives of the following groups of amino acids: Gly and/or Ser, Asp and/or Glu and/or Cys_2 , Ala and/or Met, Thr and/or Arg, Val, Leu.

Attempts to use LiBH_4 in aqueous solution

It was found that gradual addition of a solution of LiBH_4 in THF to water produced at first a vigorous effervescence and the pH of the solution rose rapidly to 9.5. Decomposition of the reagent then took place much more slowly.

Methyl hippurate (19.3 mg.; 0.1 m-mole) was dissolved in a mixture of methanol (1 ml.) and water (19 ml.) and to it was added a 0.1 M soln. of LiBH_4 in THF (2 ml.) and the mixture allowed to stand at room temp. for 3 hr. An additional portion of 2 ml. of reagent was then added, followed by two more additions of 2 ml. after 19 and 26 hr. from the beginning of the reaction. After 28 hr. had elapsed the product was hydrolysed and ethanolamine estimated as the DNP derivative as previously described. The yield was 42% of the theoretical. No improvement in the yield was obtained in the presence of cobalt sulphate, and in the presence of boric acid it was considerably decreased. These reagents are known to catalyse the decomposition of LiBH_4 in aqueous solution (Schlesinger, 1944) and it was hoped that they might also catalyse the reduction.

Lysozyme was esterified by two different procedures: first, by the action of diazomethane as described above for the formyl peptides from silk fibroin, and secondly by methanolic hydrogen chloride as described by Fraenkel-Conrat & Olcott (1945). The products were treated in aqueous methanol with LiBH_4 in a manner similar to that used for methyl hippurate, but in neither case was an appreciable quantity of DNP-leucinol obtained from the hydrolysates.

DISCUSSION

Some of the DNP-amino alcohols described in the experimental section were not obtained crystalline

but the *R* values of these products agreed well with expectations. It was not possible to achieve separation of DNP-leucinol from DNP-isoleucinol; the separations of DNP-aspartidol from DNP-glutamidiol and of DNP-phenylalaninol from DNP-*O*-methyltyrosinol were not satisfactory. The last compound would probably be produced from a tyrosine end-group if diazomethane were used as an esterifying agent. Under the conditions used for isolation from hydrolysates lysinol appeared to give a mixture of the singly and doubly substituted DNP derivatives. The clarification of these points was not pursued in view of the unsatisfactory results obtained with lithium borohydride.

It is now known that two different side reactions can occur at the peptide bond during reduction of peptides or their esters by mixed metallohydrides:

Reaction (1) was observed by Karrer & Nicolaus (1952) in reductions of peptides by lithium alu-

minium hydride under vigorous conditions. The results of the present work show that it can take place even under relatively mild conditions. It is likely to occur in amides generally (Gaylord, 1954). Reaction (2) has been observed in amides when treated with either lithium borohydride or lithium aluminium hydride (Wittig & Hornberger, 1952; Snyder & Putnam, 1954) and it appears that the nature of the substituents attached to the amide nitrogen atom determines the ease with which fission occurs. In some cases fission can be brought about under very mild conditions and the reaction can be used for the preparation of aldehydes (Wittig & Hornberger, 1952):

This reaction is analogous to scheme (2) above. Presumably the peptide bond is cleaved, yielding an aldehyde, which is then further reduced to a carbinol.

It has been shown that ethanolamine is produced from insulin by the action of lithium aluminium hydride (Fromageot & Jutisz, 1953) and of lithium borohydride (Chibnall & Rees, 1953), although glycine is not a *C*-terminal amino acid in this protein. The latter authors produced evidence to show that this may have been due to acyl migration at the glycylserine sequence, followed by reductive cleavage of the ester group:

In view of the number of different *N*-terminal residues liberated, a specific fission of this nature was unlikely to have occurred in lysozyme. It was found, however, that bonds involving the carboxyl groups of glycine or alanine were more susceptible to reduction than other peptide bonds, but this is probably due to the small size of the side chains.

As a result of this work it appears that, with the technique available at present, lithium borohydride cannot be used for the determination of the *C*-terminal residues of large peptides and proteins. This is due to the vigorous conditions which must be employed on account of the very low solubility of these large molecules in organic solvents. On the other hand, satisfactory results can be obtained on small peptides which are sufficiently soluble to allow reduction to take place at a reasonable rate of low temperature. Recently, Grassmann, Endres & Steber (1954) have suggested that procollagen contains ester bonds because it yields amino alcohols on treatment with lithium borohydride at the boiling point of THF. In a subsequent paper

Grassmann, Hörmann & Endres (1955) described the reduction of insulin and several peptides. Reductive cleavage of peptide bonds in esterified peptides was not observed under the conditions they used, although these were, apparently, more vigorous than the conditions used in the present work. On the other hand, alaninol was obtained from insulin itself in a yield of about 20 % of that obtained from the esterified protein. In view of the small degree of reduction of hippuric acid observed in the present investigation, and of the free peptides studied by Grassmann *et al.* (1955) it seems likely that free carboxyl groups of proteins would be reduced to a very small extent by lithium borohydride. It is therefore possible that part of this alaninol arose by fission of the peptide chain and not from the C-terminal residue of insulin.

SUMMARY

1. Lithium aluminium hydride and lithium borohydride were compared as reducing reagents for the C-terminal residues of benzoylated peptide esters. The latter reagent gave satisfactory results provided that the reaction was carried out at 0°.

2. Attempts to devise a stepwise degradation of peptides by means of the N, O-acyl migration reaction on the reduced peptide derivatives were unsuccessful.

3. A new method for the preparation of the DNP derivatives of amino alcohols is described. This was used for the quantitative isolation of amino alcohols from hydrolysates of proteins and peptides after reduction.

4. The value of lithium borohydride as a reagent for the determination of the C-terminal residues of large peptides and proteins was critically assessed. Owing to the low solubility of these substances in organic solvents, it was necessary to use more vigorous conditions than those required for reduction of simple peptides. Reductive cleavage of peptide bonds then took place simultaneously with the reduction of C-terminal residues. Correct identification of C-terminal residues with this reagent is therefore not possible under the conditions at present available.

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Glycine Uptake by the Proteins of the Rat Pancreatic Juice

BY L. C. U. JUNQUEIRA, G. C. HIRSCH AND H. A. ROTHCHILD

Laboratory for Cell Physiology, Faculdade de Medicina, Universidade de São Paulo, Brasil

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Although extensive research has been done on the morphology and cytophysiology of cell secretion (see reviews by Hirsch, 1931, 1939, 1955) few data exist on the chemical phenomena related to this process.

As a continuation of a series of studies on the biochemistry of cell secretion (Junqueira, 1951; Rabinovitch *et al.* 1952; Fernandes & Junqueira, 1953, 1955) experiments were performed in order to obtain data on the rate at which this process occurs.

The pancreas is a convenient organ to work with, for besides actively secreting proteins its activity can be stimulated or depressed by pharmacological agents. Furthermore, up to the present time no blood proteins have to our knowledge been proved to permeate this gland and to be excreted with pancreatic juice; if this in fact occurred, however, it would complicate the interpretation of the results obtained.

We present here the results obtained by studying the rate of appearance of radioactivity in the rat pancreatic-juice proteins after intravenous injections of radioactive glycine and radioactive plasma proteins. Furthermore, the protein-bound and 'non-protein' radioactivity of blood plasma was studied during the experiments.

MATERIAL AND METHODS

Adult male Wistar rats weighing from 250 to 380 g. (average 306 g.) were used throughout. They were starved 24 hr. previously but water was given *ad lib.* Anaesthesia was performed by injecting intraperitoneally 8.0 mg. of Numal (Roche) per 100 g. body weight.

Radioactivity in pancreatic juice after injection of glycine

In these experiments a total of nine rats were used. Pancreatic juice was collected in 10 μ l. glass pipettes from a polyethylene cannula introduced into the common biliary

and pancreatic duct 2-3 mm. above its entrance into the duodenal wall. To avoid contamination with bile a high ligation of the biliary duct was performed 5 mm. below its emergence from the liver. A continuous flow of pancreatic juice was promoted by intravenous injection into the femoral vein of 0.7 unit of secretin and 1.0 μ g. of carbamoylcholine (cerbachol) dissolved in 0.2 ml. saline per rat. This stimulus was repeated every hour up to the end of the experiment at half of the initial dose.

Approximately 5 min. after the first stimulus 3.3 μ Ci/100 g. body weight of [1-¹⁴C]glycine (specific activity, 32.5 μ Ci/mg., supplied by the Radiochemical Centre, Amersham) were injected at the rate of 1 ml./kg. body weight into the femoral vein.

Radioactivity assays. To assay the radioactivity of the pancreatic juice, 10 μ l. samples of pancreatic juice were periodically collected, mixed with 2 μ l. of 0.1 N-NaOH and spread out evenly on a stainless-steel planchet (3.11 sq.cm.) and immediately dried by heating at 40°. With this technique an average of fifteen samples of pancreatic juice and seven of plasma could be studied in each experiment. Radioactivity determinations were performed on a Tracerlab gas-flow counter. Every sample was counted three times and the counts agreed to within 3%. Samples were corrected for self-absorption by using a standard curve obtained from measurements of radioactive pancreatic-juice proteins.

Protein estimations. The amount of protein present in the samples was assayed by the method described by Lowry, Rosebrough, Farr & Randall (1951), previously checked by Kjeldahl nitrogen determinations. The average amount of protein in the samples was 261.9 μ g., with a range of 25.5-709.4.

Separation of protein-bound and non-protein radioactivity. In order to separate protein-bound radioactivity from radioactivity bound to smaller molecules (non-protein radioactivity) the following procedure was adopted. The planchets were immersed during 10 min. each in absolute ethanol, 0.5% collodion in ethanol-ether (1:1, by vol.), 70% (w/v) ethanol, 30% (w/v) ethanol and distilled water. They were then transferred to 10% (w/v) trichloroacetic acid for 3 hr. at 37° and afterwards immersed in distilled water for 12 hr. with five changes. Preliminary experiments showed that when radioactive glycine is added to non-radio-