# THE AMINOPROPYLATION OF BOVINE SERUM ALBUMIN

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*Summary*

After the reaction of reduced BSA† with BPA at pH 10·6 and subsequent hydrolysis of the protein with 6N HCl, only 40% of the lysine present in the unmodified protein can be accounted for on chromatographic analysis of the hydrolysate; a similar loss is observed of the SAPC into which the original cystine, which has totally disappeared, is presumably initially all converted. In place of these amino acids, a "neutral" ninhydrin-positive peak is observed which accounts approximately quantitatively for the missing lysine. The reaction at pH 8·6 with IPA leads to a quantitative recovery of SAPC with no loss of lysine. In contrast, reaction for the same times at both pH values with BEA leads to quantitative recovery of all amino acids including SAEC. Although the reaction of IPA with thiols at pH 8·6 is very slow compared with that of other alkylating agents, the relative absence of side reactions may be compared with the state of affairs when methyl iodide is used for an equivalent period in the same relative quantities, when there is extensive alteration of lysine, histidine, methionine, and *S*-methylcysteine.

End-group determinations on the products of the action of trypsin on BSA treated with BPA at pH 10·6 lead to the conclusion that most of the lysine residues are already altered in the modified but otherwise intact protein. The unknown observed in the acid hydrolysates is paralleled by substances observed after treating glycyllysine with BPA at pH 10·6 and then hydrolysing the product with 6N HCl. Reaction of BPA with amino acids and peptides at alkaline pH to give what are presumably polyamines appears to be a general phenomenon. Of the products to be expected from the hydrolysis of polyamines involving the ε-amino group of lysine, none so far synthesized shows chromatographic properties identical with the unknown. Attempts to convert residues of lysine *in situ* to those of 2-amino-6-hydroxyhexanoic acid, a possibility as the unknown, have led to some new observations on the action of nitrous acid on proteins.

End-group determinations on the product of action of trypsin on BSA which had been treated with IPA at pH 8·6 show that peptide bonds involving SAPC are apparently treated by the enzyme in the same way as those involving arginine and lysine.

## I. INTRODUCTION

The synthesis of 2-amino-3-(3′-amino-1′-propylthio)propionic acid (*S*-aminopropylcysteine, SAPC) from BPA and cysteine as an analogue for use in metabolic studies of SAEC will be described elsewhere. The apparent readiness of the aminopropylation reaction in triethylamine–water mixtures led to the expectation that it should be applicable to the thiol groups of the cysteine residues in reduced proteins, leading to their replacement by SAPC residues. Since SAEC (Lindley 1956)

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† Throughout this paper BSA = bovine serum albumin, BEA = 2-bromoethylamine, BPA = 3-bromo-1-propylamine, SAEC = *S*-aminoethylcysteine, SAPC = *S*-(3-amino-1-propyl)-cysteine, IPA = 3-iodo-1-propylamine, APBSA = aminopropylated bovine serum albumin, AEBSA = aminoethylated bovine serum albumin.

is a near enough analogue of lysine to act as one of the basic residues (normally only lysine and arginine) after which splitting of the peptide chain by trypsin occurs, it seemed of interest to inquire whether SAPC would also fulfil the specificity requirements of the enzyme. BSA is commonly used as a test protein in this type of study.

A series of test experiments showed that the reaction between thiols and BPA is in fact inordinately slow at 20–30°C in the pH range 8·5–9 commonly used in alkylation studies. Raftery and Cole (1963) have shown that the reaction of 2-bromoethylamine with thiols at this pH is in fact due to its slow conversion into the highly reactive ethyleneimine, the comparative reaction rate of the parent compound with thiols being very small. A comparable reaction path is not available for 3-bromo-1-propylamine.

Addition of an amount of triethylamine equimolar to and in place of the Tris or other buffering agents normally used brought about a reasonable rate of reaction between thiols and BPA at ordinary temperatures. Despite the strictures of Raftery and Cole on protein reactions at elevated pH, this procedure was therefore employed. The occurrence of some side reactions was thus inevitable but had to be accepted if the formation of SAPC, the reaction of primary interest, was to be taken to completion. The side reactions would, in any case, give an interesting clue to processes occurring to a less detectable degree under normal conditions. In the event, the side reactions proved to be rather more interesting than the main reaction.

## II. Materials and Methods

### (*a*) *Chemical Syntheses*

When slightly more than the theoretical amount of sodium iodide in acetone was refluxed with BPA hydrobromide in acetone, 3-iodopropylamine hydriodide could be recovered in nearly quantitative yield from the filtrate. Recrystallized from ethanol, it agreed in description and m.p. (167°C) with Frankel (1897).

The synthesis of certain substances that had to be considered as possibly formed from lysine by aminopropylation and hydrolysis will be described in the next few paragraphs.

The hydrochloride of 2-amino-6-chlorohexanoic acid was prepared, but not characterized, by the method of Servigne and Szarvasi (1954). The crude hydrochloride obtained by following their synthesis was dissolved in a little water and the solution brought to pH 6·5. The precipitate was repeatedly recrystallized from water to give white needles of DL-2-amino-6-chlorohexanoic acid, m.p. 189°C. Calc. for $C_6H_{12}ClNO_2$: C, 43·5; H, 7·3; Cl, 21·4; N, 8·5%. Found: C, 43·5; H, 7·4; Cl, 21·5; N, 8·2%.

Alkaline hydrolysis of 2-amino-6-chlorohexanoic acid was carried out by dissolving 165 mg ($10^{-3}$ mole) in 3 ml of 1N KOH, and allowing the solution to evaporate and stand in an open beaker for 48 hr at room temperature. For "lactonization", the solid residue was redissolved in 1 ml of conc. HCl and the process repeated. The "hydrolysate" was dissolved in water, the solution cautiously adjusted to pH 8 with dilute HCl, and made up to 10 ml as a standard solution. The acid-treated "hydrolysate" was made up directly to 10 ml with water.

Pollack (1959) has claimed the synthesis of 2-aminohex-5-enoic acid, but without giving any physical details of his product. The following synthesis, by a different principle, led to a characterized product. 4-Bromobut-1-ene (14·5 g; prepared by the method of Linstead and Rydon 1934) and diethyl acetamidomalonate (21·7 g) were dissolved in freshly distilled dimethylformamide (100 ml) and sodium methylate (5·4 g; Fluka AG, 98% pure) added. The mixture was kept 72 hr at 30–40°C and the solvent removed *in vacuo*. The residue was taken up in dry ether, inorganic salts removed by filtration, and the ether evaporated. The product eventually solidified to an oily solid, which was dried on a porous plate. Yield of crude diethyl but-3-enylacetamidomalonate, 20·6 g (76%). Recrystallized from ether–light petroleum the white waxy product had m.p. 43°C. Calc. for $C_{13}H_{21}NO_5$: C, 57·6; H, 7·8; N, 5·2; O, 29·5%. Found: C, 57·7; H, 7·9; N, 5·6; O, 29·2%.

The above crude product (19·5 g) was refluxed 6 hr with constant boiling HCl (100 ml). The clear solution was evaporated *in vacuo*, and the residual syrup dissolved in 10 ml of water and brought to pH 6 with triethylamine. Addition of excess ethanol led to the precipitation of 7·2 g of washed and air-dried crude 2-aminohex-5-enoic acid. Working up of the mother liquors gave 1·4 g more; total yield 91%. The amino acid is much more soluble in water (*c.* 0·5 g/ml) than most of its congeners and recrystallization was best effected from aqueous ethanol. Repeated recrystallizations gave shining white needles of a monohydrate, softening at 228°C and melting with decomposition over a 10°C range (heating rate 4°C/min; the m.p. is grossly dependent on heating rate). Calc. for $C_6H_{11}NO_2.H_2O$: C, 48·6; H, 8·8; N, 9·4%. Found: C, 49·0; H, 8·7; N, 9·3%.

In a repetition of the synthesis the crystallization of the diethyl but-3-enylacetamidomalonate was omitted without materially affecting the overall yield, but the product was difficult to purify from contaminating glycine which has very similar solubility and melting properties.

### (*b*) *Modification of Proteins*

The bovine serum albumin (BSA) was a sample of crystalline protein from the Commonwealth Serum Laboratories already used by Thompson and O'Donnell (1962) of this Division for the preparation of carboxymethyl-BSA. Some of the latter material was also supplied by my colleagues and has been used for comparative studies.

BSA (680 mg; $10^{-5}$ mole) was dissolved in 8M urea (50 ml, 0·1M in Tris.HCl, pH 8·6) and mercaptoethanol (0·49 ml; $7\times10^{-3}$ mole) added. The amount of mercaptoethanol used was calculated on the same basis as in Raftery and Cole's experiments (20 moles per mole of potential cysteine residue). After 2 hr, 3-bromo-1-propylamine hydrobromide (15·3 g, $7\times10^{-2}$ mole; Aldrich Chemical Co.) and triethylamine (24 ml, about $17·5\times10^{-2}$ mole) were added. The tightly stoppered flask was kept 16 hr at 28°C, the nitroprusside reaction then being negative; the indicated pH (glass electrode) of the aqueous layer was 10·6. The whole mixture was then poured into a Cellophane dialysis bag and dialysed 24 hr against running tap water. Aminoalkylated BSA separates from solution at this point. It redissolves on bringing the pH to 3·5–4 with HCl and subsequently remains in solution. Dialysis

was then continued 4 days at 1°C against frequent changes of distilled water in a rocking dialyser. The solution was then lyophilized and the dry protein powder allowed to equilibrate in air. Yield 650 mg, nearly quantitative allowing for mechanical losses.

A comparison sample of aminoethylated BSA was prepared identically except for the replacement of the bromopropylamine hydrobromide with an equimolar quantity of 2-bromoethylamine hydrobromide.

The conditions for the reaction between IPA and BSA at pH 8·6 were modified from the above by incorporating Tris buffer according to the directions of Raftery and Cole (1963). To conserve material, the excess of mercaptoethanol over protein thiol was reduced to 10 times and that of alkylating agent over total thiol also to 10 times. Under these conditions with IPA, the nitroprusside reaction became negative after 6–7 hr at 28°C; for methyl iodide this point came at approximately 2 hr (for comparison, it comes after 5–10 min with iodoacetate) but the reaction was continued until 6–7 hr had elapsed. A further comparative sample of AEBSA was also prepared at pH 8·6.

Deamination of BSA was carried out according to the general directions of Philpott and Small (1938). BSA (680 mg, $10^{-5}$ mole) was dissolved in 10 ml of 0·5M sodium acetate buffer, pH 4·0 at 1°C, and sodium nitrite (690 mg) added, bringing the nitrite concentration to 1M. This represents a 15-fold excess of nitrite over lysine but even so, according to Maurer and Heidelberger (1951), the degree of deamination to be expected for ovalbumin would not exceed 50%; for BSA, some 60% of the lysine in fact disappeared (Table 1). After 24 hr at refrigerator temperature, solid "deaminated" BSA was prepared by dialysis and lyophilization as for APBSA. The preparation was bright orange-yellow in colour and in agreement with Maurer and Heidelberger's observations for ovalbumin consisted of "soluble" and "insoluble" fractions; in the dialysate before lyophilization, at pH 5·8, 70% of the material was insoluble. However, no attempt was made to separate these fractions in the preparation of total "deaminated" protein which was used for hydrolysis and analysis.

### (c) *Analytical Procedures*

Hydrolyses of the proteins (24 hr, 6N HCl in a sealed tube at 105°C) and subsequent amino acid analyses (Spinco amino acid analyser) were carried out by Mr. A. S. Inglis. A number of qualitative experiments, in which the comparative chromatographic behaviour of known amino acids and unknown reaction products were to be compared, were carried out on the multicolumn amino acid analyser,* which separates the amino acids in the same sequence as the Spinco machine.

The results of a number of analyses carried out for various purposes are set out in Table 1 so that comparison allows some idea of the general reproducibility of analyses and gives a background against which disappearance or appearance of a given amino acid in a given sample can be assessed. Apart from this the data of Table 1 are not meant to be considered *in toto*. To avoid questions of moisture content and non-protein impurities from incomplete dialysis, the analyses have been

* Paton Industries, Adelaide.

expressed in terms of leucine (the most stable amino acid, and the second most abundant in BSA) = 1000.

The relevant portions of certain elution curves are set out in Figure 1. The chromatographic behaviour and ninhydrin colour factor of SAEC were already known, and those of SAPC were determined by incorporating an authentic sample into an amino acid calibration mixture. It is of interest that the elution peaks of SAEC and SAPC spaced considerably further apart than might have been expected *a priori*.

Tryptic digestion of alkylated BSA was carried out by suspending known weights of protein in 1% ammonium carbonate solution and adding trypsin (Worthington Biochemical Corporation, twice-crystallized, 1% on the weight of protein). The aminoalkyl proteins were initially insoluble and some hours of gentle shaking at 28°C were required before they went into solution. Enzyme action was allowed to continue 24 hr, thymol being added as an antiseptic: even the most refractory of "specific" bonds should have been cleaved by this time. Some "unspecific" bond cleavage would be expected to take place, but it should be approximately the same for BSA and all its derivatives, and relative rather than absolute values were required. At the end of the reaction period, water and ammonium carbonate were removed by lyophilization.

The procedure for converting all terminal amino groups into phenylthiohydantoins according to Fraenkel-Conrat, Harris, and Levy (1955) was applied to the total sample. No attempt was made to extract the final phenylthiohydantoins into organic solvents, the optical density in a suitably diluted aliquot being read against a blank made by carrying an equivalent quantity of undigested protein through the same process. Phenylthiohydantoin concentration was determined by using the value of 16,000 for the mean molecular extinction coefficient of amino acid phenylthiohydantoins at 267·5 mμ.

Tryptic digests of alkylated BSA were also subjected to "fingerprint" analysis, using two-way, high-voltage electrophoresis at pH 3·5 and 6·5. The method and apparatus were those of Thompson and O'Donnell (1962) and the "fingerprint" of carboxymethyl BSA agreed in all essential points with the one that they have published.

Paper electrophoresis of amino acids, peptides, and their derivatives was carried out in the conventional way in the Beckmann version of the Durrum cell, with a potential gradient of approximately 12 V/cm.

## III. Results

### (*a*) *Amino Acid Analyses of BSA Aminoalkylated at pH 10·6*

The amino acid analysis of the acid hydrolysate of APBSA prepared with BPA at pH 10·6 (Table 1), when compared with that of unmodified BSA, shows the disappearance of cystine and its partial replacement by SAPC, and the disappearance of some 60% of the lysine. About 60% of the SAPC expected to be formed from cystine is also not accounted for. A "neutral" unknown, before and partially overlapping valine (Fig. 1), accounts, when the apparent excess amount of valine is added to it, for nearly the whole of the lost lysine.

## TABLE 1

ANALYSIS OF BSA AND CERTAIN MODIFIED SERUM ALBUMINS

All values are in molecular ratios based on leucine = 1000. Tryptophan was not determined and no correction has been applied for destruction of amino acids during hydrolysis, etc. Differences are calculated using the mean of all values for a given amino acid that have not been modified by one of the treatments applied

| Amino Acid | Calc. from Data of Tristram and Smith (1963) | Mean Values from Present Analyses | BSA | | Aminopropyl-BSA [pH 10·6; $Br(CH_2)_3NH_2$] | | Aminopropyl-BSA [pH 8·6; $I(CH_2)_3NH_2$] | | Aminoethyl-BSA [pH 10·6; $Br(CH_2)_2NH_2$] | | Methyl BSA [pH 8·6; $CH_3I$] | | Deaminated BSA | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Found | Diff. from Mean Value | Found | Diff. from Mean Value | Found | Diff. from Mean Value | Found | Diff. from Mean Value | Found | Diff. from Mean Value | Found | Diff. from Mean Value |
| Glycine | 259 | 251 | 235 | −16 | 240 | −11 | 252 | +1 | 266 | +15 | 256 | +5 | 259 | +8 |
| Alanine | 749 | 724 | 733 | +9 | 727 | −3 | 720 | −4 | 722 | −2 | 726 | +2 | 718 | −6 |
| Serine | 430 | 378 | 387 | +9 | 380 | +2 | 364 | −14 | 405 | +27 | 351 | −27 | 379 | +1 |
| Threonine | 523 | 504 | 514 | +10 | 509 | +5 | 496 | −8 | 520 | +16 | 494 | −10 | 494 | −10 |
| Proline | 441 | 469 | 480 | +11 | 470 | +1 | 467 | −2 | 474 | +5 | 449 | −20 | 477 | +8 |
| Valine | 540 | 560 | 548 | −2 | 659 | +99 | 564 | +4 | 550 | −10 | 577 | +17 | 556 | −4 |
| Isoleucine | 213 | 218 | 215 | −3 | 218 | 0 | 219 | +1 | 220 | +2 | 216 | −2 | 218 | 0 |
| Leucine | 1000 | 1000 | 1000 | | 1000 | | 1000 | | 1000 | | 1000 | | 1000 | |
| Phenylalanine | 427 | 427 | 427 | 0 | 450 | +23 | 417 | −10 | 440 | +13 | 418 | −9 | 411 | −16 |
| Tyrosine | 299 | 312 | 312 | 0 | 290 | −22 | 306 | −6 | 314 | +2 | 339 | +27 | 252 | −60 |
| ½ Cystine | 525 | 496 | 478 | −18 | 000 | −496 | 032 | −464 | 000 | −496 | 057 | −439 | 514 | +18 |
| *S*-Methylcysteine | | | | | | | | | | | 222 | +222 | | |
| *S*-Aminoethylcysteine | | | | | | | | | 518 | +518 | | | | |
| *S*-Aminopropylcysteine | | | | | 238 | +238 | 513 | +513 | | | | | | |
| Methionine | 57 | 56 | 55 | −1 | 56 | 0 | 55 | −1 | 63 | +7 | 33 | −23 | 52 | −4 |
| Aspartic acid | 875 | 848 | 864 | +16 | 860 | +12 | 827 | −21 | 870 | +22 | 844 | −4 | 823 | −25 |
| Glutamic acid | 1197 | 1238 | 1263 | +25 | 1263 | +25 | 1212 | −26 | 1235 | −3 | 1207 | −31 | 1246 | +8 |
| Ammonia | 594[1] | | 1502 | | 1581 | | 674 | | 683 | | 866 | | | |
| Arginine | 362 | 361 | 332 | −29 | 355 | −6 | 369 | +8 | 372 | +11 | 360 | −1 | 376 | +15 |
| Histidine | 275 | 251 | 228 | −23 | 236 | −15 | 284 | +33 | 248 | −3 | 189 | −62 | 258 | +7 |
| Lysine | 938 | 946 | 959 | +13 | 377 | −569 | 864 | −82 | 1015 | +69 | 691 | −247 | 383 | −563 |
| "Neutral" unknown | | | | | 433 | +433 | | | | | | | | |
| Basic unknowns 1 | | | | | | | 18[2] | +18 | | | 201[5] | +201 | | |
| 2 | | | | | | | | | | | 43[6] | +43 | | |
| 3 | | | | | | | | | | | 75[6] | +75 | | |
| 4 | | | | | | | | | | | Trace[7] | | | |
| Neutral unknowns 1 | | | | | | | Trace[3] | | | | Trace[8] | | 221[9] | +221 |
| 2 | | | | | | | Trace[4] | | | | Trace[8] | | 72[10] | +72 |
| 3 | | | | | | | | | | | Trace[8] | | 37[11] | +37 |
| 4 | | | | | | | | | | | Trace[8] | | Trace[12] | |
| | | | | | | | | | | | | | Trace[12] | |

[1] Amide-$NH_2$. [2] Before lysine. [3] Before glycine. [4] After cystine. [5] Between lysine and histidine; probably *N*-methyllysine. [6] Before lysine. [7] ? A methylhistidine. [8] Traces after serine, proline, cystine, and pseudo-carboxymethylcysteine. [9] Major unknown before tyrosine, probably 2-amino-6-nitrohexanoic acid. [10] 2-Amino-6-hydroxyhexanoic acid. [11] Peak with shoulder before tyrosine, probably mainly 2-amino-6-chlorohexanoic acid. [12] Two small strongly acid peaks; possibly tyrosine derivatives.

For AEBSA prepared with BEA at pH 10·6 (Table 1), there are no such complications. Cystine has disappeared from the hydrolysate and been replaced by an equivalent amount of SAEC, but within experimental error no other changes are noticeable.

To explain these observations, a working hypothesis was constructed to explain how BPA and BEA could have such very different effects; most of the rest of this paper will be devoted to testing the hypothesis outlined below.

The rate of formation of ethyleneimine from bromoethylamine will rise with pH and it is reasonable to suppose that the reaction mechanism will be the same at pH 10·6 as that established at pH 8·6. Although 3-halogeno-1-propylamines cyclize to

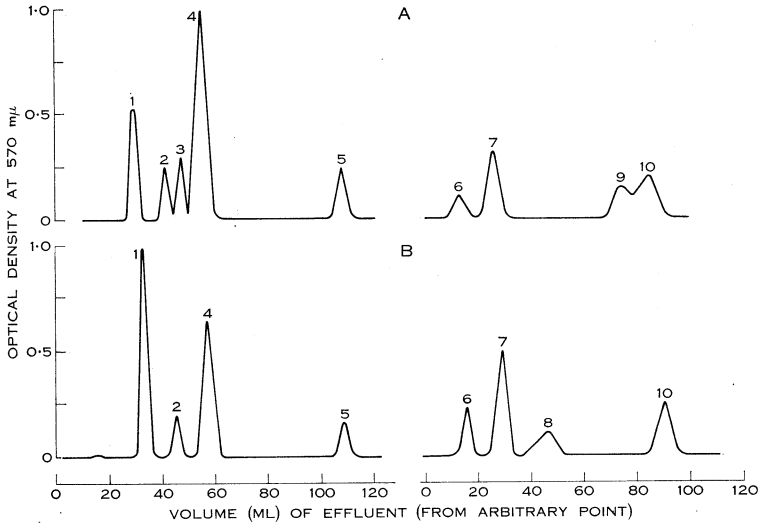


Fig. 1.—Transcriptions from chart of Spinco amino acid analyser. 1–5, basic amino acids; 6–10, glycine to valine. *A*, BSA aminopropylated at pH 10·6. *B*, unmodified BSA; values in region 1–5, 0·59 times the true values in relation to rest of the graph. 1 = lysine, 2 = histidine, 3 = aminopropylcysteine, 4 = ammonia, 5 = arginine, 6 = glycine, 7 = alanine, 8 = cystine, 9 = unknown, 10 = valine.

azetidines in alkaline solution (Gabriel and Lauer 1890), the rate is much slower than for the corresponding 2-haloethylamines (Freundlich and Kroepelin 1926; Kharasch and Fuchs 1945). The principal competing process is linear polymerization; Gibbs and Marvel (1935) have shown that for 3-bromo-1-dimethylaminopropane the ratio of cyclic product and polymer depends on concentration, the linear polymer being favoured except at extreme dilutions. Azetidine, if formed, is not a reactive molecule and, unlike ethyleneimine, will be removed from the reaction process. In addition both bromopropylamine and polymers can react with the free amino groups of terminal amino acids, lysine and aminopropylcysteine. A variety of reaction paths are thus available to a mixture of protein and 3-bromopropylamine in alkaline solution. Under Raftery and Cole's conditions, reaction with all thiol present in the

system still leaves about 65 bromopropylamine molecules present for every (lysine+aminopropylcysteine) residue in BSA.

The results of Table 1 and Figure 1 may therefore be explained by supposing that at pH 10·6 lysine and aminopropylcysteine residues are converted to residues bearing the side chains $CH_2CH_2CH_2CH_2(NHCH_2CH_2CH_2)_nNH_2$ and $CH_2SCH_2CH_2CH_2(NCH_2CH_2CH_2)_nNH_2$. Depolymerization of such polymers by heating in concentrated hydrochloric acid under conditions similar to those used in hydrolysis is well known (cf. Gibbs and Marvel 1935; Kharasch and Fuchs 1945). There are two possibilities for such a reaction RNHR′ giving RCl(ROH) and $R'NH_2$ or else $RNH_2$ and RCl. For lysine and aminopropylcysteine the possibilities are apparently such that only 40–50% of the polymer reverts to the original amino acid, and the rest is transformed to $HOOC.CH(NH_2)CH_2CH_2CH_2CH_2Cl$ (2-amino-6-chlorohexanoic acid), or $HOOC.CH(NH_2)CH_2SCH_2CH_2CH_2Cl$, or the corresponding hydroxy compounds, or possibly to further transformation products of these species. These compounds would have properties very similar to the "unknown" compound.

One consequence of this hypothesis, that the free terminal amino group in BSA would be altered, at least in part, is beyond the experimental accuracy of present procedures to test. Peters, Logan, and Stanford (1958) have shown that the *N*-terminal amino acid of BSA is aspartic acid. Partial or total elimination of one aspartic acid residue in 55 would not materially alter the data of Table 1.

### (*b*) *Amino Acid Analyses of BSA Alkylated at pH 8·6*

The rate of reaction of halides, RX, with thiols decreases sharply in the order $(X =)I > Br > Cl$, and it seemed possible that the replacement of BPA by IPA might lead to a measurable increase in the reaction rate. At the same time, since both lysine residues and XPA molecules will be present mainly in uncharged forms at pH 10·6, and in the mutually repulsive charged forms at pH 8·6, the rate of alkylation of lysine might show at worst no great increase, and at best a sharp decrease.

The data of Table 1 show that these expectations are amply confirmed; BSA has been converted into an APBSA which on hydrolysis shows a quantitative replacement of cystine by SAPC. There is an apparent loss of lysine (8·5%), but it is difficult to know what significance is to be attached to it. Inspection of the analysis of AEBSA shows that in this case there was an apparent *gain* in lysine of the same order (7%); analytical variation about the mean is high for this particular amino acid, and the loss observed in APBSA is perhaps not outside the expected range of experimental error. The appearance of a small unknown "basic" peak in the position to be anticipated for ε-aminopropyllysine is evidence that some alkylation may have taken place but the absence of detectable amounts of the neutral unknown or of increase in apparent valine shows that the gross degree of transformation encountered at pH 8·6 has not taken place. Later calculations (Tables 2 and 3) give consistent answers when based on the assumption that there has been no significant loss of lysine in APBSA prepared at pH 8·6, but a small percentage decrease in lysine would not in fact materially affect the calculations. The specimen of AEBSA prepared at

pH 8·6 was not subjected to amino acid analysis, since the data of Section II(*a*) and of Raftery and Cole (1963) lead to no expectation of amino acid modifications.

The results for methylated BSA (Table 1) show that the amino acid residues of BSA have not lost their capacity to be alkylated as such under the conditions used for alkylation with IPA; lysine, histidine, methionine, and *S*-methylcysteine have all decreased in amount and a number of unknown peaks of which only a few are tentatively identified, have appeared. The rate of alkylation by methyl iodide is of the same order as that observed by Yankeelov and Koshland (1965) for iodoacetamide under similar conditions; the side reactions are similar. Although the neutral $CH_3I$ and the positively charged $IPAH^+$ obviously react with groups other than thiols at very different rates, nothing other than a general explanation can be offered in the absence of detailed kinetic information.

TABLE 2

BASIC GROUPS PRESENT IN $10^5$ G OF VARIOUS SUBSTITUTED DERIVATIVES OF BSA

The numbers have been calculated assuming a basic molecular weight of 68,000 for BSA which is increased by an amount corresponding to the total substitution of all the thiol groups in reduced BSA by the alkylating agent

| Substituent | Arginine | Lysine | SAEC | SAPC | Total |
|---|---|---|---|---|---|
| Carboxymethyl | 33 | 85 | | | 118 |
| Aminoethyl | 33 | 86 | 50 | | 169 |
| Aminopropyl | | | | | |
| Theoretical | 33 | 85 | | 49 | 167 |
| From analytical data of Table 1; pH 10·6 reaction | 33 | 32 | | 23 | 88 |
| Methyl | | | | | |
| Theoretical | 34 | 87 | | | 121 |
| From analytical data of Table 1 | 34 | 64 | | | 98 |

### (*c*) *Trypsin Action and End-group Determination*

The analytical figures of Tristram and Smith (1963) plus the presence of approximately one cysteine residue per molecule of BSA (Leach 1960) lead to the following number of expected residues per $10^5$ g: lysine, 87·9; arginine, 33·9; ½cystine+cysteine, 50·7. If these values are rounded off and allowance made for the increase in molecular weight on substitution, the expected number of basic residues in variously substituted BSA may be calculated. The results are set out in Table 2.

The data of Table 2 may also be taken as giving the number of bonds susceptible to trypsin cleavage per $10^5$ g, provided that the bond subsequent to an SAPC residue may be assumed to be one of these. However, if the structure envisaged for APBSA prepared at pH 10·6 is true and all available $-NH_2$ groups are considered to be polysubstituted, then the number of trypsin-susceptible bonds falls to 33 (the number of arginine residues) per $10^5$ g, since residues of the type $RNHCH_2CH_2NH.....NH_2$ will not be expected to fit the narrow specificity of trypsin.

The determination of the exact number of end-groups liberated per molecule of protein by trypsin requires the concurrent elucidation of the purity of the protein. The degree to which "specific" and "unspecific" bonds are split must be determined by identifying the actual end-groups liberated as well as the rate of liberation of these end-groups as phenylthiohydantoins and their contribution to the total optical density. The data of Table 3 have been organized in a way that circumvents these difficulties. The effect of all these factors on carboxymethyl BSA and AEBSA (the sample prepared at pH 8·6 was used) should be nearly the same. A factor can be calculated representing the ratio of the number of end-groups per $10^5$ g protein found by the phenylthiohydantoin method to the number of appropriate basic residues present.

TABLE 3

NUMBER OF AMINO END GROUPS RELEASED, ESTIMATED BY THE PHENYL THIOHYDANTOIN METHOD, FROM $10^5$ G OF ALKYLATED BSA THROUGH TRYPTIC DIGESTION

| Substituent | Estimated End Groups/$10^5$ g | Basic Residues/$10^5$ g | Ratio |
|---|---|---|---|
| | *Experiment* 1 | | |
| Carboxymethyl | 49 | 118 | 0·414 |
| Aminoethyl | 74 | 169 | 0·438 |
| Aminopropyl (pH 10·6 reaction) | 22·6 | 53* | 0·426* |
| | *Experiment* 2 | | |
| Carboxymethyl | 67 | 118 | 0·568 |
| Aminoethyl | 90 | 169 | 0·533 |
| Aminopropyl (pH 8·6 reaction) | 95 | 167 | 0·569 |
| Methyl | 18·2 | 32·5* | 0·557* |

* Basic residues calculated from the mean value of the ratio derived from the other alkylated BSA's, rather than vice versa.

If this is identical for the two substituted BSA's of known structure, then it can be applied to other substituted BSA's of unknown structure to determine approximately the true number of "sensitive" basic residues present. In two different experiments the value of the ratio was in fact the same within experimental error for both carboxymethyl BSA and AEBSA (Table 3).

Comparison of Tables 2 and 3 demonstrates that the estimated number of end-groups (53) liberated per $10^5$ g in APBSA prepared at pH 10·6 is only 20 per $10^5$ g more than the number of arginine residues present (33 per $10^5$ g). Not only can nothing be said about whether SAPC constitutes a "sensitive" basic residue fulfilling the specificity requirements of trypsin, but is also obvious that most of the lysine residues (85 per $10^5$ g) are no longer effective. On the other hand, the ratio (0·569) between the total estimated number of end-groups liberated per $10^5$ g from BSA aminopropylated at pH 8·6 (95) and the total sum of arginine, lysine, and SAPC residues ($33+85+49 = 167$) per $10^5$ g equals within experimental error those for carboxymethylated ($67/118 = 0·568$) and aminoethylated ($90/169 = 0·533$) BSA.

Not only must the lysine residues be unblocked, *but SAPC must constitute a "sensitive" basic residue.*

The result for methylated BSA shows that there are many fewer liberated end-groups than the total of arginine+residual lysine residues. As a purely *ad hoc* explanation, it may be suggested that in this case that charged sulphonium ion side-chains derived from *S*-methylcysteine and methionine have considerably affected the susceptibility of the protein chain to enzyme attack.

### (*d*) *Fingerprinting*

When the "fingerprints" of the tryptic digests of carboxymethyl BSA and AEBSA, prepared by two-way high-voltage electrophoresis at pH 3·5 and 6·5, were compared it was obvious that they agreed nowhere except for a few spots attributable to such "basic" entities as free lysine and arginine. The observed differences are exactly what might have been predicted *a priori;* both fingerprints consist of a large number of well-resolved spots, tending to concentrate in the "basic" region of the AEBSA fingerprint and the "acidic" region of the carboxymethyl BSA fingerprint. The former in fact has both an absolutely greater number of spots and a virtual absence of spots in the "acidic" region so that the spots tend to overlap and smear in the "basic" region. In both fingerprints there were too many spots for a meaningful census to be taken.

The data of Table 3 suggest that the tryptic digest of APBSA (pH 10·6) would give a much reduced number of larger (on the average) peptides and that the average charge on these peptides would be about the same as for AEBSA. The tryptic digest of APBSA (pH 8·6) should give a fingerprint almost indistinguishable from that of AEBSA. The latter expectation was fully realized and the fingerprints were identical on casual inspection. The fingerprint from APBSA (pH 10·6) consisted of a few well-resolved "acid" and "ultra-basic" spots and an unresolved smear covering the same area as the basic peptides from AEBSA. Table 3 suggests that not all the relevant basic groups are ineffective in this APBSA; the position of these groups will almost certainly differ from molecule to molecule of BSA in a completely random way. This would lead to a very large number of peptides of very similar properties, a supposition in accordance with the actual appearance of the fingerprints.

### (*e*) *Aminopropylation of a Lysine Peptide*

Attempts to repeat the transformation of lysine by aminopropylation in a simpler system than intact BSA are not likely to give interpretable results with the free amino acid. The α-amino group will be at least as susceptible to substitution as the ε-amino group, and a mixture of products will result. The simplest analogue at the next level of complexity, with blocked α-amino group, is the dipeptide glycyllysine.

Since small molecules cannot be removed by dialysis when the reaction is complete, as in the case of BSA, a practical limit was set to the amount of BPA that could be present. Amino acid or peptide (0·001 mole) and BPA (0·002 mole) were dissolved in water (2 ml) and triethylamine (0·5 ml) added; the final pH was 10·6–10·8. After 24 hr at 28°C, a sample was transferred to an electrophoresis strip, and the

remainder lyophilized. Constant boiling HCl (2 ml) was now added and the solution refluxed 24 hr. Renewed lyophilization and dissolution of the residue in water (2 ml) gave a new specimen for electrophoresis.

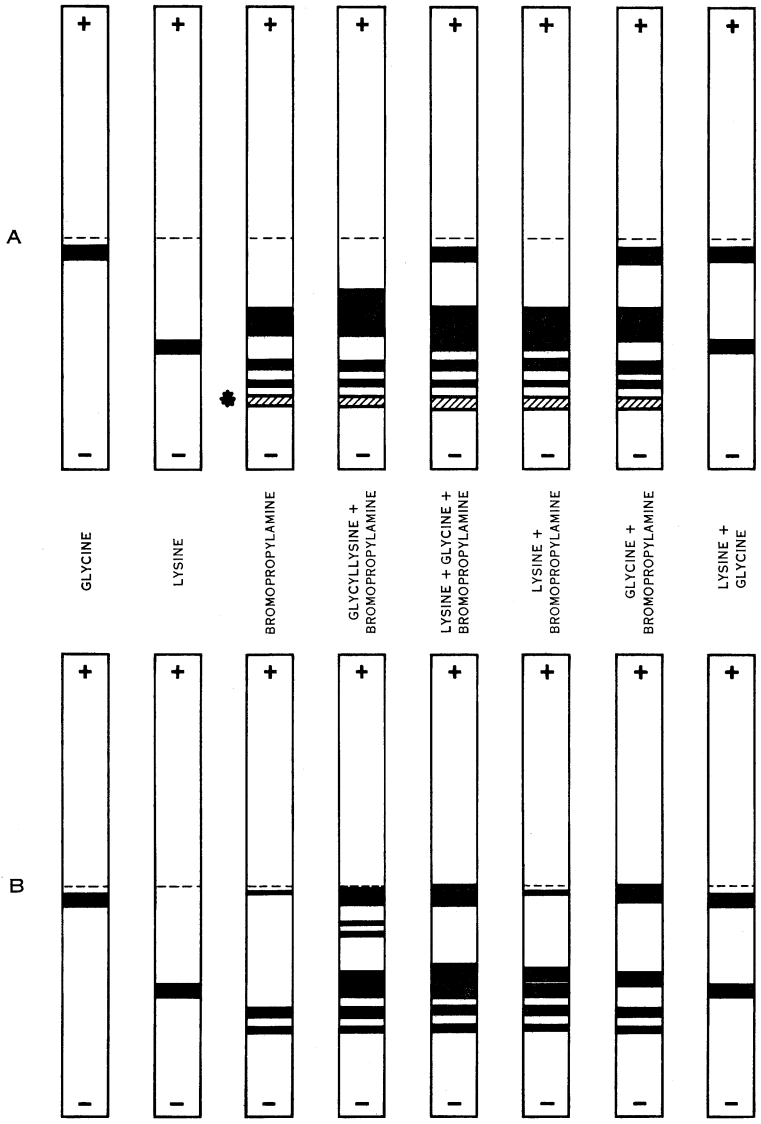


Fig. 2.—Electrophoresis on 30 by 3 cm Whatman No. 1 filter-paper strips in McIlvaine buffer, pH 5·0, diluted 1 in 10, for 1 hr using a gradient of 14 V/cm. Strips dried at 100°C, dipped in an 0·25% solution of ninhydrin in acetone, and heated. Series *A*, 24 hr in aqueous triethylamine; series *B*, after removal of triethylamine and refluxing for 24 hr in 6N HCl. * Yellow band corresponding in position to an authentic sample of azetidine.

The results of these experiments are given in Figure 2. It is clear that both glycine and lysine are alkylated, and that basic products of this alkylation, not

identical with the original amino acids nor the breakdown products of BPA, survive hydrolysis conditions. Glycyllysine also is alkylated; but in this case there are other hydrolysis products than the basic ones. Two bands appear between the "glycine" and "basic" positions; on the evidence these must be transformation products of the $\epsilon$-alkylated lysine; products from the alkylation of the glycyl moiety should be indistinguishable from those from alkylated glycine. The general behaviour of the substances producing these bands is not quite that of the unknown from BSA, which as a "neutral" should be nearly indistinguishable from glycine under the electrophoretic conditions used. The original degree of polysubstitution of the lysine residues may be expected to be much greater in the experiments with BSA in view of the much greater excess of BPA used. Very heavy application to the paper of the hydrolysis product of the reaction mixture of lysine alone and BPA sometimes showed faint traces of these bands. This is what might be expected if some small amounts of lysine substituted on the $\epsilon$-amino group only are formed by the reaction.

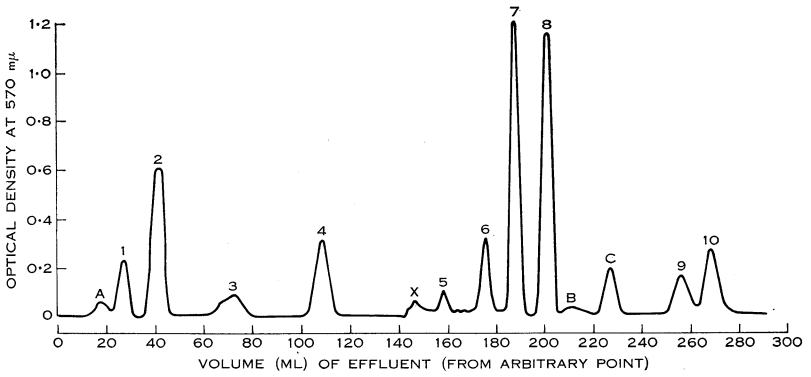


Fig. 3.—Transcription from chart of Spinco amino acid analyser of analysis of hydrolysate of "deaminated" BSA. 1 = glycine, 2 = alanine, 3 = cystine, 4 = valine, 5 = methionine, 6 = isoleucine, 7 = leucine, 8 = norleucine (standard), 9 = tyrosine, 10 = phenylalanine. *A* = 2-amino-6-hydroxyhexanoic acid, *B* = ? 2-amino-6-chlorohexanoic acid, *C* = 2-amino-6-nitrohexanoic acid. *X* is a false peak caused by buffer change.

### (*f*) *Reaction between Nitrous Acid and BSA*

Gaudry (1948) has synthesized 2-amino-6-hydroxyhexanoic acid and Gingras, Pagé, and Gaudry (1947) have shown it to be identical with the "anaemia factor" demonstrated in "deaminated" casein by Hogan, Powell, and Guerrant (1941). This compound was an obvious possibility for the unknown transformation product of lysine, and it seemed possible to avoid Gaudry's tedious synthesis by "deaminating" BSA with nitrous acid, hydrolysing the deaminated protein, and picking up the position of the new neutral ninhydrin-positive substance.

In the event, three unknown ninhydrin-positive substances were found to be present in the hydrolysate (Fig. 3). A second reading of the literature showed that only minute amounts of 2-amino-6-hydroxyhexanoic acid had in fact ever been

isolated from "deaminated" proteins, and that its textbook identification as the only or even the major product of the reaction between the $\epsilon$-amino group of lysine residues and nitrous acid rests only on analogy. Pagé, Gaudry, and Gingras (1948) quote a private communication from Dent who showed that their 2-amino-6-hydroxyhexanoic acid was chromatographically identical with a substance that he had isolated in "minute amount" from deaminated casein. Of the three unknown peaks, peak A may confidently be identified as 2-amino-6-hydroxyhexanoic acid; besides being produced from the deamination of lysine, it is the major product of the alkaline hydrolysis of 2-amino-6-chlorohexanoic acid (Fig. 4) and stands chromatographically in the same relation to norleucine that serine does to alanine.

In identifying peaks B and C, the mechanism of the deamination reaction must be considered. From $RCH_2CH_2NH_2$ the reactive intermediate is $R–CH_2CH_2^+$ which may lose a proton to give the olefin $RCH=CH_2$, or react with various species $X^-$ to give $RCH_2CH_2X$ [Austin (1961) summarizes the available reaction mechanisms]. Under the reaction conditions $X^-$ might be $CH_3COO^-$ or $NO_2^-$, but the 6-acetic or nitrous esters would be converted to the 6-hydroxy compound on hydrolysis. Since the chromatographic position of peak C does not correspond with 2-aminohex-5-enoic acid, and branched-chain hexenoic acids will come off the column even more rapidly (cf. norleucine and leucine), the most likely candidate for this unknown, which accounts for most of the lost lysine, is therefore 2-amino-6-nitrohexanoic acid. Identification of the minor peak B has not been attempted. It coincides very nearly in position with 2-amino-6-chlorohexanoic acid and may easily have arisen from chloride ion contaminating the protein (microanalysis showed about 0·15% chloride in the sample).

When a solution of 2-amino-6-chlorohexanoic acid (0·001 mole) and sodium nitrite (0·0015 mole) in water (3 ml) is boiled under reflux (15 min), examination of the product by paper chromatography shows that 2-amino-6-chlorohexanoic acid has been completely replaced by a new ninhydrin-positive substance.

However, amino acid analysis showed two components (Fig. 4) of which one was in the position of component C and one in that of phenylalanine. The two components were present in roughly equal amounts, a state of affairs that might be predicted from the results of Takayama *et al.* (1961) for the distribution of the product between nitroparaffin and nitrous ester in this type of reaction. Prolonged boiling destroys the pseudo-phenylalanine component and replaces it with one in the expected position for 2-amino-6-hydroxyhexanoic acid (Fig. 4); it may therefore be identified with the nitrite ester. The acid-stable component corresponding in position to component C is then tentatively identified as 2-amino-6-nitrohexanoic acid. Positive chemical identification of this component must await the unambiguous synthesis, not so far successfully accomplished, of a pure sample of this acid, and the demonstration of the appearance of a single peak in the component C position when some of it is added to the hydrolysate of "deaminated" BSA. We may, however, tentatively conclude that the main conversion product of the lysine residues of BSA in acetate buffer at pH 4·1 and in the presence of excess nitrite is 2-amino-6-nitrohexanoic acid. If this is so its instability towards alkali (Fig. 4) may have prevented its recognition in the past.

It is apparent (Table 1) that the only other amino acid showing a significant decrease in amount under the reaction conditions is tyrosine; cystine and arginine

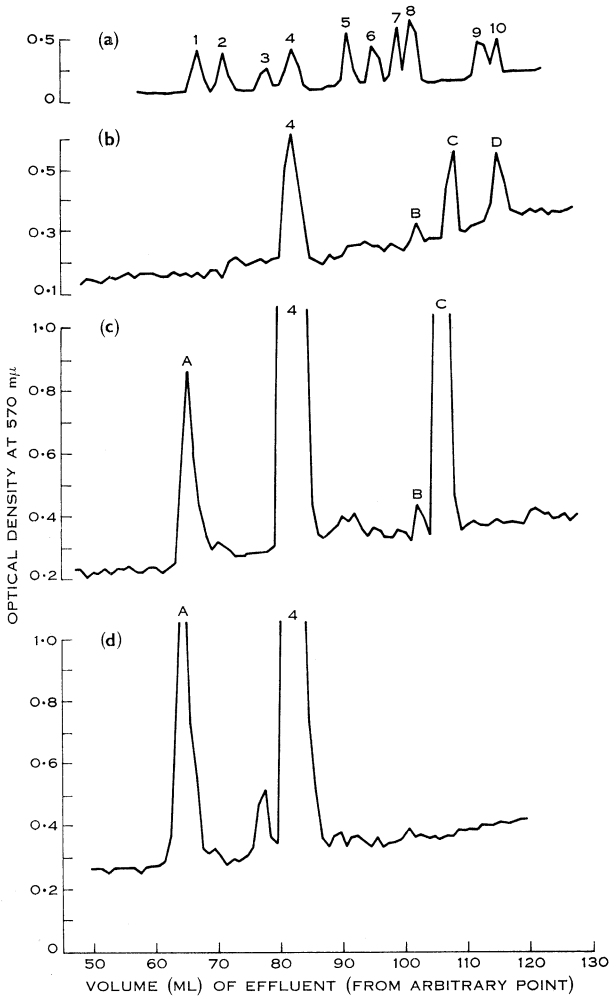


Fig. 4.—Transcriptions from chart of multicolumn analyser. (*a*) Standard amino acid mixture. (*b*) Reaction mixture from 2-amino-6-chlorohexanoic acid and 50% excess of sodium nitrite, boiled 10 min. (*c*) Same as (*b*), but boiled 6 hr. (*d*) Same as (*c*) but left 48 hr at room temperature in 0·1N NaOH. Valine was added as a marker to (*b*), (*c*), and (*d*); all traces except (*a*) were shifted so that the valine peaks correspond exactly. 1 = glycine, 2 = alanine, 3 = cystine, 4 = valine, 5 = methionine, 6 = isoleucine, 7 = leucine, 8 = norleucine, 9 = tyrosine, 10 = phenylalanine. *A*, *B*, *C*, *D* are 2-aminohexanoic acids: *A* = 6-hydroxy, *B* = 6-chloro, *C* = ? 6-nitro, *D* = ? 6-nitrosyloxy-2-aminohexanoic acid.

residues, which have often been thought to be labile to prolonged treatment with nitrous acid, are not significantly affected. In the absence of a survey of the action

of nitrous acid on BSA, using modern methods of amino acid analysis, it is pointless to speculate about these observations. The necessary experiments are being undertaken and will be reported elsewhere.

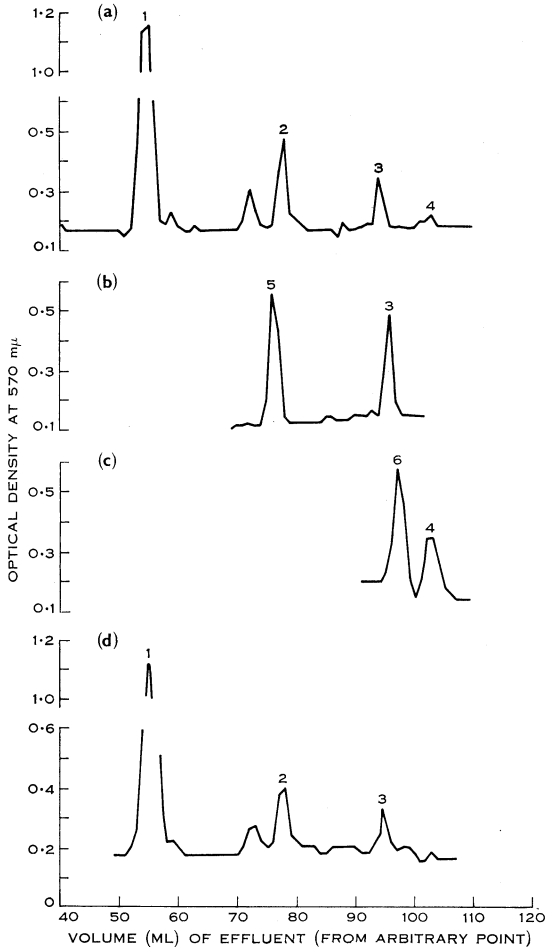


Fig. 5.—Transcriptions from chart of multicolumn analyser. (*a*) Basic hydrolysate of 2-amino-6-chlorohexanoic acid. (*b*) Valine+2-aminohex-5-enoic acid. (*c*) Norleucine+2-amino-6-chlorohexanoic acid. (*d*) "Lactonized" preparation from (*a*). 1= 2-amino-6-hydroxyhexanoic acid, 2 = pipecolic acid, 3 = 2-aminohex-5-enoic acid, 4 = 2-amino-6-chlorohexanoic acid, 5 = valine, 6 = norleucine.

### (*g*) *The Unknown Product from the Hydrolysis of APBSA*

Figure 5 shows the products of the alkaline hydrolysis of 2-amino-6-chlorohexanoic acid; identifications have been made by comparison with known material. Pipecolic, 2-amino-6-hydroxyhexanoic, and 2-aminohex-5-enoic acids are all expected products of this reaction and their chromatographic behaviour excludes them from

consideration as the lysine transformation product from the hydrolysis of APBSA prepared at pH 10·6. Compound X, on the other hand, shows chromatographic behaviour identical with this unknown. A possible structure that assumes their chemical identity and is consistent with the evidence is the ϵ-lactone of 2-amino-6-hydroxyhexanoic acid, the formation of which would be enhanced by the acidic conditions of protein hydrolysis. However, the curve from the "lactonized" hydrolysis product, also set out in Figure 5, shows no change in the relative proportions of any of the major products. Two minor peaks of unknown origin appear to have been suppressed but they are irrelevant to this discussion.

If the two unknowns are considered identical on chromatographic evidence alone, compound X is a neutral compound produced both by acid hydrolysis from (?poly)aminopropylated lysine residues in BSA and by alkaline hydrolysis of 2-amino-6-chlorohexanoic acid. The simplest substances filling this role [2-amino-6-hydroxy- (or chloro-) hexanoic, 2-aminohex-5-enoic, and pipecolic acids] are eliminated by the chromatographic evidence. The next order of complexity than can be envisaged leads to substances such as the ether

$$\begin{array}{l} \diagup\ CH_2CH_2CH_2CH_2CH(NH_2)COOH \\ O \\ \diagdown\ CH_2CH_2CH_2CH_2CH(NH_2)COOH \end{array}$$

the synthesis of which will not be simple. Moreover their chromatographic behaviour is difficult to predict and the chance of disappointment will be high. In these circumstances synthetic procedures do not seem to be very economical. An attempt is therefore being made to separate the unknown products from both hydrolyses in sufficient quantities to demonstrate their identity, or otherwise, as well as for analysis and, if possible, identification. Preliminary results show that compound X can be readily separated on a preparative column from the hydrolysis products of 2-amino-6-chlorohexanoic acid.

## IV. Discussion

The original purpose of this paper, quantitative aminopropylation of the cysteine residues of a reduced protein, and demonstration that the SAPC residues in the modified protein give rise to trypsin-sensitive bonds has been achieved in the APBSA prepared with IPA at pH 8·6. A number of further questions have been raised, however, that cannot be answered within the confines of a single paper. They may be listed here without suggesting answers:

(1) Is the appearance of what appears to be 2-amino-6-nitrohexanoic acid in deaminated BSA prepared by the "mild" procedure of Philpott and Small (1938) a function of the large excess of nitrite present, or is it always formed in greater amounts than 2-amino-6-hydroxyhexanoic acid? Since the attack on arginine and cystine appears to be negligible and the observed partial attack on tyrosine could probably be decreased by changing pH and reagent concentration, can the reaction of nitrous acid with BSA be confined to certain specific lysine residues? The direction of the necessary changes in conditions to achieve this object — higher pH, lower concentrations of total ions, specifically lower nitrite concentration — seems fairly obvious.

(2) The most obvious candidates for the "unknown" ninhydrin-positive substance presumably arising from lysine residues on aminopropylation at pH 10·6 and subsequent acid hydrolysis are not identical with it chromatographically and its nature remains unknown. Until the compound has been identified it is impossible to advance any firm hypotheses capable of test about the nature of its precursors in the aminopropylated protein. The experimental section of this paper has been organized around what is purely a working hypothesis and every link in the chain of reasoning will require confirmation.

(3) In the aminopropylation reaction at pH 10·6, a large amount of the SAPC initially formed was subsequently destroyed. The fate of this material is not clear except that it is quite insufficient in amount to be the source of the unknown, which corresponds closely in amount to the lost lysine. The latter has therefore been taken as its source. The transformation product of SAPC may not be ninhydrin-positive; alternatively, the assumed value for the colour factor of the unknown may be in error and the transformation products of SAPC and lysine may coincide chromatographically. It does not seem possible at this stage to decide between these alternatives without resorting to tracer experiments using labelled BSA.

(4) If there is any reaction between IPA and lysine residues at pH 8·6, it must be quite small. It is in fact confined to a few residues, it may be expected that the very different chemical reaction mechanisms of IPA and BEA will lead to very different findings as to which are the "reactive" lysine residues in any given protein. The attractive possibilities that accrue from this hypothesis deserve further investigations.

## V. Acknowledgment

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