enrichment of gelatin with both tyrosine and glutamic acid yielded an antigen that elicited more antibodies than a polytyrosyl gelatin with a similar tyrosine content. The antibodies to pGluTyrGel are specific. This is in agreement with the common assumption that the polar groups strongly influence the antigenic specificity of proteins. The only substance that interacted with, and even precipitated, these antibodies was a copolymer of glutamic acid and tyrosine, thus indicating that all the antigenic specificity lies in the peptide chains attached to gelatin.

Electrophoretic experiments have shown that the antigen which is not precipitated in the equivalence zone is bound to antibodies in the form of a soluble complex. This proves the existence of both precipitating and non-precipitable antibodies in the antisera to gelatin and its various derivatives.

It appears from this investigation that the attachment of a very limited amount of tyrosine residues enhances dramatically the antigenicity of gelatin, without essentially changing its antigenic specificity. This may serve indirectly as an additional proof of the antigenicity of unmodified gelatin. On the other hand, an increase in the amount of tyrosine, or tyrosine and glutamic acid, bound to gelatin results in changes not only in the extent of antigenicity but also in the serological specificity of the gelatin derivatives obtained. The question arises in these cases whether gelatin still contributes in any way towards the immunological properties of the new antigens. An answer to this question may come from a study of the antigenic properties of synthetic molecules composed of peptides or copolymers, but containing no protein moiety.

SUMMARY

1. Three polytyrosyl gelatins differing in their tyrosine content, as well as a gelatin enriched with both tyrosine and glutamic acid, were synthesized and tested for their antigenicity in rabbits. All antisera gave positive precipitin tests.

2. The extent of the antigenicity of the gelatin derivatives investigated depends on the amount of tyrosine attached. As little as 2% of tyrosine suffices to enhance strongly the antigenicity of gelatin.

3. The serological specificity of the gelatin derivatives changes strongly as a function of their tyrosine content. The specificity of compounds rich in tyrosine resides almost exclusively in the peptide chains attached, whereas it resides essentially only in the gelatin moiety in the derivative containing only 2% of tyrosine residues.

4. The attachment to gelatin of the polar glutamic acid in addition to tyrosine yielded an antigen of very narrow specificity.

5. Electrophoretic experiments have shown that the antigen which is not precipitated in the equivalence zone is bound to non-precipitable antibodies in the form of a soluble complex.

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Chromogenic Groupings in the Lowry Protein Determination

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Folin & Ciocalteu (1927), extending earlier work by Wu (1922), showed that a complex reagent containing molybdate, tungstate and phosphoric acid would react with proteins to yield a blue colour approximately proportional to the tyrosine and tryptophan content. Pressman (1943) defined some of the variables influencing colour yield. Herriott (1941) discovered that pretreatment with cupric ion greatly intensified the blue colour given by proteins and peptides, but had no effect upon the colour yield from tyrosine. Lowry, Rosebrough, Farr & Randall (1951) further refined and systematized these observations into a practical method of protein assay which, because of its

simplicity and sensitivity, has been widely adopted. Yet the molecular basis of the colour production has remained fairly obscure.

Our purpose was to investigate the nature of the chromogenic groups in proteins, and the mechanism whereby they reduce the phenol reagent in the presence of copper. It was found that whereas any peptide bond yields some colour, certain amino acid sequences are far more chromogenic than others, and these largely account for the colour yield of a protein. A substance presumed to be an oxidized product was obtained in small yield after reaction of histidylhistidine with the Lowry reagent; some of its properties and behaviour suggested a plausible mechanism of copper-catalysed electron transfer from dipeptide to phenol reagent. Finally, some of the conclusions derived from studies with simple peptides were shown to be valid for the B chain of insulin and its peptic-hydrolysis products.

EXPERIMENTAL

Colour reaction. This was performed as described by Lowry *et al.* (1951). Each lot of Folin–Ciocalteu phenol reagent (Braun–Knecht–Heimann, San Francisco) was tested as described by Oyama & Eagle (1956) to ascertain the optimum dilution. A barely visible amount of phenol red was added to each sample, which was then neutralized before the addition of reagents. To 1 ml. of sample was then added 5 ml. of Lowry's alkaline copper solution (in some experiments, as noted, copper was omitted). After 5–10 min. 0·5 ml. of properly diluted phenol reagent was jetted in. The blue colour was read 30 min. later in the Klett–Summerson colorimeter with red (no. 66) filter. A Klett unit (K.u.) is the amount of chromogenic material in 6·5 ml. of final reaction mixture which gives a Klett reading of unity. Data presented here have been corrected for reagent blanks (usual Klett reading 15–20). The term 'colour increment' is used to denote colour yielded by peptides and proteins in excess of that attributable to their component amino acids.

General procedures. Peptides and proteins were hydrolysed completely in 6N-HCl (sealed tubes) at 100° for 20 hr. and recovered by drying *in vacuo* over P_2O_5 and NaOH. A ninhydrin spray was used to locate amino acids or peptides on paper after chromatography or electrophoresis; narrow strips were sprayed and used as guides for elution of bands. The Pauly reaction was used to follow the imidazole ring of histidine on paper (Brown, Sanger & Kitai, 1955) or in solution (Macpherson, 1942). L-Cysteinylglycine and L-cysteinyl-L-valine were prepared through the benzyloxycarbonyl intermediates, as described by du Vigneaud & Miller (1952); identities were established by paper chromatography of the compounds and their hydrolysis products.

Paper electrophoresis was carried out in the Spinco–Durrum apparatus on strips of Whatman 3MM paper or on 31 cm. squares of such paper when large amounts of material were to be separated. The runs were at 13 v/cm. in collidine–acetic acid buffer (Lockhart & Abraham, 1954); to 9 ml. of γ-collidine were added 1 ml. of acetic acid and water to 1 l., pH 7·8 (Beckman model G pH meter). The duration of runs was varied to achieve optimum separation of bands in each case but was usually 2 hr. Bands were eluted with 0·1N-HCl and dried as described above.

For separation and identification of amino acids from hydrolysates, two-dimensional paper chromatograms were done on 22 cm. squares of Whatman 3MM paper, ascending technique, 3·5 hr. Solvents were ethanol–butanol–water–propionic acid (10:10:5:2, by vol.) and butanol–acetone–water–di*cyclo*hexylamine (10:10:5:2, by vol.). Papers were sprayed with 0·25% solution of ninhydrin in acetone containing 7% of acetic acid, and developed for 3 min. at 75°. The characteristic colours and positions of the various amino acids described by Hardy, Holland & Nayler (1955) were confirmed in standard runs.

Isolation of an oxidized reaction product. The Lowry reaction was carried out with 30 mg. of His.His and all reagent volumes were 100 times greater than usual. The resulting 650 ml. of blue solution was poured onto a Dowex-50 (H) column (2·6 cm. × 50 cm.) prepared according to Moore & Stein (1951). The run-through (containing all the blue colour), the water-wash (300 ml.) and an aq. 0·1N-NH_3 soln. eluate (100 ml.) gave no Pauly reaction and were discarded. When the eluting solution was changed to aq. 1N-NH_3 soln., two fractions were obtained: an acidic fraction (20 ml.) and an alkaline fraction (100 ml.). Both fractions gave positive Pauly reactions. Each was dried *in vacuo*, subjected to paper electrophoresis at pH 7·8 and further prepared as described in the text.

Experiments with the B chain of insulin. Crystalline ox insulin was split into its two chains by Sanger's (1949) performic acid procedure. Chain B (50 mg.) was then dissolved in 25 ml. of 0·01N-HCl containing 2 mg. of pepsin (Nutritional Biochemicals Corp.). After 24 hr. at 37° the mixture was heated to 100° for 2 min., an equal volume of 10% trichloroacetic acid was added and, after standing overnight at 5°, the precipitate was removed by centrifuging. The supernatant was extracted four times with equal volumes of anhydrous ether and the digested products were recovered by lyophilization. Ten peptide bands were separated by paper electrophoresis at pH 7·8. After elution, a portion of each was tested for colour yield directly, and also after complete acid hydrolysis. A portion of the hydrolysate was also subjected to two-dimensional paper chromatography for identification of its component amino acids. The amino acid composition and the known points of cleavage by pepsin (Neurath, 1957) allowed us to assign unambiguous structures to nearly every peptide. The colour increment of each peptide was given by the colour yield before and after hydrolysis, but the exact amount of each peptide was not ascertained.

Abbreviations. For definition of the abbreviations used for amino acids in this paper see *Biochem. J.* (1957), **66**, 6.

RESULTS

Molecular configuration and colour yield. Preliminary complete hydrolysis reduced the colour-yielding property of albumin by more than two-thirds, and that of insulin by more than one-half (Table 1). Of the twenty common L-amino acids, only tyrosine, tryptophan, cysteine (and cystine) and, to a lesser extent, histidine gave significant

Table 1. *Effect of complete hydrolysis on colour yield of proteins*

The colour reaction was performed as described in the Experimental section.

		Crystalline* bovine albumin	Insulin†
Amount of protein	μg.	62·3	47·0
	μm-mole	0·904	8·20
Klett reading initially		103	96
K.u./μmole		114 000	11 700
K.u./μg.		1·65	2·04
Klett reading after complete hydrolysis		29	40
K.u./μmole		32 000	4 880
Colour remaining (%)		27	42

* Armour Laboratories, Kankakee, Ill. (mol.wt. taken as 69 000).

† Gift from Eli Lilly and Co., Indianapolis, Indiana (mol.wt. taken as 5733).

Table 2. *Colour yields of amino acids*

The colour reaction was performed as described in the Experimental section.

Amino acid	Amount (μmoles)	Klett reading	K.u./μmole
Tyrosine	0·1	126	1260
Tryptophan	0·1	112	1120
Cystine	1·0	128	128
Cysteine	2·0	122	61
Histidine	10·0	149	15
All others	5·0	<35	<7

colour (Table 2). Amino acid mixtures gave strictly additive colour values. D-Amino acids had the same colour yields as the L-isomers. The expected colour for the amino acids of the albumin hydrolysate, calculated from the data of Moore & Stein (1949), is 31 600 K.u./μmole; that from an insulin hydrolysate, based on the data of Ryle, Sanger, Smith & Kitai (1955), is 5450 K.u./μmole. The experimentally determined hydrolysate colours (Table 1) were in good accord with these expectations. It follows that the component amino acids account for but a fraction of the colour yield of a protein in the Lowry determination.

Data on the colour yields of peptides (Table 3) may be summarized as follows. All dipeptides developed some colour, even when their constituent amino acids did not. The colour increment due to a single peptide bond increased systematically with side-chain length in the series glycine, alanine, valine, leucine. Functional side chains (serine, threonine, methionine, asparagine) caused marked colour increments in both the glycine and alanine series. On the other hand, tyrosine and tryptophan, which yield considerable colour by themselves, underwent suppression of colour yield when linked to glycine or leucine. Cysteine dipeptides gave about the expected colour for cysteine alone. Dipeptides of histidine showed consistent colour increments. Tripeptides containing glycine or leucine gave very high colour increments, but one containing cysteine (glutathione) did not. Glycine tetrapeptide yielded much less colour than the tripeptide but still very much more than the dipeptide.

Table 3. *Colour yields of some peptides*

The colour reaction was performed as described in the Experimental section.

	K.u./μmole	
Peptide*	Found	Calc. from sum of amino acids
Dipeptides with *N*-terminal glycine		
Gly.Gly	14	0
Gly.DL-Ala	22	0
Gly.L-Val	26	0
Gly.DL-Val	28	0
Gly.L-Leu	40	0
Gly.DL-Leu	30	0
Gly.DL-Ser	65	3
Gly.DL-Thr	160	5
Gly.DL-Met	60	2
Gly.L-Asp–NH_2	270	6
Gly.D-Asp–NH_2	276	6
Gly.L-Tyr	990	1260
Gly.L-Try	676	1120
Dipeptides with *C*-terminal glycine		
Gly.Gly	14	0
DL-Ala.Gly	20	0
L-Leu.Gly	26	0
D-Leu.Gly	23	0
DL-Leu.Gly	21	0
L-CySH.Gly†	78	61
Dipeptides with *N*-terminal alanine		
DL-Ala.DL-Ala	40	0
DL-Ala.DL-Val	50	0
DL-Ala.L-Leu	6	0
DL-Ala.DL-Ser	14	3
DL-Ala.DL-Met	78	2
DL-Ala.L-Phe	28	4
DL-Ala.D-Asp–NH_2	329	6
DL-Ala.DL-Asp–NH_2	324	6
Other peptides		
L-CySH.L-Val†	64	61
L-His.Gly‡	138	12
L-His.L-Ala§	200	12
L-His.L-Leu§	256	12
L-His.L-His	760	24
L-Leu.L-Tyr	1090	1260
D-Leu.L-Tyr	960	1260
Gly.Gly	14	0
Gly.Gly.Gly	455	0
Gly.Gly.Gly.Gly	169	0
L-Leu.Gly.Gly	490	0
γ-L-Glu.L-CySH.Gly (glutathione)	76	61

* Unless otherwise noted, peptides were obtained from Nutritional Biochemicals Corp.

† These compounds were prepared in this Laboratory.

‡ Kindly supplied by Dr Emil L. Smith.

§ Kindly supplied by Dr Robert W. Holley.

A limited number of histidine dipeptides was available with blocked terminal groups. The data (Table 4) indicate that blocking the free amino group abolished the high colour increment but left an amount of colour approximately that to be expected from an ordinary peptide bond. Additionally blocking the free carboxyl group had no effect, but we lack information on histidine dipeptides blocked in this position alone. An important special case was that of His.His, the colour of which was reduced by blocking the terminal groups but still greatly exceeded the sum of colour yields from two histidine residues and the peptide bond.

Penicillin may be regarded as a curiously cyclized Cys.Val with a blocked terminal amino group. It yielded considerably more colour than Cys.Val (Table 4). After cleavage of the internal peptide bond by penicillinase (leaving the two amino acids linked through the cysteine S), the colour unexpectedly increased. Since desthiopenicillin gave no colour, the S must be essential to the reaction. Finally, penicillamine ($\beta\beta$-dimethylcysteine) yielded less colour than cysteine alone.

When the colour reaction was carried out in the absence of copper, as in the original Folin method, compounds fell into three categories (Table 5). Category I includes all peptides which yield more colour than their component amino acids and with these peptides copper was indispensable for the colour increment. Cystine, and to a lesser extent histidine, also depended upon copper for the colour they produced. Category II comprises peptides of tyrosine and tryptophan. These gave no colour increments. Tyrosine peptides yielded somewhat less colour than tyrosine alone and the colour was largely unaffected by copper. The only tryptophan peptide examined gave considerably less colour than the free amino acid and still less in the absence of copper. Category III consists of cysteine and its peptides, which gave much less colour in the presence of copper than in its absence.

Finally, the blue reaction product was found to have the same spectrum ($\lambda_{max.}$ 745, $\lambda_{min.}$ 405) for

Table 4. *Colour yields of some peptide derivatives*

The colour reaction was performed as described in the Experimental section. Cbz-, the benzyloxycarbonyl derivative. Enzymic hydrolysis of penicillin: 40 mg. of penicillinase (Nutritional Biochemicals Corp.) was incubated with 20 μmoles of penicillin-G sodium at pH 7·0, at 37° for 1 hr., followed by treatment with cold 5% trichloroacetic acid and colour determination on the supernatant. It was shown in an assay with *Sarcina lutea* that this procedure destroyed more than 99% of the antibacterial potency of the penicillin. Desthiopenicillin-G: this compound was prepared from penicillin-G with Raney nickel catalyst and recrystallized to constant melting point (105°) (Kaczka & Folkers, 1949).

Compound	K.u./μmole
Cbz-L-His.L-Ala*	32
(L-His.L-Ala)	200
Cbz-L-His.Gly ethyl ester†	38
(L-His.Gly)	138
Cbz-L-His.L-Leu methyl ester*	26
(L-His.L-Leu)	256
Cbz-L-His.L-His methyl ester†	208
(L-His.L-His)	760
Cbz-L-His.Gly.Gly ethyl ester†	306
Penicillin-G sodium	412
(L-CySH.L-Val)	64
Penicillinase-treated penicillin	580
Desthiopenicillin	0
Penicillamine HCl	16

* Kindly supplied by Dr Robert W. Holley.
† Kindly supplied by Dr Emil L. Smith.

Table 5. *Influence of copper on colour yield*

The colour reaction was performed as described in the Experimental section, except that special Lowry reagent was prepared without copper.

	Compound	K.u./μmole With Cu	K.u./μmole Without Cu	Difference (%)
I.	Crystalline bovine albumin	134 000	26 600	−80
	L-His.L-His	760	37	−95
	Gly.Gly.Gly	455	0	−100
	Penicillin-G sodium	412	10	−98
	L-Cystine	128	3	−97
	L-Histidine	15	4	−73
II.	L-Leu.L-Tyr	920	970	+5
	Gly.L-Tyr	990	980	−1
	L-Tyrosine	1 320	1 210	−8
	Gly.L-Try	676	368	−46
	L-Tryptophan	1 190	1 030	−13
III.	γ-L-Glu.L-CySH.Gly (glutathione)	62	700	+1 030
	$\beta\beta$-Dimethylcysteine (penicillamine)	16	256	+1 500
	L-Cysteine	61	194	+218

all types of reactant (amino acid, dipeptide, protein, Na_2SO_3), as would be expected if the final common step were reduction of the phenol reagent.

Nature of the oxidized product after reaction of His.His. We sought to isolate an oxidized product of the reaction of His.His with copper and phenol reagent as described under Methods. The principal component of the reaction mixture appeared in the alkaline eluate and was evidently unchanged His.His. It was identified by comparison with authentic His.His in paper electrophoresis and two-dimensional chromatography, by electrophoretic and chromatographic behaviour of its single hydrolysis product and by its ability to yield colour again in the Lowry reaction. The amount of unreacted peptide thus recovered was as great as 95% of the starting material in some experiments. Thus the colour yielded by His.His arises from oxidation of a very small fraction of the peptide initially present.

The only other component of the reaction mixture which gave a positive Pauly reaction for imidazole represented about 1% of the original His.His. This compound was eluted from the Dowex-50 (H) column in the acidic fraction, it gave a negative ninhydrin reaction and its electrophoretic migration was 6·0 cm. toward the anode in 2 hr. at pH 7·8. The Pauly colour of the band was typically pink, in contrast with the muddy brown obtained from an equimolar mixture of copper and His.His. Moreover, a direct H_2S test on the unknown band was negative, although copper in a ratio of 1 mole/imidazole group would have been detected readily. Evidently the unknown compound is not a copper complex. Its early elution from Dowex-50 (H), its negative ninhydrin reaction and its net negative charge at pH 7·8 indicate that the primary amino group has been removed or blocked.

The unknown band was eluted from paper, hydrolysed with 6N-HCl (100°, 20 hr.) and dried and subjected again to electrophoresis under the same conditions. Two bands were now obtained. One was identified as histidine. The other reacted with ninhydrin, but not with the Pauly reagent, and it migrated 8·8 cm. toward the cathode (cf. histidine 1·6 cm., His.His 1·3 cm.). Thus the unknown hydrolysis product appears to lack the imidazole ring, possesses a free amino group and has acquired a stronger net positive charge than histidine at pH 7·8.

Further studies of the presumed oxidized product were not undertaken and it would be premature to suggest any structure. However, the apparent absence of a free amino group in the unknown, and the recovery of intact histidine as a hydrolysis product, suggest that oxidation of His.His in the Lowry reaction may involve the removal of an electron pair from the primary amino group and imidazole ring of the *N*-terminal histidine residue.

Colour yields of peptides derived from insulin. When crystalline ox insulin was oxidized with performic acid to obtain chains A and B, there was a loss of about one-third of the total colour yield. By comparing the colour obtained after complete

Table 6. *Colour yields and colour increments of peptides obtained by peptic hydrolysis of chain B of insulin*

The colour reaction was performed as described in the Experimental section. The 10 bands are from paper electrophoresis of a peptic hydrolysate of insulin chain B.

Band	Total K.u.*	Percentage of total K.u.	Total K.u.* after complete hydrolysis	Percentage of colour remaining	Peptide structure†
1	210	0·6	0	0	Val.Glu
2	3 440	9·9	45	1·3	Val.Glu.Ala
3	1 160	3·3	50	4·3	Val.Glu.Ala.Leu
4	1 000	2·9	155	15·5	Leu.Val.Cy-SO_3H.Gly.Glu.Arg.Gly
5	3 940	11·4	375	9·5	Leu.Val.Cy-SO_3H.Gly.Glu.Arg.Gly.Phe
6	1 920	5·5	370	19·3	Tyr.Leu.Val.Cy-SO_3H.Gly.Glu.Arg.Gly.Phe
7	1 760	5·1	400	22·7	Val.Asp.Glu.His - - - - - - - - Glu.Arg.Gly.Phe‡
8	11 440	33·0	3 665	32·0	Indistinguishable from band 7‡
9	1 840	5·3	360	19·6	Val.Asp.Glu.His.Leu.Cy-SO_3H.Gly.Ser.His.Leu
10	7 950	23·0	4 020	50·4	Tyr.Thr.Pro.Lys.Ala
	34 660	100·0	9 440	27·2	—
K.u./μmole	4 820	—	1 310	—	—

* For material obtained from 7·19 μmoles of insulin B, initial colour yield 4540 K.u./μmole.

† Deduced from amino acid composition, expected points of cleavage and known sequences according to Ryle, Sanger, Smith & Kitai (1955).

‡ Bands 7 and 8 appear to be practically the whole chain less the terminal sequence of band 10. Phe is present in both but which of the three Phe residues are present in each cannot be stated.

hydrolysis of insulin with that found after complete hydrolysis of chains A and B it could be shown that part of the colour loss in the performic acid procedure was due to splitting of disulphide bridges and oxidation of cysteine residues to cysteic acid. Another part of the colour loss was due to partial oxidation of tyrosine residues.

The peptic digestion of chain B caused no further loss of chromogenicity. Paper electrophoresis yielded ten peptide bands whose colour yields and colour increments could be related to their amino acid sequences. The data (Table 6) show that the sum of the colour yields of the peptides was in good agreement with the colour yield of intact chain B. Peptic hydrolysis did not diminish chromogenicity because none of the points of cleavage is in a chromogenic sequence. Splitting at one of the histidine residues might well have caused a considerable colour loss.

The column of the table showing percentage of colour remaining after complete hydrolysis supports the following generalizations: (1) Sequences of the type Val.Glu, Val.Glu.Ala and Val.Glu.Ala.Leu give very large colour increments, although their contribution to the total colour yield of the protein is probably small. (2) Certain other sequences not represented among the synthetic peptides examined earlier are obviously chromogenic. In bands 4, 5 and 6, where the colour increments are considerable, we would suspect the Glu.Arg.Gly. The greater colour increment of band 5 than of band 4 implicates Gly.Phe as chromogenic. (3) The relatively high colour increment of band 9 can be attributed to its two sequences containing histidine. (4) The colour increment of band 10 shows that the sequence Thr.Pro.Lys.Ala must be chromogenic. This was confirmed by partial acid hydrolysis of band 10 (6N-HCl, 37°, 3 days), isolation of the tetrapeptide and determination of its colour yield before and after complete hydrolysis. (5) When tyrosine occurs in a large peptide (as in band 6) it reduces the colour increment (cf. band 5), as expected, but does not make a major contribution to the total colour. In a small peptide, on the other hand (as in band 10), tyrosine accounts for a much greater fraction of the total colour and the colour increment becomes correspondingly much smaller. (6) Finally, the residual colour yield of chain B after complete hydrolysis is accounted for almost entirely by its two tyrosine residues, as indicated by the total colour remaining in bands 6, 7, 8 and 10.

DISCUSSION

Tyrosine, tryptophan, cysteine and, to a very minor extent, histidine are capable of reducing the phenol reagent directly. Copper has little effect upon the colour yields of tyrosine and tryptophan but suppresses the reactivity of cysteine and its peptides, presumably by blockade of the sulphydryl group. Cystine produces colour only in the presence of copper. Any dipeptide yields some colour, provided that copper is present and the terminal amino group is free. The colour is greatly intensified by the presence of a side-chain nitrogen or carboxyl group on at least one of the component amino acids. Tripeptides are highly chromogenic even in the absence of functional side chains.

As to the mechanism of colour production, our observations suggest the following hypothesis, based upon the known capacity of peptides to form copper chelates (Martell & Calvin, 1952, pp. 525–531). In a dipeptide, the copper can be held in a coplanar tridentate chelate involving the free amino nitrogen, the peptide-bond nitrogen and the free carboxyl group. The additional presence of a side-chain nitrogen (as in asparagine or histidine) permits formation of a more stable, quadridentate chelate. In a tripeptide, a quadridentate chelate can be formed even in the absence of a functional side-chain group because the nitrogen atom of the second peptide bond is now available for co-ordination to copper. Chelation may be expected to facilitate electron removal from the peptide, as, for example, in the oxidation of ascorbic acid and the action of copper oxidases (Martell & Calvin, 1952, pp. 387–392).

As demonstrated for His.His, the colour yield does not represent complete oxidation of substrate, but rather the resultant of two simultaneous processes: the copper-catalysed electron removal from substrate, and the rapid destruction of electron acceptor in alkaline solution. Since the phenol reagent has a half-life of only 8 sec. at the alkaline pH of the reaction (Lowry *et al.* 1951), the rate of electron transfer will obviously be critical in determining the total colour produced. Our findings suggest that the tightness of binding of copper, especially the ability to form a quadridentate chelate, determines the rate of electron removal from substrate.

In the specific case of His.His our attempts to determine the structure of an oxidized product were inconclusive, but the partial information obtained is consistent with the reaction mechanism postulated above. Molecular models (Stuart–Briegleb models, Arthur S. LaPine Co., Chicago) demonstrated that copper can be co-ordinated in a quadridentate chelate to the free amino nitrogen, the peptide nitrogen, and a nitrogen atom of each imidazole ring. Evidence has been presented which suggests that an electron is then donated by the primary amino nitrogen, but the source of the second electron is uncertain.

Complete hydrolysis of a protein decreases its chromogenicity by one-half to two-thirds, and the

residual colour is almost wholly accounted for by its tyrosine and tryptophan content. Thus the major part of the colour yielded by proteins must arise from chromogenic amino acid sequences. It had seemed possible, at the outset of this investigation, that the large colour increments of proteins might be ascribed to a rather restricted set of sequences. Had that proved to be the case, the Lowry determination might conceivably have been used specifically to detect such sequences in polypeptides. However, the results with chain B of insulin, as well as those with simple dipeptides, point to the existence of a considerable number of different chromogenic sequences in proteins.

SUMMARY

1. In the Lowry protein determination the phenol reagent is reduced to yield a blue colour. The colour yield of a completely hydrolysed protein is due to its content of tyrosine, tryptophan and cysteine, the only amino acids that react significantly.

2. Preliminary treatment with alkaline copper solution leads to a great increase in the colour yield of proteins. We have shown with simple peptides as well as with chain B of insulin that this large colour increment is largely attributable to sequences of amino acids containing functional side chains. Particularly chromogenic are dipeptides containing a histidine, arginine or glutamic acid residue, the nature of the second residue being relatively unimportant.

3. The behaviour of a compound obtained in small yield and presumed to be the oxidized product of His.His suggests that electron removal from the dipeptide to the phenol reagent involves the amino nitrogen and imidazole ring of the *N*-terminal residue.

4. Efficient copper catalysis of electron transfer from a chromogenic grouping in protein to the phenol reagent is believed to require formation of a coplanar quadridentate chelate. The colour yield depends critically upon the rate of electron transfer since the phenol reagent is rapidly destroyed in the alkaline solution.

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A Titrimetric Method for the Determination of Creatine Phosphokinase

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Creatine phosphokinase is usually assayed by measuring the amount of creatine or creatine phosphate formed in a standard time period or the initial rate of formation of one of these products (Askonas, 1951; Banga, 1943; Chappell & Perry, 1954; Ennor & Rosenberg, 1954; Kuby, Noda & Lardy, 1954; Narayanaswami, 1952; Rosenberg & Ennor, 1955). Oliver (1955) has described a method based on the coupling of adenosine triphosphate formation with reduction of triphosphopyridine