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THE NUTRITIONAL VALUE OF PROTEINS WITH SPECIAL REFERENCE TO:

A) THE AVAILABILITY OF AMINO ACIDS IN MEAT MEALS TC CHICKS, AND

B) THE CHEMICAL CHANGES WITH HEAT-DAMAGE OF PURE PROTEINS

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Margaret Nellie Wilson

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ABSTRACT

Meat meals are highly variable in protein quality and this may be due in part to heat-damage. This possibility was investigated by estimating the available lysine content of meat meals by chick growth assay. In addition, the combined urinary and faecal excretion of dietary amino acids by chicks, fed a meat meal as the sole source of protein, was determined, and by subtraction from the amount consumed, values for the apparent retention of dietary amino acids were obtained.

In a second part of the study, the mechanism of heat-damage to pure proteins was investigated. Since cross-linkages may form during heat-damage of proteins, enzymatic digests were examined for the presence of peptides with enzyme-resistant linkages. Samples of unheated and heat-damaged haemoglobin or globin were digested with either trypsin, or exhaustively, using several proteolytic enzymes.

Chick growth assay for lysine. A chick growth assay for lysine was developed using wheat gluten as the protein source in a semi-purified diet. The meat meals were added to the basal diet either at the expense of starch or by isonitrogenous substitution of wheat gluten.

Estimated potencies based on weight gain varied with the method of meat meal addition to the basal diet. This variation was probably due to an effect on the appetite of the chicks as estimates based on food conversion efficiency did not differ significantly with the method of meat meal inclusion.

The percentage of lysine biologically available in eight meat meals ranged from 61 to 105%, suggesting that some meals had been heat-damaged.

Apparent retention of dietary amino acids. Estimates of the apparent retention of essential amino acids in six meat meals ranged from 79 to 100%. The apparent retention of lysine was generally much higher than

the estimated potencies by chick growth assay. The difference in the two biological estimates indicated that other factors, apart from digestibility, and absorption and urinary excretion of peptides and amino acids, must be responsible for the reduced availability of lysine in heat-damaged proteins.

Tryptic digests of unheated and heated haemoglobin and globin. Several large fragments were isolated from digests of heated globin which were not present in digests of unheated globin. The fragments had more than one amino-terminal but individual peptides could not be separated. It was not possible to determine if cross-linkages were present.

Exhaustive enzyme digests of unheated and heated globin. A peptide was isolated from digests of heated globin which was not present in unheated globin digests. Results obtained indicated that the peptide was a cyclic tetrapeptide composed of equal quantities of lysine and aspartic acid. It was suggested that the peptide was the result of cross-linkages formed during heat-damage of globin, between the β-carboxyl groups of aspartic acid and the ε-amino groups of lysine.

It is considered that the formation of covalent bonds with the ε-amino groups would account for an appreciable proportion of the decreased availability of lysine in heat-damaged proteins.

PREFACE

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CHAPTER I

INTRODUCTION

Although meat meals, which by general usage includes meat and bone meals, are important protein concentrates in chick and layer rations, they are well known to be highly variable in protein quality (Bunyan and Price, 1960; Boyne, Carpenter and Woodham, 1961; Grace and Richards, 1964). Part of the variation is undoubtedly due to the miscellany of raw materials used in their manufacture (Atkinson and Carpenter, 1970) and considerable differences have been reported in the amino acid compositions of meals (Pritchard and Smith, 1957; Eastoe and Long, 1960). However, as meat meals are heat processed under a wide range of conditions, it is probable that heat-damage also contributes to the variation in protein quality.

The reduction in the nutritional value of proteins for poultry and other non-ruminant animals with heat processing was first reported by McCollum and Davis (1915) and later by Osborne and Mendel (1917). Subsequent studies have shown that the presence of other food components may influence the nature and degree of heat-damage to proteins. In particular, the presence of reducing sugars is known to produce a decrease in the nutritional value of mildly heated proteins (Henry, Kon, Lea and White, 1947-8; Carpenter, Morgan, Lea and Parr, 1962; Miller, Carpenter and Milner, 1965a). The reducing sugar reacts with the free amino groups, and apparently other functional groups in the protein (Henry et al, 1947-8; Patton, Hill and Foreman, 1948; Evans and Butts, 1949; Lea and Hannan, 1950), probably in a manner similar to the Maillard reaction between free amino acids and reducing sugars (Reynolds, 1965). Binding of lysine occurs most rapidly (Lea and Hannan, 1950) with a consequent decrease in the nutritional value of the protein (Henry and Kon, 1950) but little change in digestibility (Henry et al, 1947-8). More severe conditions result in the

decreased availability of other amino acids which can be largely accounted for by the low digestibility of the heat-damaged protein (Miller et al, 1965a; Boctor and Harper, 1968; Valle-Riestra and Barnes, 1970). An analogous reaction may also occur between proteins and the carbonylic secondary decomposition products of oxidised lipids (Lea, Parr and Carpenter, 1958, 1960), although the aldehydes are usually rapidly destroyed in other reactions. These Maillard-type reactions may contribute to heat-damage of meat meals but these concentrates would not contain appreciable amounts of reducing sugars.

However, the nutritional value of essentially carbohydrate-free proteins may also be decreased with heat processing (Morgan, 1931; Clandinin, 1949; Carpenter, Ellinger, Munro and Rolfe, 1957; Miller et al, 1965a; Anwar, 1965), although more severe conditions are required than when reducing sugars are present (Carpenter et al, 1962; Miller et al, 1965a). These changes with heat-damage of pure proteins probably contribute to the low protein quality of some meat meals. The chemical changes which occur are not understood although the effects of such heat-damage have been extensively investigated. Heat-damage of proteins has been shown to effect:

1. Amino acid composition.
2. Availability of amino acids.
3. Nitrogen and amino acid digestibility.
4. Rate of digestion, *in vitro* and *in vivo*.
5. Absorption and urinary excretion of amino acids.

These aspects will be discussed in the following sections. The discussion will be limited to essentially carbohydrate-free proteins, and studies with concentrates such as soybean meal, which contain appreciable carbohydrate, will not be considered.

Effect of heat-damage on carbohydrate-free proteins

1. Amino acid composition of heat-damaged proteins

Some destruction of lysine may occur when proteins are severely heat-damaged (Pader, Melnick, and Oser, 1948; Carpenter et al, 1957; Miller et al, 1965a; Bjarnason and Carpenter, 1970), and considerable losses of cystine have been reported (Beuk, Chornock and Rice, 1948; Hankes, Riesen, Henderson and Elvehjem, 1948; Evans and Butts, 1949; Miller et al, 1965a; Bjarnason and Carpenter, 1970). Bjarnason and Carpenter (1970) suggested that the destruction of lysine may result from a Maillard-type reaction with carbonyl degradation products of cystine, although these workers found little correlation between the amount of lysine destroyed and the initial cystine content of the heated proteins. The destruction of amino acids with heat-damage would however, be of little nutritional significance especially as the losses would be reflected in the amino acid analysis of the protein. Nutritionally more important are the effects of heat-damage on the availability of amino acids to animals as such changes are not readily detected or estimated. In this thesis the availability of amino acids in meat meals to chicks was a major interest.

2. Availability of amino acids from heat-damaged proteins

The results of several studies have indicated that a series of protein concentrates such as meat meals, fish meals or whale-meat meals, all of which have been heat processed, may have similar amino acid compositions but differ widely in their nutritive value (Clandinin, 1949; Ford, 1962; Waterworth, 1964; Bunyan and Woodham, 1964). Ford (1962) and Waterworth (1964) suggested that this discrepancy might be due to differences in the availability of certain or all constituent amino acids. According to the definition of amino acid availability by De Muelenaere, Chen and Harper (1967b) this would be the result of a difference in:

> that portion of an amino acid present in a protein which is used for growth, development, and maintenance of an animal in so far as it is dependent on the digestibility of the protein; presence of enzyme-resistent peptide linkages; enzyme-inhibiting substances; and rate of release of the amino acid in the intestinal tract.

The availability of lysine has been shown to be particularly readily reduced since the nutritional value of heated proteins could often be considerably improved by supplementation with lysine (Greaves and Morgan, 1934; Cook, Morgan, Singer and Parker, 1951; Carpenter et al, 1957). With heat-damage of proteins, the ε-amino groups of lysine residues seem to be bound in some manner. This has been demonstrated by the method developed by Carpenter and Ellinger (1955) and Carpenter (1960), for the estimation of the amount of lysine in proteins with the ε-amino groups free to react with fluorodinitrobenzene, FDNB-reactive lysine. The method has been used widely and many studies have shown that the FDNB-reactive lysine value decreased with heat-damage of proteins (Carpenter et al, 1957; Carpenter et al, 1962; Miller et al, 1965a; Ford, 1965; Anwar, 1965; Boctor and Harper, 1968; Bjarnason and Carpenter, 1970). In addition, the nutritional values of proteins were also shown to be reduced with a decrease in the number of free ε-amino groups of lysine (Henry and Kon, 1950; Carpenter et al, 1957; Anwar, 1965).

The results of chick growth assays confirmed that the availability of lysine was decreased, by as much as 48% in one experiment, when proteins were heat-damaged (Carpenter, March, Milner and Campbell, 1963; Miller et al, 1965a), and these estimates agreed quite closely with the corresponding FDNB-reactive lysine values. However, results obtained by microbiological assays, suggested that the nutritional availability of lysine in heat-damaged proteins may be even lower than that indicated by the FDNB-reactive lysine value (Ford, 1965; Ford and Salter, 1966; Stott and Smith, 1966; Dvořák, 1968). As a result of their studies, Ford (1965) and Stott and Smith (1966) considered that it was probable that,

apart from the possible binding of the ε-amino groups of lysine, other factors, such as the extent or rate of digestion, may be important in determining lysine availability.

Availabilities of other amino acids have also been shown to be reduced in heat-damaged proteins. The availability of methionine for chicks was reduced to a similar extent to that for lysine when cod meal was heated (Miller et al, 1965a), and the availability of both methionine and tryptophan for chicks was reduced (approximately 52 and 34% respectively) when beef fillet was autoclaved (Atkinson and Carpenter, 1970). Considerable variation in the availability of methionine for chicks in a series of meat meals was reported by Guttridge and Lewis (1964) and, for a series of whale-meat meals and other protein concentrates, by Miller, Carpenter, Morgan and Boyne (1965b). Microbiological assays have also shown that the availabilities of several essential amino acids, including lysine, are reduced when proteins are heat-damaged. The results obtained by Ford (1965) and Ford and Salter (1966) indicated that the availability of lysine was most severely decreased with heat-damage of cod meal, but Miller et al (1965a) concluded that the availabilities of lysine and the other amino acids estimated, were reduced to a similar extent.

The results obtained by microbiological assays have generally shown reasonable agreement with results obtained by growth assays. The most widely used method is that developed by Ford (1960, 1962, 1964, 1965) using Streptococcus zymogenes. This organism has an absolute requirement for eight amino acids, seven of which are essential for animals (methionine, arginine, histidine, leucine, isoleucine, tryptophan, valine plus glutamic acid). The availability of methionine estimated with Strep. zymogenes closely agreed with that obtained by chick growth assay (Miller et al, 1965a; Miller et al, 1965b). In addition, rat growth assay values, obtained by Henry and Porter and reported by Ford and Salter (1966), for

methionine and isoleucine, were similar to estimates with Strep. zymogenes. However, Atkinson and Carpenter (1970) reported that the values for available tryptophan with Strep. zymogenes were lower than those obtained by chick growth assay, and they stressed the need for care in the interpretation of microbiological assays. The assay with Strep. zymogenes requires that the test protein be pre-digested with papain and the conditions are precisely defined. The amino acid availability values obtained are not maximum values, as after prolonged incubation of the protein with pepsin, pancreatin and erepsin, the values increased and were greater than the estimates by rat growth assay (Ford and Salter, 1966).

Other micro-organisms have also been used to assay the availability of certain amino acids. The availability of methionine estimated with the protozoa, Tetrahymena pyriformis, was similar to the value obtained with Strep. zymogenes in the study by Stott and Smith (1966). However, Boyne, Price, Rosen and Stott (1967) found that the values obtained with the two organisms did not always agree. Another organism, Streptococcus durans (Streptococcus faecalis R) was used by Ford and Salter (1966) to estimate the available lysine content of unheated and heated cod meal and the values obtained also agreed closely with rat growth assay values, especially for the heated meal.

3. Nitrogen and amino acid digestibility of heat-damaged proteins

Although Miller and Carpenter (1964) and Valle-Riestra and Barnes (1970) found that there was a close relationship between the nitrogen digestibility of heat-damaged proteins and the availability of certain amino acids, the results of other studies indicated that the decrease in availability was much greater than the reduction in digestibility (Miller et al, 1965a; Ford and Salter, 1966; Neshein and Carpenter, 1967). Similarly, the decrease in nitrogen digestibility was insufficient to explain the lowered nutritional values of heat-damaged proteins (Morgan,

1931; Donoso, Lewis, Miller and Payne, 1962; Miller et al, 1965a). In addition, estimates of the digestibility of individual amino acids in heat-damaged proteins have usually been higher than availability values for the amino acids (Miller et al, 1965a; Ford, Henry and Porter, 1967).

These results indicate that the decrease in nutritional value and lowered availability of specific amino acids with heat-damage of proteins cannot be accounted for by a reduction in the digestibility of the protein or of the individual amino acids. A possible explanation for this apparent discrepancy, is that the activity of the intestinal micro-organisms may influence the amount of nitrogen or amino acid that is excreted. This possibility was investigated by Kuiken (1952) who found that the faecal excretion of amino acids by rats was unchanged when succinyl-sulphathiazole was added to the diet. A similar result was reported by Payne, Kifer, Snyder and Combs (1971) with sulphasuxidine added to the diets of chicks, but plate counts showed that the treatment had had no significant effect on the viable microbial population.

Valle-Riestra and Barnes (1970) fed autoclaved egg albumen labelled with ^{14}C-lysine, to conventional and antibiotic treated rats, and from the results obtained, concluded that the bacteria in the lower gut participated in the digestion of heat-damaged protein. However, the percentage recovery of lysine radioactivity in the gut (mainly the caecum and the large intestine) and the total radioactivity recovered in the faeces, were exactly the same for both groups of rats. This result suggested that there was no detectable absorption of lysine or of its degradation products from the indigestible residue in the caecum and colon, although deamination could have occurred. In a further investigation by Salter and Coates (1971), the digestion of freeze-dried and heat-damaged egg albumen labelled with ^{14}C, was compared in normal and germ-free chicks. With both diets, but particularly with the heat-damaged protein, there was a higher

^{14}C:Nitrogen ratio in the lower gut of conventional chicks suggesting that bacterial deamination had occurred. In addition, the total nitrogen content in the excreta of conventional chicks was less than in corresponding samples from germ-free chicks which indicated that some nitrogen released by bacterial activity may have been absorbed. There was no indication that the activity of the intestinal micro-organisms had improved the availability of the protein to the chicks. Miller (1967) also found that the net protein utilisation value for a casein-gelatine mixture was the same for both germ-free and normal chicks.

Caecectomised chickens have been used in some studies since the caecum is the site of greatest bacterial proliferation in the chick. Nesheim and Carpenter (1967) found that caecectomy reduced the apparent digestibility of heat-damaged cod meal (from 77 to 68%) although the digestibility of the unheated meal was unchanged. They suggested that with proteins resistant to digestion, as shown with heat-damaged cod meal, a portion of the protein may be fermented in the caecum and the nitrogen absorbed as ammonia or "in some other form with no nutritional value". Payne, Combs, Kifer and Snyder (1968) also found that caecectomy lowered the apparent digestibilities for several amino acids when good quality fish meal was fed to chickens. Their explanation for this result differed to that given by Nesheim and Carpenter (1967), in the suggestion that, for normal birds, the higher apparent digestibilities were due to the combined effect of bacterial proteolytic activity and absorption from the caeca, of endogenous amino acids which were lost in the caecectomised chicks.

The recovery of amino acids from the ileal contents has also been examined in an attempt to avoid possible interference by the intestinal micro-organisms in the estimation of protein digestibility (Payne et al, 1968). Varnish and Carpenter (1971) found that the digestibility of amino acids estimated by either ileal or faecal analysis were similar for

the control protein, but that when heat-damaged protein was fed, the ileal digestibility was slightly lower than indicated by faecal analysis. However, when good to poor quality fish meals were fed to chicks, the recovery of amino acids from the ileal contents did not reflect the big differences in availability observed in growth assays (Soares and Kifer, 1971).

4. Rate of digestion of heat-damaged proteins

a. In vitro digestion studies

A number of studies have shown that when proteins are heat-damaged they are less susceptible to in vitro digestion, but, as pointed out by Ford (1965), the results so obtained may reflect more closely the specificities of the enzymes chosen for the digestion rather than the relative susceptibilities of the different amino acids to enzymic release.

Heat-damage of casein was shown by Eldred and Rodney (1946) to reduce the amount of lysine released by digestion with pepsin, trypsin and chymotrypsin, even though the overall digestibility of the casein was little changed. A similar result was obtained by Pader et al (1948), and they also showed that the rate at which the lysine was released from the heated casein, by digestion with pancreatin, was reduced. In another study, Hankes et al (1948) observed that when casein was heat-damaged, and digested with pepsin, pancreatin and erepsin, the release of several amino acids, including lysine, was decreased. Similar reductions in the release of amino acids with heat-damage were reported for pork (Beuk et al, 1948), herring meal (Clandinin, 1949), cod meal (Ford, 1965), and beef serum albumin (Dvořák, 1968). However, digestion of soybean protein with erepsin and trypsin, indicated that autoclaving the protein, mainly reduced the amount of lysine released, with little change in the release of other essential amino acids (Evans and Butts, 1949).

By fractionation of pepsin-pancreatin digests, using Sephadex gels,

Ford (1965) showed that there was much more high molecular weight material in digests of heat-damaged cod meal than digests of freeze-dried meal. The high molecular weight fraction also contained considerable quantities of lysine which, by microbiological assay, was not nutritionally available. Further digestion of this fraction with papain did not improve the availability of lysine although the availabilities of other amino acids were improved.

In a further study, Ford and Salter (1966) digested freeze-dried or heated cod fillet, with either pronase alone, pepsin and papain, or pepsin, pancreatin and erepsin, and again fractionated the digests using a Sephadex gel. The release of all amino acids estimated, was reduced when the cod fillet was heat-damaged but, in particular, with increasing severity of heating, the 'free amino acid' component of the digests became progressively more deficient in lysine and the sulphur amino acids, relative to their concentration in the original unheated meal. Similar results were obtained with each digest. The authors concluded that there was "a marked differential effect of heating in retarding *in vitro* the enzymic release of several amino acids". Such an effect would alter the nutritional value of the protein since:

> for optimum utilisation of food proteins all essential amino acids must not only be available for absorption but must also be liberated during digestion *in vivo* at rates permitting mutual supplementation (Melnick, Oser and Weiss, 1946).

b. *In vivo* digestion studies

Studies of the *in vivo* digestion of proteins have shown that heat-damage alters the time-course of digestion. Heat-damaged proteins were found to be retained for a longer time in the stomach than were the undamaged proteins (Rogers, Chen, Peraino and Harper, 1960) but this may not be significant, as the rate at which proteins leave the stomach depends on many factors and appears to be unrelated to the nutritional value of the protein (Rogers and Harper, 1966).

Nesheim and Carpenter (1967) reported that when cod meal was heat-damaged, much higher levels of nitrogen were present in the intestinal contents of rats than when unheated cod meal was fed. A similar result was reported by Porter and Rolls (1971), and by filtration on Sephadex gel, the soluble nitrogenous material was separated into 'protein', 'peptide' and 'free amino acids'; greater amounts of all three fractions were obtained with heated cod meal. Amino acid analysis of the peptide fractions showed this to often have a higher content of lysine and glutamic acid than the original protein. The authors pointed out that if certain amino acids were concentrated in enzyme-resistant peptides, even if these were eventually hydrolysed, the utilisation of the protein would be altered and could contribute to the lowered nutritional value of heat-damaged proteins. Buraczewski, Buraczewska and Ford (1968) also suggested that the accumulation of the intermediate products of protein digestion in the intestine may saturate the absorption sites involved in the uptake of amino acids into the mucosal cells, and this may explain the high level of free amino acids present in the intestine when heat-damaged protein was fed.

5. Absorption and urinary excretion of amino acids and peptides from heat-damaged proteins

Heat-damaged proteins were found to produce lower rises in portal plasma amino acid levels, particularly of lysine, than when unheated protein was fed (Guggenheim, Halevy and Friedmann, 1960; Goldberg and Guggenheim, 1962; Smith and Scott, 1965a; Hill and Olsen, 1967); in addition, the peak in concentration was delayed (Wheeler and Morgan, 1958). These changes probably reflect a decreased rate of absorption of amino acids from heat-damaged protein, but the response of the plasma amino acid pattern to dietary protein is complicated by the influence of other factors such as the metabolic activity of the intestinal tissue (Dawson and Holdsworth, 1962; Dawson and Porter, 1962).

Although it is generally accepted that amino acids are the main protein digestion products which are absorbed, there is some evidence that peptides may be an important component of the nitrogen absorbed from the gut (Craft, Crampton, Lis and Matthews, 1969; Nixon and Mawer, 1970). It is possible that the mucosal uptake of peptides involves an independent mechanism to that of amino acid absorption (Lis, Matthews and Crampton, 1972).

Dawson and Holdsworth (1962) found no evidence of absorbed peptides in portal blood, but the possible absorption of peptides is supported by some studies on nitrogenous urinary excretion. Sauberlich, Pearce and Baumann (1948) observed that when mice were fed poor quality proteins such as arachin, the urinary excretion of amino acids was greater than when good quality protein (e.g. lactalbumin) was ingested, and they also found that a considerable proportion of the amino acids were bound. Large amounts of threonine containing peptides were found in the urine as well as the faeces when rats were fed a 10% rice glutelin diet and these peptides were completely resistant to papain and pancreatin hydrolysis in vitro (Kiriyama, 1970). Ford and Shorrock (1971) found that the urinary excretion of peptide-bound amino acids as well as of free amino acids, increased when rats were fed heat-damaged cod fillet rather than the unheated protein. The composition of the peptide-bound amino acids was also changed, with lysine, glutamic and aspartic acids accounting for 70% of the residues. However, the combined losses of lysine in the peptide bound and free amino acid fractions of the urine were only 1.5% of the total lysine ingested. The effects of similar heat treatment of casein were less marked; there was no increase in the total urinary bound amino acid excretion and the increase in the lysine content of the peptide component was small.

Ford and Shorrock (1971) suggested that the increase in urinary amino acid excretion, when heated cod fillet was fed to rats, could be the result

of the peptides lowering the renal threshold for free amino acids; i.e. in an analogous manner to that postulated by Buraczewski et al (1968), for the possible effect of undigested peptides in the intestine saturating the sites involved in amino acid absorption. As an alternative, they considered that the free amino acids might be derived from the hydrolysis of unavailable 'peptide' within the kidney. An acyl-lysine deacylase (EC 3.5.1.17) is present in mammalian kidney tissue and could participate in the hydrolysis of absorbed peptides, although Paik and Benoitin (1963) found that the rat enzyme could not utilise ε-N-peptides of lysine. The rat can utilise both ε-N-formyl and acetyl lysine but not ε-N-propionyl lysine (Bjarnason and Carpenter, 1969), which is consistent with the known specificity of the deacylase (Leclerc and Benoitin, 1968). However, ε-N-propionyl lysine had about 80% of the activity of lysine for chicks (Varnish and Carpenter, 1970). Other enzymes, which were suggested by Ford and Shorrock (1971) as likely to hydrolyse absorbed unavailable peptides, were ureidosuccinase (EC 3.5.1.7), β-ureidopropionase (EC 3.5.1.6) and aminoacylase (EC 3.5.1.14).

Conclusion

The results of these various studies do not clearly indicate how heat-damage reduces the nutritional values of proteins. The availability of several amino acids have been shown to be lowered but it is not certain if the availability of all constituent essential amino acids are equally reduced by heat-damage. The reduced availability of lysine is presumably due, partly at least, to the binding of the ε-amino group, but the decreased availability of other amino acids such as methionine cannot be explained in a similar manner. The rate of digestion is probably a very important factor as this has been shown to be reduced when proteins are heated and this would alter the nutritional value of the protein. In contrast, digestibility, in terms of nitrogen or of individual amino acids, does not apparently reflect the decreased availability of amino acids with heat-damage. The discrepancy may possibly be due to the activity of the

intestinal micro-organisms although the evidence is not conclusive, or it may be due to the absorption of enzymatically resistant peptides.

In the present study, the possible absorption of amino acids in an unavailable form was investigated by measuring the combined urinary and faecal excretion of amino acids when chicks were fed diets containing meat meal as the sole source of protein. The difference between the intake and the excretion of an amino acid would give the apparent retention of the amino acid, and this in turn, would reflect the availability of the amino acid, although the effect of other factors, such as the rate of digestion, would not be included. The values obtained for the apparent retention of lysine were compared to those obtained by chick growth assay of the same meat meals. A chick growth assay for lysine was developed as, although meat meals should be good sources of lysine, the amino acid may not be fully available to chicks if heat-damage has occurred. These methods and the results obtained are described in Part I. A second part of the study was an investigation into the products of enzymatically digested heat-damaged haemoglobin or globin, in an attempt to elucidate the chemical change which occurs with heat-damage of pure proteins; this is reported in Part II.

CHAPTER II

PART I : A, CHICK GROWTH ASSAY FOR LYSINE

INTRODUCTION

The essential feature of growth assays is that both the standard and the test protein should produce an increase in weight gain, or some other measure of the growth of the animal, by the same active component, the amino acid to be assayed. The response of the test animals must be linearly related to the amount of the amino acid added to the basal diet, or to some function of such doses. A basal diet is formulated which is nutritionally complete except for the amino acid to be assayed, and graded amounts of the amino acid, or of the test protein, are added to form the standard and test diets respectively. By comparing the growth of animals fed the standard diets to those supplied the test diets, an estimate of the relative potency of the test protein is obtained.

A variety of biological methods have been described for measuring the availability for growth of individual amino acids in proteins. In many of the bioassays a basal diet has been formulated using a protein, or a mixture of proteins, deficient in the amino acid to be assayed (Deshpande, Harper, Collins and Elvehjem, 1957; Calhoun, Hepburn and Bradley, 1960; Carpenter et al, 1963; Miller et al, 1965b). Some studies have reported the use of a mixture of purified amino acids, complete except for the one under study, for the protein portion of the basal diet (Smith and Scott, 1965b; De Muelenaere et al, 1967a and b; Smith, 1968; Netke and Scott, 1970). A combination of a protein basal diet deficient in the amino acid to be assayed and a crystalline amino acid mixture was used in the assay procedure of Uwaegbute and Lewis, (1966).

In some investigations the test protein has been added to the basal

diet at only one level and the potency estimated directly by comparison of the response with that obtained with the standard diets. This procedure has assumed that the test material and the standards behave in a similar manner which may not be justified. Other assays have included more than one level of the test protein so that the data could be statistically analysed to test the validity of the assay and to provide information on the precision of the potency estimated (Carpenter et al, 1963; Guttridge and Lewis, 1964; Miller et al, 1965b; Uwaegbute and Lewis, 1966). However, although statistical analysis may detect a lack of similariety in behaviour of the standards and the test protein, it may not detect systematic errors resulting from the influence of factors in the test material other than the amino acid to be estimated.

Bioassays for an amino acid are difficult to design free of distortion as with the addition of the test protein to the basal diet, other nutrients, mainly amino acids, will also be contributed apart from the amino acid to be assayed. In some bioassays an attempt was made to keep the diets isonitrogenous when the test protein was added (Deshpande et al, 1957; Carpenter et al, 1963) but in many assays the test material was added at the expense of the starch component of the basal diet (Gupta and Elvehjem, 1957; Calhoun et al, 1960; Guttridge and Lewis, 1964; Miller et al, 1965b; De Muelenaere et al, 1967a and b). Uwaegbute and Lewis (1966) attempted to maintain both the nitrogen level and the amino acid balance of the test diets by removing an equivalent amount of the crystalline amino acid mixture included in the basal diet, to the amount of test protein added. The growth response has been reported to be influenced by the level of the test protein added (Gupta and Elvehjem, 1957; De Muelenaere et al, 1967b) and by the amino acid balance (De Muelenaere et al, 1967b) although Guttridge, Lewis and Morgan (1961) and Schweigert and Guthneck (1954) both found that the addition of a mixture of amino

acids excluding the one to be assayed, had no effect on growth. Alteration of the calorie-protein ratio influenced growth response (De Muelenaere et al, 1967b) and in addition, this was altered by the type of carbohydrate present (Gupta, Dakroury, Harper and Elvehjem, (1958).

Gupta et al (1958) found that the influence of the type of carbohydrate used on the growth response could be removed if growth was related to the amount of the amino acid eaten rather than to the percentage of the amino acid in the supplement. Similarly, the growth depressing effect, of additional protein lacking in the amino acid to be assayed, was removed if these response and dose metameters were used (Carpenter et al, 1963). These workers also reported that there was no difference in the potencies estimated by this method to those obtained using feed conversion efficiency (FCE, g gain/g food consumed) as the response metameter and % amino acid in the supplement as the dose metameter; similar results were obtained by Miller et al, (1965b). Uwaegbute and Lewis (1966) considered that the potencies based on FCE as the response metameter might be over estimated and they preferred body nitrogen retention as the response. Other response metameters have also been used in some assays, for example, empty body weight gain (Calhoun et al, 1960; De Muelenaere et al, 1967a and b), body water content and carcass amino acid content (De Muelenaere et al, 1967a).

In the assay procedure to be described for the estimation of the biologically available lysine content of meat meals, a basal diet was formulated using wheat gluten which is deficient in lysine. The meat meals were added to the basal diet by either isonitrogenous substitution of the gluten or by replacement of a portion of the starch component, and the potency estimates, obtained by these two methods of test

material addition, compared. The potency estimates were calculated by using both weight gains and FCE values as response metameters, and % supplementary lysine as the dose metameter.

MATERIALS AND METHODS

Management and conditions of chick experiments

White Leghorn or White Leghorn-Australorp chicks of either or both sexes were used. At hatching, after sexing, the chicks were wingbanded and assigned at random to compartments in tiered, electrically heated, wire floored, battery brooders, situated in a windowless, semi-controlled environmental house. Each tier of a battery had a common heating element and was divided into four compartments, each with its own feed trough but with a common water trough between two adjacent compartments; the compartments held 14 - 16 chicks. Feed and water were supplied *ad libitum*, the feed weighed out at frequent intervals to reduce spillage. White light was used for the first week and then red lights to reduce cannabalism and toe pecking, with 14 hours of light per day provided. A detailed daily diary was kept of all activities and to record any deaths or ailing chickens. All chicks that died or were culled were *post mortemed* (by the Animal Health Department, Massey University), and a report received. Shed temperatures were maintained between 60 - 70°, particularly during the first two weeks after hatching, Brooder temperatures were lowered progressively with age.

Chicks were weighed individually at various stages of experiments, on a Mettler P3 balance, reading to within 1 g. A metric spring scale was used to weigh feed out and to weigh back remaining feed at the end of the experimental period. With all experiments, a preliminary

period of 10 - 11 days from hatching was imposed before commencing the experiment. This was to allow the yolk reserves to be utilised and to permit development of the chicks thermo-regulatory mechanisms. A commercial chick starter ration was fed during this preliminary period.

Diets

Semi-purified diets were used in all experiments and were composed of wheat gluten, starch, corn or soybean oil, cellulose, vitamins and minerals, and amino acid supplements. The source of these ingredients and the purity of the amino acids, are described in Table A, Appendix 1. The protein content and the FDNB-reactive lysine values for the three batches of gluten used, are given in Table B, Appendix 1 and the amino acid composition of one batch (batch 1) in Table C, Appendix 1.

Preparation of diets

The main problem in the preparation of the diets was the incorporation of the cellulose. The fine powder tended to clump and to ensure even distribution of cellulose in the rations, a mixture of 6 parts cellulose with 4 parts oil and 90 parts starch was prepared. After mixing and grinding in a hammer mill, the mixture was used to provide 50% of the diet, i.e. all diets contained 2% oil and 3% cellulose.

During the preparation of the diets, care was taken to ensure an even distribution of the ingredients, and mixing, both mechanically and by hand, was repeated several times. As some settling out of the ration components could occur with storage, the diets were hand mixed before being weighed into the feed troughs.

Meat meals

The eight meal meals (coded Mm) assayed in these experiments are described in Table A, Appendix 1, the proximate composition, FDNB-

reactive lysine and calcium content given in Table B, Appendix 1, and amino acid composition in Table C, Appendix 1.

Chemical analysis

Proximate analysis and calcium estimation. The water, protein (N x 6.25), crude fat, ash and calcium content were determined by the methods of the Association of Official Agricultural Chemists (1960).

Amino acid analysis. The amino acid composition of protein concentrates was estimated by hydrolysing 0.5 - 1.0 g samples in 950 cm^3 6 M HCL for 24 hours. The solution was filtered and made up to 1 l with distilled water. An aliquot of this solution was evaporated and a sample, 0.1 - 0.5 cm^3, analysed with a Beckman 120C Amino Acid Analyser; norleucine was included as an internal indicator. All samples were analysed in duplicate or triplicate.

FDNB-reactive lysine. The FDNB-reactive lysine value of the protein concentrates was determined by the method of Carpenter (1960), with the application of the factor 1.09 to correct for the loss of dinitrophenyl lysine during hydrolysis. The 2,4-dinitrofluorobenzene (FDNB), ε-(2,4-dinitrophenyl)-L-lysine hydrochloride and methyl chloroformate, were supplied by B.D.H. Ltd., England.

Statistical analysis

The potency of the meat meals as sources of lysine for chicks was estimated by slope ratio analysis (Finney, 1964), using the method adopted by Clarke (1952) for multiple assays. The dose metameter was % supplementary lysine and the response metameters were either weight gain or weight gain, g/g food eaten (FCE). The slope ratio procedure was used for analysis as the response was found to be linearly related to increments of lysine or of the test proteins. Although in most growth assay experiments three standard and three test diets were included

in each assay, the values for only two doses, evenly spaced, of each series were used for the analysis. For potency estimates using FCE values as the response, the upper dose level was omitted, but for estimates based on weight gain, the lower dose level was omitted in some experiments. Fieller's equation was used for the calculation of fiducial limits with the modification recommended by Finney (1964), that when the assymmetry of the upper and lower limits was small it was replaced by approximate symmetrical limits indicated by a single estimation of the standard error.

Values for missing chick weight gains were calculated, by the missing plot technique as outlined by Snedecor and Cochran (1967) for the statistical analysis of results. Standard analysis of variance procedures were used to test significant differences in weight gains or FCE values between treatments. Minimum significant difference values, (MSD, 5%) to establish which specific comparisons were significant after a significant F ratio was established, were computed according to Snedecor and Cochran (1967).

EXPERIMENTS WITH CHICKS AND RESULTS

The first two experiments with chicks were conducted to develop a suitable basal diet to use in growth assays for lysine. In four further experiments, eight meat meals were assayed for lysine potency by the growth assay method.

Experiment I, development of basal diet

A basal diet deficient in lysine was formulated using wheat gluten as the protein source. Wheat gluten was included in the basal diet to provide 20% protein (N x 6.25) which would supply 0.4% lysine compared to the recommended chick requirement of 1% by the United States National Research Council (USNRC, 1963). Corn or soybean oil, to supply essential fatty acids, a low level of cellulose, and comprehensive vitamin and mineral premixes, were included in the basal diet, and starch made up the remainder. The composition of this basal diet A is given in Table D, Appendix 1, and of the vitamin and mineral premixes in Table E, Appendix 1. The amount of $Ca_3(PO_4)_2$ added to diets varied in each experiment so this was not included in the basal diet; in this experiment 3.06% $Ca_3(PO_4)_2$ was added, so that all diets contained 1.4% calcium.

Comparison of the amino acid content of basal diet A with the recommendations for chicks by the USNRC (1963), indicated that, apart from lysine, the levels of methionine, arginine and possibly threonine, were deficient. The effect of supplementing basal diet A with these amino acids was investigated in this experiment, and the growth of chicks fed these semi-purified diets compared to those fed a commercial starter ration. The four diets were:

1. Basal diet A, unsupplemented.
2. Basal diet A supplemented with 0.6% lysine, 0.42% arginine, and 0.35% methionine.

3. Basal diet A supplemented with 0.6% lysine, 0.42% arginine, 0.35% methionine and 0.03% threonine.

4. Commercial chick starter ration, (20% protein).

These treatments were arranged in a randomised block design of four treatments over two blocks, a tier of the battery brooder forming a block. There were two replicates per treatment, with 16 chicks (equal sexes) per replicate, a total of 128 chicks. All chicks were fed a commercial starter ration (which was used throughout for treatment 4) to 10 days of age. The chicks were then supplied the experimental diets for a pre-experimental period of 4 days to permit them to become accustomed to the different textured feed. At 14 days, the chicks were weighed individually and fresh feed weighed into the troughs. At the end of the fourth week the chicks were weighed again and the food consumption recorded.

Results

The mean weight gains and food consumption (g/chick) are shown in Table I. Supplementation of basal diet A with lysine, arginine and methionine markedly increased growth and this was further improved by the addition of threonine. The gain in weight of chicks fed the fully supplemented diet, diet 3, was only slightly less than for chicks fed the commercial starter ration. There were no deaths during the experimental period and the chicks fed the unsupplemented diet, diet 1, remained healthy.

Experiment 2, development of basal diet and preliminary bioassay

Although in experiment 1 the fully supplemented basal diet A, diet 3, supported satisfactory growth of chicks, the effect of additional supplements of amino acids was investigated in this experiment to ensure that the basal diet was growth limiting for lysine only. A basal diet B was used which was the same as basal diet A except that 0.5% methionine,

TABLE I

Mean weight gain and food consumption, g/chick,

14 - 28 days, experiments 1 and 2

Experiment	Diet code	Diet description	Weight gain	Food consumption
1	1	Basal diet A	23.1	162.9
	2	Basal diet A with lysine, arginine and methionine	143.3	338.8
	3	Basal diet A with lysine, arginine, methionine and threonine	161.9	354.8
	4	Commercial chick starter	175.4	428.2
2		Supplements to basal diet B:		
	1	None	28.2	173.1
	2	0.56% lysine, 0.05% arginine	183.8	357.7
	3	0.56% lysine, 0.05% threonine	178.4	355.7
	4	0.56% lysine, 0.05% tryptophan	177.8	354.2
	5	0.14% lysine }	74.5	251.6
	6	0.28% lysine } standard diets	129.2	319.2
	7	0.56% lysine }	171.2	334.7
	8	1.50% Mm B }	61.6	234.3
	9	3.01% Mm B } test diets	78.3	260.3
	10	6.02% Mm B }	113.5	292.0
		* MSD (5%)	17.2	52.7

* experiment 2, excluding diets 5 - 7.

0.42% arginine and 0.05% threonine were included; the composition of this diet is given in Table D, Appendix 1. The levels of methionine and threonine included in basal diet B were slightly greather than the levels used in experiment 1 to provide a margin of safety. This basal diet B was supplemented with 0.56% lysine so this would not be limiting for growth, and the effect of further supplements of arginine and threonine investigated. In addition, one diet contained a supplement of tryptophan as values in the literature indicated that this might become limiting when the basal diet was otherwise fully supplemented. The four diets were:

1. Basal diet B.
2. Basal diet B supplemented with 0.05% arginine.
3. Basal diet B supplemented with 0.05% threonine.
4. Basal diet B supplemented with 0.05% tryptophan.

Other treatments were included in this experiment to provide a preliminary trial with bioassay techniques. Diet 1 above was used as the negative control diet and three standard diets were included, with supplements of 0.14, 0.28 and 0.56% lysine. The meat meal assayed, Mm B, was included in the basal diet B at three levels calculated on the basis of the total lysine content of the meal, to provide comparable amounts of lysine to that in the standard diets. The lysine supplements and the meat meal were added to basal diet B at the expense of starch. As the meat meal contained only a small amount of calcium, 2.5% $Ca_3 (PO_4)_2$ was added to all diets, including diets 1 - 4. The standard and test diets were:

5. Basal diet B supplemented with 0.14% lysine
6. Basal diet B supplemented with 0.28% lysine } standard diets
7. Basal diet B supplemented with 0.56% lysine
8. Basal diet B supplemented with 1.50% Mm B
9. Basal diet B supplemented with 3.01% Mm B } test diets
10. Basal diet B supplemented with 6.02% Mm B

The levels of supplements added to basal diet B for these ten diets are given in Table F, Appendix 1.

The procedures used for the experiment were similar to those used for experiment 1. There were two replicates of 14 chicks (equal sexes) per treatment, a total of 280 chicks. Treatments 1 - 4 were arranged as a randomised block design, over two tiers of one battery brooder. The treatments for the bioassay, (treatments 5 - 10), were arranged in a completely random design over three tiers in a separate battery unit. All chicks were fed a commercial starter ration for 11 days after hatching. A complete semi-purified diet, diet C, was then fed for three days; diet C was basal diet B supplemented with 0.56% lysine and 3.00% $Ca_3 (PO_4)_2$, and the composition is given in Table D, Appendix 1. The chicks were weighed at 14 days and the experimental diets supplied for two weeks. Chicks and the feed remaining in the feed troughs were weighed at 21 and 28 days.

Results

The mean weight gains and food consumption per chick, 14 - 28 days, are shown in Table I. The additional supplements of arginine and threonine to the basal diet did not improve growth and there was no benefit from the addition of tryptophan. It was concluded that basal diet B supplemented with 0.56% lysine would support satisfactory growth of chicks and that no other amino acid was limiting for growth. As basal diet B appeared to be nutritionally adequate except for the deficiency in lysine, this diet was used in future bioassay experiments.

The results for the negative control diet, diet 1, and the three standard diets, diets 5 - 7, show that each increment of lysine significantly ($P < 0.05$) increased the weight gain of the chicks; the response was linear for the two lower dose levels but not for the highest level of supplementary lysine, 0.56% (Figure 1). The weight gains of

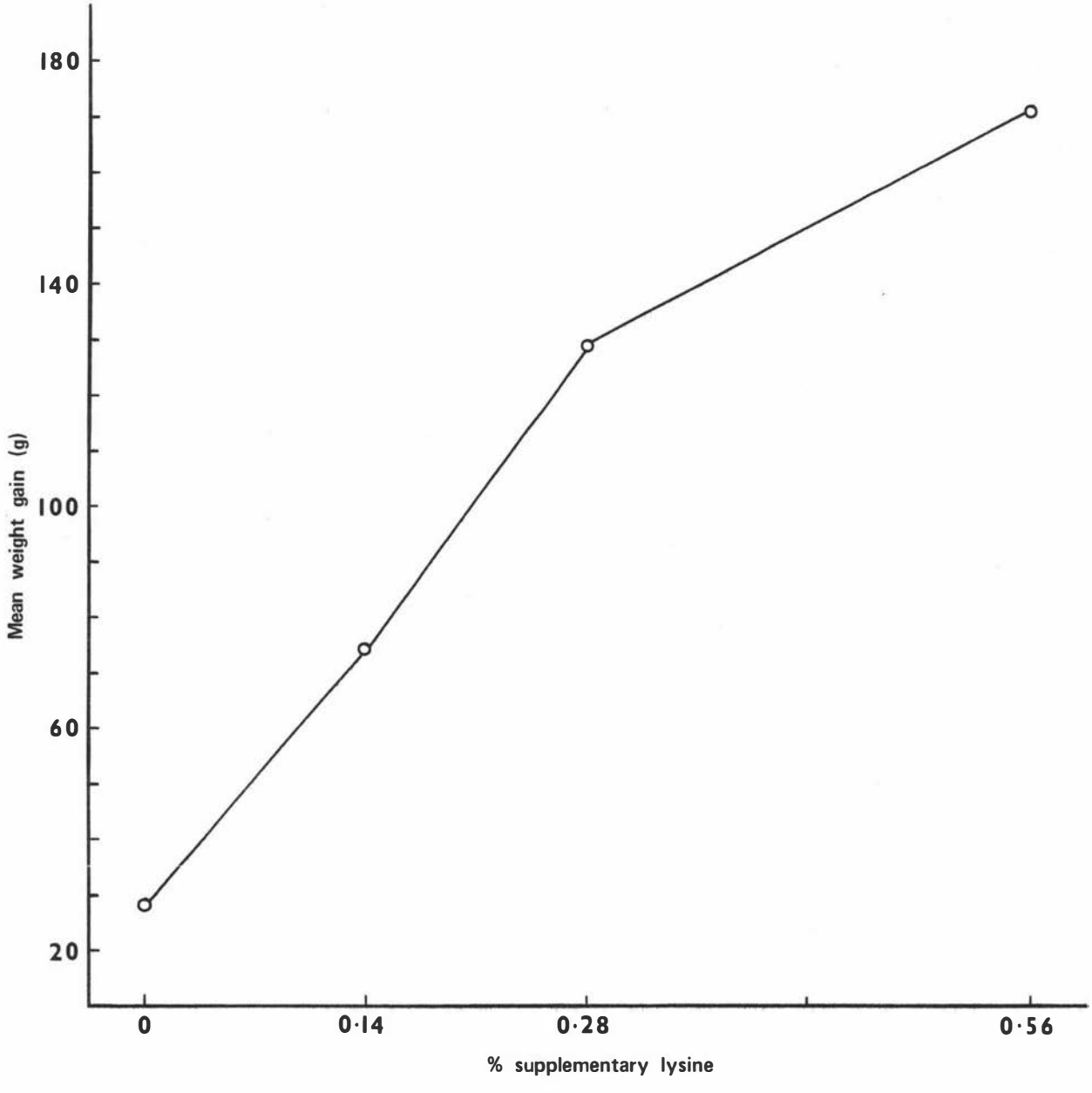


FIGURE 1. Effect of supplementary lysine on mean weight gains with standard diets, experiment 2.

chicks fed the test diets containing Mm B, diets 8 - 10, were however, much lower than the responses obtained with the standard diets, and the weight gains of chicks fed the two lower levels of Mm B were not significantly different. These unsatisfactory results for the meat meal were due to a technical error, a value of 9.3% lysine had been used for the calculation of the test diets but the meal actually contained 4.9% lysine. Because of the difference in dose levels of the standard and test diets, these results for the bioassay of Mm B have been excluded from discussion. However, the inclusion of these treatments in the experiment, provided useful information on the techniques required for bioassays.

Experiment 3, bioassay of Meat meals, A, B and C

This experiment involved the bioassay of three meat meals for lysine potency. The levels of lysine included in the three standard diets were reduced to 0.12, 0.24 and 0.48% as the response to the upper dose level, 0.56%, used in experiment 2 had exceeded the linear portion of the dose-response curve. The meat meals assayed were included at three levels of addition to basal diet B to provide similar amounts of lysine to that present in the standard diets on the basis of the FDNB-reactive lysine value for the meal multiplied by 1.2; this factor was included to ensure that the dose would not exceed the linear portion of the dose-response curve. The meat meal replaced either starch or isonitrogenous amounts of gluten in the basal diet. The latter procedure required the restitution of an equivalent amount of lysine present in the removed gluten, and was calculated using the FDNB-reactive value for gluten.

The three meat meals assayed were Mm A, Mm C, and Mm B as the assay of this meal, in experiment 2, was invalid. In this experiment, Mm B was assayed by either inclusion in the test diets at the expense of starch as had been the case in experiment 2 or by the isonitrogenous replacement

of gluten. Mm A and Mm C were both added to the test diets by substitution of the gluten. The 16 diets are shown in Table II and the levels of the supplements to basal diet B, the gluten content and the calculated protein content of the diets, are given in Table G, Appendix 1. As Mm C contributed appreciable amounts of calcium, the amount of $Ca_3 (PO_4)_2$ added was adjusted so that all diets contained 1.6% calcium.

For this experiment, 14 cockerels per compartment were used, (a total of 448 chicks), housed in three, three tiered battery brooders, with the 16 treatments randomly assigned; there were two replicates per treatment. All chicks were fed a commercial starter ration for the first 11 days and then the semi-purified diet, diet C, for 3 days. The chicks were then weighed and the experimental diets allocated. The chicks were weighed at 21 and 28 days of age and the feed consumption recorded weekly.

Results

The chick responses in terms of weight gain, food consumption and feed conversion efficiency (FCE) are shown in Table III. The mean weight gains and FCE values were significantly different ($P < 0.05$) for each of the standard diets and for each of the test diets in a series. A linear response in terms of weight gain was obtained for the standard diets (Figure 2) but for FCE, the response was linear only up to the addition of 0.24% supplementary lysine (Figure 3). These standard diets were used without modification in most subsequent bioassays.

The weight gains of chicks fed the test diets were similar to those fed the standard diets. Weight gain and FCE values with test diets containing Mm B were slightly less, at the lowest dose, when the meat meal replaced gluten rather than starch in the basal diet, but at the higher dose levels, the responses were similar. The estimated potencies of these meat meals, using either weight gain or FCE as the response and excluding the top dose, are given in Table X, together with the 95% approximate

TABLE II

Diets : experiment 3

1.	Basal diet B	
2.	Basal diet B supplemented with 0.12% lysine	standard diets
3.	Basal diet B supplemented with 0.24% lysine	
4.	Basal diet B supplemented with 0.48% lysine	
5.	Basal diet B supplemented with 2.94% Mn B	starch replaced
6.	Basal diet B supplemented with 5.88% Mn B	
7.	Basal diet B supplemented with 11.76% Mn B	
8.	Basal diet B supplemented with 2.94% Mn B	gluten replaced
9.	Basal diet B supplemented with 5.88% Mn B	
10.	Basal diet B supplemented with 11.76% Mn B	
11.	Basal diet B supplemented with 4.55% Mn A	gluten replaced
12.	Basal diet B supplemented with 9.10% Mn A	
13.	Basal diet B supplemented with 18.20% Mn A	
14.	Basal diet B supplemented with 5.13% Mn C	gluten replaced
15.	Basal diet B supplemented with 10.26% Mn C	
16.	Basal diet B supplemented with 20.52% Mn C	

TABLE III

Mean weight gain, food consumption (g/chick) and feed conversion efficiency (g gain/g food eaten) of chicks, 14-28 days; bioassay of Mn A, Mn B and Mn C, experiment 3

Diet code	Supplements	Substituted material	Weight	Food consumption	Feed conversion efficiency
Standard diets:					
1	None		27.9	177.4	0.149
2	0.12% lysine	Starch	65.9	222.0	0.284
3	0.24% lysine	Starch	96.6	250.4	0.386
4	0.48% lysine	Starch	166.5	326.7	0.508
Test diets:					
5	2.94% Mn B	Starch	58.4	215.5	0.267
6	5.88% Mn B	Starch	93.0	237.3	0.373
7	11.76% Mn B	Starch	169.8	316.4	0.535
8	2.94% Mn B	Gluten	42.6	203.9	0.199
9	5.88% Mn B	Gluten	91.0	256.3	0.353
10	11.76% Mn B	Gluten	168.9	329.3	0.512
11	4.55% Mn A	Gluten	45.8	193.0	0.225
12	9.10% Mn A	Gluten	87.9	241.9	0.324
13	18.20% Mn A	Gluten	169.1	336.3	0.483
14	5.13% Mn C	Gluten	48.7	197.8	0.234
15	10.26% Mn C	Gluten	102.1	271.5	0.367
16	20.52% Mn C	Gluten	183.2	357.8	0.512
		MSD (5%)	19.2	44.9	0.081

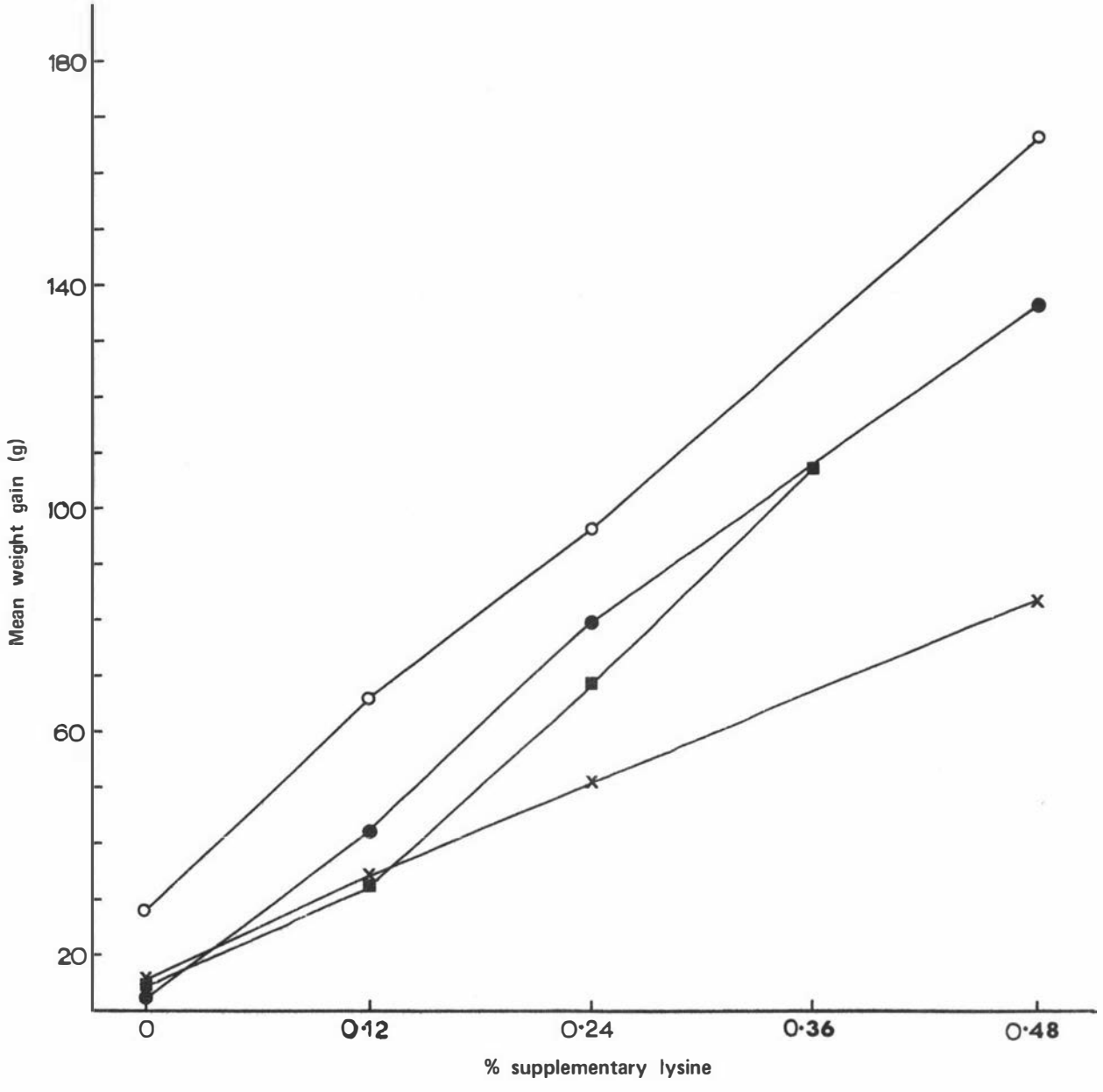


FIGURE 2. Effect of supplementary lysine on mean weight gains with standard diets, experiments 3–6. O—O experiment 3, ●—● experiment 4, ✕—✕ experiment 5, ■—■ experiment 6.

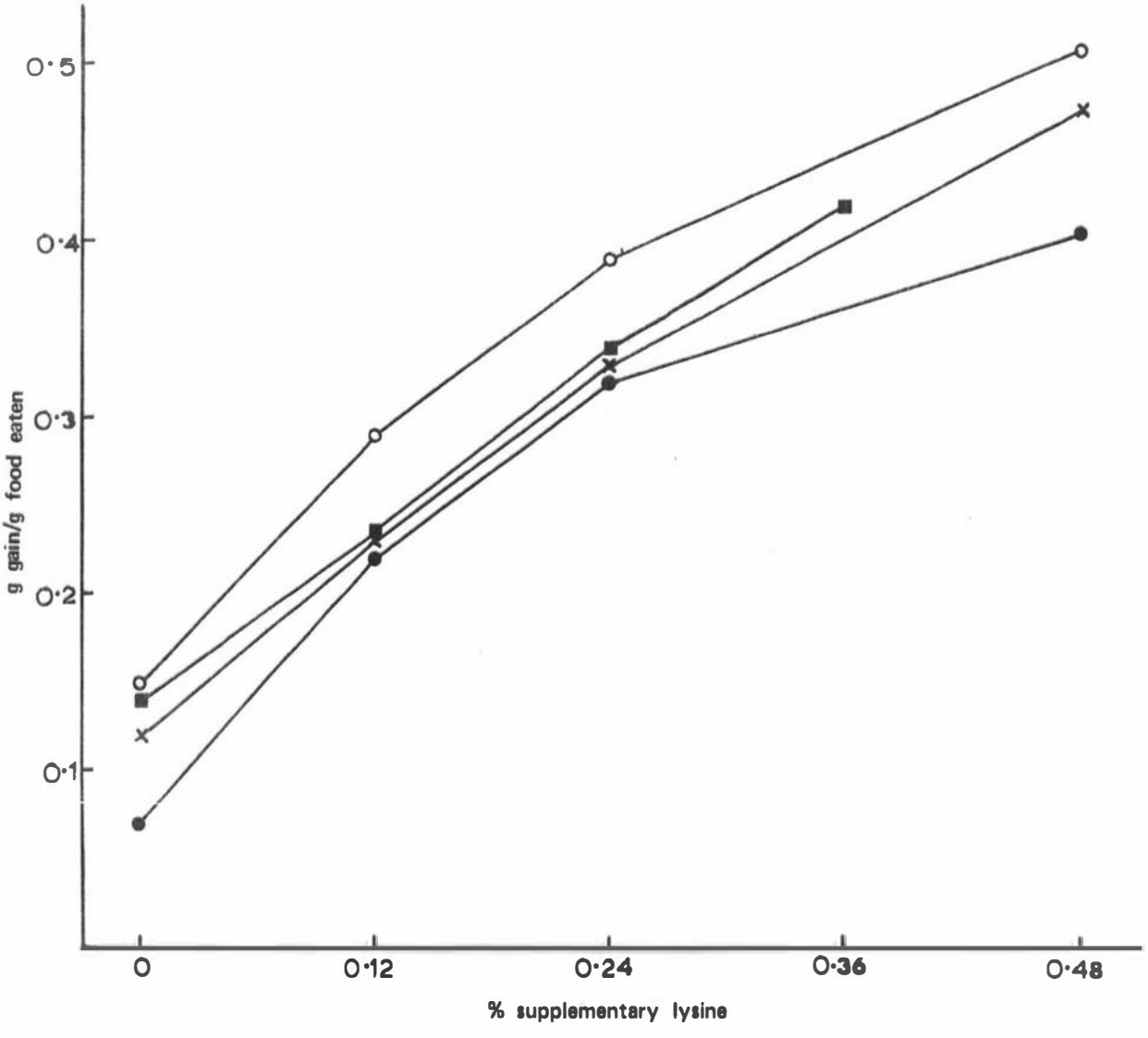


FIGURE 3. Effect of supplementary lysine on the feed conversion efficiency of standard diets, experiments 3–6. ○—○ experiment 3, ●—● experiment 4, ×—× experiment 5, ■—■ experiment 6.

fiducial limits. The analysis of variance for these assays are shown in Table A, Appendix 2. The estimated potency of Mm B with weight gain as the response, was the same for either method of meat meal addition to the test diets, but with FCE as the response the two estimates differed considerably, although this was not quite significant statistically. With all three meat meals the estimated potencies were greater when weight gain was the response metameter than with FCE as the response.

An outbreak of Salmonella pullorum disease occurred amongst the chicks in this experiment, with 21 deaths amongst the 448 chicks. The disease may have affected results although a mortality of 5% is not unusual for chicks to three weeks of age. (The disease was brought on to the farm through hatching eggs sent in as part of a Random Sample Test series, and the cockerels, normally not required, were used in this trial).

Experiment 4, bioassay of Meat meal, H

As the estimated potency of Mm B, using FCE values, varied with the method of meat meal inclusion in the test diets in experiment 3, in this experiment, another meat meal, Mm H, was assayed either by replacement of starch or by substitution of gluten in the test diets. The ten diets are shown in Table IV. The amount of $Ca_3(PO_4)_2$ included in the diets was adjusted so that all diets contained 1.4% calcium. The level of supplements, gluten content and the calculated protein content of the diets are given in Table G, Appendix 1.

Two replicates, each of 14 pullet chicks, (a total of 280 chicks) were randomly assigned to 20 compartments, housed in two battery brooders, one of two tiers and the other, of three tiers. The same regime as in experiment 3 was followed, that is, the chicks were fed a commercial starter ration post hatch, followed by diet C for a preliminary period of 3 days, and the experimental diets supplied at 14 days for two weeks, the chicks weighed

TABLE IV

Diets : Experiment 4

1.	Basal diet B	
2.	Basal diet B supplemented with 0.12% lysine	standard diets
3.	Basal diet B supplemented with 0.24% lysine	
4.	Basal diet B supplemented with 0.48% lysine	
5.	Basal diet B supplemented with 2.89% Mn H	starch replaced
6.	Basal diet B supplemented with 5.78% Mn H	
7.	Basal diet B supplemented with 11.56% Mn H	
8.	Basal diet B supplemented with 2.89% Mn H	gluten replaced
9.	Basal diet B supplemented with 5.78% Mn H	
10.	Basal diet B supplemented with 11.56% Mn H	

and the food consumption recorded at weekly intervals.

Results

The mean weight gains, food consumption and FCE values are shown in Table V, and the estimated potencies of the meal, excluding the values for the lower dose level for the estimates based on weight gain, are given in Table X, together with the approximate 95% fiducial limits. The analysis of variance for the assays are shown in Table B, Appendix 2. The response to the standard diets was linear when weight gain was the response (Figure 2) but not with FCE as the response (Figure 3). The estimated potencies of Mm H, based on weight gain, were significantly greater when the meal was included in the test diets by isonitrogenous substitution of gluten than when starch was replaced, but with FCE as the response metameter, the two estimates were similar.

Experiment 5, bioassay of Meat meals, D, E, F and G

As the method of meat meal addition to the test diets did not consistently influence the potency estimates of meals assayed in experiments 3 and 4, two other meat meals, Mm D and Mm F, were assayed by both methods of inclusion, in this experiment. The lysine potency of two other meat meals, Mm G and Mm E, was also estimated, both by addition to the test diets at the expense of starch. The 22 diets are listed in Table VI. The amount of $Ca_3 (PO_4)_2$ added provided 1.4% calcium in all diets. The level of the supplements, gluten content and the calculated protein content of the diets are given in Table H, Appendix 1.

There were two replicates of 14 pullets per treatment (a total of 616 chicks) and the 44 compartments were randomly assigned over three battery brooders. The same procedures were followed as in previous experiments, but an illness affected the chickens in the pre-experimental period. The main symptom was foot tenderness and the chicks slowly

TABLE V

Mean weight gain, food consumption (g/chick) and feed conversion efficiency (g gain/g food eaten) of chicks, 14 - 28 days; bioassay of Mn H, experiment 4

Diet code	Supplements	Substituted material	Weight gain	Food consumption	Feed conversion efficiency
Standard diets:					
1	None		11.3	157.5	0.068
2	0.12% lysine	Starch	42.1	190.2	0.221
3	0.24% lysine	Starch	80.0	251.1	0.318
4	0.48% lysine	Starch	136.5	336.1	0.406
Test diets:					
5	2.89% Mn H	Starch	33.8	168.7	0.200
6	5.78% Mn H	Starch	69.0	212.4	0.325
7	11.56% Mn H	Starch	125.9	270.4	0.466
8	2.89% Mn H	Gluten	37.8	171.5	0.221
9	5.78% Mn H	Gluten	71.3	236.1	0.302
10	11.56% Mn H	Gluten	139.8	286.8	0.487
		MSD (5%)	24.4	47.2	0.063

TABLE VI

Diets : experiment 5

1.	Basal diet B	
2.	Basal diet B supplemented with 0.12% lysine	standard diets
3.	Basal diet B supplemented with 0.24% lysine	
4.	Basal diet B supplemented with 0.48% lysine	
5.	Basal diet B supplemented with 5.0% Mm D	starch replaced
6.	Basal diet B supplemented with 10.0% Mm D	
7.	Basal diet B supplemented with 20.0% Mm D	
8.	Basal diet B supplemented with 5.0% Mm D	gluten replaced
9.	Basal diet B supplemented with 10.0% Mm D	
10.	Basal diet B supplemented with 20.0% Mm D	
11.	Basal diet B supplemented with 4.0% Mm E	starch replaced
12.	Basal diet B supplemented with 8.0% Mm E	
13.	Basal diet B supplemented with 16.0% Mm E	
14.	Basal diet B supplemented with 3.45% Mm F	starch replaced
15.	Basal diet B supplemented with 6.90% Mm F	
16.	Basal diet B supplemented with 13.80% Mm F	
17.	Basal diet B supplemented with 3.45% Mm F	gluten replaced
18.	Basal diet B supplemented with 6.90% Mm F	
19.	Basal diet B supplemented with 13.80% Mm F	
20.	Basal diet B supplemented with 4.17% Mm G	starch replaced
21.	Basal diet B supplemented with 8.33% Mm G	
22.	Basal diet B supplemented with 16.66% Mm G	

recovered, with only 5 deaths amongst the 616 chicks. However, the pre-experimental period was extended by one week to maximise recovery, and the experimental diets were supplied for only one week, from 21 to 28 days of age; the chicks were weighed at the beginning and end of this period and the food consumption recorded.

Results

The mean weight gains, food consumption and FCE values per chick, are given in Table VII. The response to the standard diets was linear for all dose levels when weight gain was the response (Figure 2) but in terms of FCE values the response was linear for only the two lower dose levels (Figure 3). For both Mm D and Mm F the weight gains at the upper dose level, were greater, although not significantly, when the meat meal was added at the expense of gluten rather than of starch. The estimated potencies for the meals, excluding the values for the lower dose level when weight gain was the response, are given in Table X together with the approximate 95% fiducial limits, and the analysis of variance for the assays, in Table C, Appendix 2. The estimated potencies of Mm D based on weight gain, were significantly different for the two methods of meat meal addition to the test diets; those for Mm F were almost significant. However, the potency estimates for these two meals based on FCE values, were similar for the two methods of meal inclusion in the test diets. There was no consistent difference between the potency estimates based on weight gain to those based on FCE values for the four meals assayed.

Experiment 6, the effect of added oil on the bioassay of Meat meal, F

In previous experiments, the estimated potencies of meat meals based on weight gain, were lower for three of the four meals, when starch, rather than gluten, was replaced by the meat meal in the test diets; estimates using FCE values generally showed closer agreement. Food

TABLE VII

Mean weight gain, food consumption (g/chick) and feed conversion efficiency (g gain/g food eaten) of chicks, 21-28 days; bioassay of Mn D, Mn E, Mn F and Mn G, experiment 5

Diet code	Supplements	Substituted material	Weight gain	Food consumption	Feed conversion efficiency
Standard diets:					
1	None		15.7	118.3	0.117
2	0.12% lysine	Starch	34.0	129.3	0.230
3	0.24% lysine		50.2	150.7	0.333
4	0.48% lysine		83.3	170.8	0.474
Test diets:					
5	5.0% Mn D	Starch	35.0	127.9	0.268
6	10.0% Mn D		46.5	131.8	0.319
7	20.0% Mn D		88.2	172.9	0.499
8	5.0% Mn D	Gluten	32.2	125.0	0.245
9	10.0% Mn D		51.4	144.8	0.337
10	20.0% Mn D		101.2	204.3	0.495
11	4.0% Mn E	Starch	30.1	122.8	0.239
12	8.0% Mn E		47 9	138.2	0.324
13	16.0% Mn E		78.4	153.2	0.505
14	3.45% Mn F	Starch	34.3	129.0	0.252
15	6.90% Mn F		54.7	149.3	0.359
16	13.80% Mn F		94.4	171.8	0.549
17	3.45% Mn F	Gluten	32.8	129.3	0.240
18	6.90% Mn F		54.6	148.9	0.367
19	13.80% Mn F		102.8	201.5	0.494
20	4.17% Mn G	Starch	29.8	125.1	0.229
21	8.33% Mn G		45.5	133.7	0.318
22	16.66% Mn G		80.1	157.5	0.473
		MSD (5%)	13.8	34.4	0.086

consumption as well as weight gains, tended to increase, particularly with the highest dose level, when gluten was substituted than when starch was replaced in the test diets. This indicated that part of the variation in the potency estimates based on weight gains, with the method of meat meal inclusion, was due to an effect on the appetite of the chicks. As the metabolisable energy content of wheat gluten would probably be less than starch (no value has been found in the literature) this difference in food consumption with the two methods of meat meal addition may be due to a difference in the energy content of the test diets, the starch replaced test diets possibly having a lower energy content than the gluten replaced diets. This possibility was investigated in this experiment by the inclusion of additional corn oil in the test diets. The meat meal, Mm F, which had been assayed in experiment 5, was added to the test diets by either isonitrogenous substitution of gluten, or by replacement of starch, and both assays were repeated with the inclusion of 2% corn oil in the test diets. The amount of Mm F in the test diets was calculated on the basis of the potency estimate obtained in experiment 5 using FCE values. The meat meal was assayed at only two levels of addition, calculated to provide 0.12 and 0.24% lysine. The upper dose level (0.48%) of the standard diets was omitted and replaced by a diet containing 0.36% supplementary lysine, so that the linearity of response in terms of FCE at this level could be determined. The twelve diets are listed in Table VIII. Sufficient $Ca_3 (PO_4)_2$ was included to provide 1.4% calcium in all diets. The level of supplements, gluten content and the calculated protein content of the diets are shown in Table H, Appendix 1. The additional corn oil in diets 7, 8, 11 and 12, was incorporated by preparing a mix of 16 parts oil, 12 parts cellulose and 72 parts starch, and including this to provide 25% of the diets.

Three replicates per treatment, instead of two as in previous

TABLE VIII

Diets : experiment 6

1.	Basal diet B	
2.	Basal diet B supplemented with 0.12% lysine	standard diets
3.	Basal diet B supplemented with 0.24% lysine	
4.	Basal diet B supplemented with 0.36% lysine	
5.	Basal diet B supplemented with 2.79% Mm F	starch replaced
6.	Basal diet B supplemented with 5.58% Mm F	
7.	Basal diet B supplemented with 2.79% Mm F, 2% oil	starch replaced
8.	Basal diet B supplemented with 5.58% Mm F, 2% oil	
9.	Basal diet B supplemented with 2.79% Mm F	gluten replaced
10.	Basal diet B supplemented with 5.58% Mm F	
11.	Basal diet B supplemented with 2.79% Mm F, 2% oil	gluten replaced
12.	Basal diet B supplemented with 5.58% Mm F, 2% oil	

experiments, were used to improve the precision of the potency estimates based on FCE values. There were 14 cockerels per replicate (a total of 504 chicks), and the 36 compartments were randomly assigned to three, three tiered battery brooders. The procedures used were similar to previous assays with a preliminary period followed by a two week experimental period, the chicks weighed at 2, 3 and 4 weeks and the food consumption recorded each week.

Results

The mean weight gains, food consumption and FCE values per chick are given in Table IX, and the estimated potencies for Mm F, by each assay, in Table X, together with the approximate 95% fiducial limits. The analysis of variance for these assays are shown in Table D, Appendix 2. The slope ratio analysis using weight gains as the response was not statistically valid as the mean square for blanks was significant ($P < 0.05$); this was probably due to the rather erratic weight gains recorded for the standard diets (Figure 2). In terms of FCE, the response was linear for all dose levels of the standard diets (Figure 3). Comparing the results of assays with no added oil, food consumption and weight gains were slightly less when the meat meal replaced gluten than when starch was replaced. The potency estimates of the meal based on either weight gain or FCE values reflected these differences, with slightly higher values obtained when starch was substituted than when gluten was replaced. These results were in contrast to those of previous experiments, in which food consumption, weight gains and potency estimates based on weight gains, were generally lower when the meat meal had replaced starch, rather than gluten, in the test diets.

The addition of 2% oil to the test diets had little effect on the values obtained with the isonitrogenous test diets but with the starch replaced diets, food consumption and weight gains were slightly reduced.

TABLE IX

Mean weight gain, food consumption (g/chick) and feed conversion efficiency (g gain/g food eaten) of chicks, 14-28 days; bioassay of Mm F, with or without added corn oil, experiment 6

Diet code	Supplements	Substituted material	Weight gain	Food consumption	Feed conversion efficiency
Standard diets:					
1	None		16.6	117.1	0.142
2	0.12% lysine	Starch	32.4	141.9	0.229
3	0.24% lysine		69.7	207.4	0.336
4	0.36% lysine		107.5	256.9	0.419
Test diets:					
5	2.79% Mm F	Starch	37.7	162.5	0.231
6	5.58% Mm F		63.0	186.8	0.337
7	2.79% Mm F + 2% oil		32.4	134.7	0.240
8	5.58% Mm F + 2% oil		56.8	172.6	0.329
9	2.79% Mm F	Gluten	32.6	145.6	0.223
10	5.58% Mm F		58.3	184.6	0.316
11	2.79% Mm F + 2% oil		35.5	146.6	0.240
12	5.58% Mm F + 2% oil		56.9	179.5	0.317
		MSD (5%)	10.3	41.9	0.052

TABLE X

Estimated potencies of meat meals (Mm A - Mm H), g lysine/16 g N, with approximate 95% fiducial limits, calculated with either a) weight gain or b) feed conversion efficiency (FCE), as the response; experiments 3 - 6

Experiment	Supplement	Substituted material	Estimated potency based on: a) Weight gain	b) FCE
3	Mm B	Starch	5.9 ± 0.41	5.4 ± 1.01
		Gluten	5.9 ± 0.41	4.5 ± 0.95
	Mm A	Gluten	4.4 ± 0.30	3.6 ± 0.80
	Mm C	Gluten	5.2 ± 0.36	3.6 ± 0.70
4	Mm H	Starch	5.7 ± 0.47	6.3 ± 0.95
		Gluten	6.3 ± 0.50	6.0 ± 0.93
5	Mm D	Starch	4.3 ± 0.42	4.2 ± 0.86
		Gluten	5.0 ± 0.46	4.4 ± 0.88
	Mm F	Starch	6.6 ± 0.61	6.7 ± 1.30
		Gluten	7.2 ± 0.64	6.7 ± 1.30
	Mm G	Starch	4.6 ± 0.47	4.5 ± 0.98
	Mm E	Starch	5.0 ± 0.50	5.3 ± 1.10
6	Mm F	Starch	6.7 ± 0.97*	7.2 ± 1.09
	Mm F + oil	Starch	5.8 ± 0.92*	7.1 ± 1.08
	Mm F	Gluten	6.0 ± 0.77*	6.5 ± 1.04
	Mm F + oil	Gluten	6.0 ± 0.77*	6.8 ± 1.07

* Not statistically valid at 5% level of significance

These results do not indicate that the lowered food consumption and weight gains obtained in previous experiments when the meat meal was added to the test diets at the expense of starch, compared to when gluten was replaced, were due to a lower energy level of the diets. It is possible that the effect of added oil may have been different and more marked, if higher dose levels of the meat meal, such as used in other experiments, had been included.

The addition of oil to the starch replaced diets also reduced the estimated potency of the meal based on weight gains, but as the estimates using weight gains were not statistically valid, these results are probably not meaningful. Similarly, the improved agreement between the potency estimate for the starch substituted diets with the addition of oil, with the potencies, also based on weight gains, obtained with the gluten replaced diets is probably not significant.

For all assays, the potencies based on FCE values were higher than the potency estimates with weight gain as the response. The potency estimates with FCE as the response metameter however tended to exceed the total lysine content of the meal (6.5 g/16 g N). The estimates based on FCE values, particularly for those assays in which gluten was replaced, agreed quite closely with the estimates obtained in experiment 5 for Mm F.

CHAPTER III

PART I : B, APPARENT RETENTION OF DIETARY AMINO ACIDS

INTRODUCTION

Relatively few studies have investigated the digestibility of the individual amino acids, or even of nitrogen, in proteins for poultry, as this requires surgical modification of the birds to separate the faeces from urine. Bragg, Ivy and Stephenson (1969) compared the amino acid excretion of normal and surgically modified birds fed the same diets and concluded that the method using the normal chick was to be preferred since the amino acid availability values obtained with the surgically modified chicks were sometimes greater than 100%. In this study the combined urinary and faecal excretion of amino acids was estimated when different meat meals were fed to chicks as the sole source of protein. The value obtained would therefore reflect not only the digestibility and absorption of the amino acids from the protein but also would include any amino acids or peptide-bound amino acids which may have been absorbed in a non-utilisable form and excreted in the urine.

Little information is available on the endogenous urinary excretion of amino acids. Teekell, Richardson and Watts (1968) found that the urinary excretion of amino-nitrogen by surgically modified layers remained rather constant with diets containing from 0.0 - 13.7% protein from dried whole egg. A similar result was reported by Kiriyama (1970) for rats fed a protein-free or a 15% casein diet but the distribution of urinary amino acids was distinctly different for the two diets. In both studies, the daily total urinary amino-nitrogen or amino acid excretion was low (5 - 7 mg per bird and less than 2 mg per rat,) so that the endogenous urinary excretion of amino acids would probably have little effect on the

calculation of the excretion of the dietary amino acids.

The metabolic faecal excretion of amino acids may, however, account for an appreciable proportion of the total faecal amino acid excretion, especially when low levels of dietary protein are supplied (Harper and De Muelenaere, 1963). The metabolic losses of amino acid, or nitrogen, represent the unabsorbed residues resulting from proteolysis of the digestive enzymes secreted into the intestine, as well as losses due to slough off of cells from the intestinal wall, and losses of bacterial protein. These metabolic losses are usually estimated by measuring the excretion of amino acids, or of nitrogen, when a protein-free diet is fed, or when very low levels of a highly digestible protein is included in the diet.

The metabolic faecal nitrogen excretion was found to be constant per gram of food consumed (with constant fibre content) by Mitchell (1924) and similar results were obtained by Kuiken and Lyman (1948) for the endogenous faecal amino acid excretion; however, Boas Fixsen and Jackson (1932) claimed the metabolic faecal nitrogen was independent of the food intake. There is also disagreement on the effect of dietary protein on metabolic losses. Mitchell and Bert (1954) concluded that the metabolic faecal nitrogen was independent of the dietary protein level, as the value obtained with a low protein diet was the same as that estimated by extrapolating a series of nitrogen levels to zero. However, Twombly and Meyer (1961) found that the value obtained by extrapolation was lower than that obtained when rats were fed a protein-free diet. In addition, Crampton and Rutherford (1954) reported that there was a slight increase in faecal protein with increasing levels of highly digestible protein which they suggested was probably bacterial in origin.

In this study the metabolic excretion of amino acids was estimated by feeding either a protein-free or low-protein diets containing only 2 or 4%

protein. The values obtained were used to correct the excretion of amino acids when the test protein was the sole source of protein in the diet. The difference between the combined excretion and the intake of the amino acid would represent the apparent retention of the amino acid by the chick. In experiment 7, wheat gluten was the test protein and in experiment 8, the apparent retention of amino acids in six meat meals was estimated.

MATERIALS AND METHODS

Management and conditions of chick experiments

Many of the procedures with chicks in this part of the experimental work have been described in sections concerned with the growth assay experiments. The chicks were fed a commercial chick starter ration for the first 14 days after hatching, followed by three feeding regimes over three consecutive seven day periods, I, II and III. The metabolic excretion was determined in periods I and III, while the test proteins were fed in the middle period, (II). The chicks were weighed at the beginning and end of each period and food consumption recorded for each period.

Diets

Semi-purified diets were used, with the same components as used in the growth assay experiments, and mixed by the same procedure. A basal diet, D, was prepared consisting of soybean oil, cellulose, vitamin and mineral premixes (as for the growth assay, see Table E, Appendix 1), and starch; the composition of this diet is given in Table D, Appendix 1. All diets contained 0.3% chromium sesquioxide (Cr_2O_3) to act as a marker to estimate the amount of food ingested, corresponding to the excreta samples; the preparation and purity of the Cr_2O_3 is described in Table A, Appendix 1. In experiment 7, 3.06% $Ca_3(PO_4)_2$ was included in all diets, and this

amount was also included in the protein-free and low-protein diets used in experiment 8 for the estimation of metabolic amino acid excretion. Varying amounts of $Ca_3(PO_4)_2$ were added to the test diets in experiment 8 depending on the amount of calcium contributed by the meat meal, but all diets contained 1.6% calcium. The calcium salt was added to the basal diet D at the expense of starch in both experiments.

Collection of excreta

The excreta was collected for the last three days of each period, i.e., two samples were collected from each replicate for the estimation of the metabolic excretion, and one sample when the test protein was fed. Brown waxed paper was laid on the dropping trays each day of the collection period. As the dropping tray was shared by four compartments, a wooden divider was used to section off the areas corresponding to each compartment; any spilt food or feathers were removed or avoided when the samples were collected. Quantitative collection of the excreta was not required. The excreta was collected daily in waxed cartons and immediately frozen. The samples were later dried in a vacuum oven at 80°, ground through a 40-mesh/inch screen, and stored in air tight containers for analysis.

Analysis of the excreta samples

The amino acid composition of the excreta samples was determined as described in the section on chemical analysis for the growth assay experiments. The Cr_2O_3 content of the excreta was estimated by the method of Czarnocki, Sibbald and Evans (1961).

Calculation of amino acid excretion and apparent retention of dietary amino acids

The amino acid excretion/100 g food eaten was calculated from the following relationship:

$$\text{amino acid excreted/g food eaten} = \text{amino acid/g excreta} \times \frac{Cr_2O_3/\text{g diet}}{Cr_2O_3/\text{g excreta}}$$

The amino acid excretion/100 g food eaten when the test proteins were fed, was corrected for metabolic losses by subtracting the value obtained when the low-protein or protein-free diets were fed. The % apparent retention of a dietary amino acid was calculated as:

$$100 - \left[\frac{\text{corrected amino acid excreted/g food eaten}}{\text{dietary amino acid/g diet}} \times 100 \right]$$

Tests of significance between excretion or apparent retention values for amino acids were made using Duncan's multiple range test (Duncan, 1955).

EXPERIMENTS WITH CHICKS AND RESULTS

Two experiments, (7 and 8), were conducted to investigate the apparent retention of dietary amino acids by chicks. Similar procedures were used in both experiments.

Experiment 7, the apparent retention of amino acids in wheat gluten

This was a preliminary experiment to examine the feasibility of using the combined excretion of chicks fed a single source of protein, for estimating the apparent retention of dietary amino acids. The metabolic excretion of amino acids was estimated with 2 or 4% protein diets (coded 2 or 4% LPD) fed to chicks; the protein source was casein which has a high nutritional value and should be readily digested. The casein is described in Table A, Appendix 1. The results obtained with the two low-protein diets were compared, as the breakdown of tissue, which would occur particularly with the 2% protein diet, may increase the excretion of amino acids; similarly some excretion of dietary amino acids may occur when the 4% protein diet was fed. These low-protein diets were fed before and after the period in which the test proteins were supplied so that the effect of an increase in body weight could be

assessed.

The test protein in this experiment was wheat gluten. The apparent retention of the lysine present in gluten was of primary interest because a value for the availability of lysine was required in the growth assays where meat meals were added to the test diets by isonitrogenous substitution of gluten. The wheat gluten was included in the test diets of experiment 7 to supply either 10 or 12.5% protein so that the effect of the dietary protein level on the excretion of amino acids could be determined. In addition, the effect of supplementing the wheat gluten with the limiting amino acids, lysine, methionine and arginine was investigated. Supplementation would improve the utilisation of the protein and may therefore reduce the excretion of amino acids. A further treatment compared the excretion of dietary amino acids from gluten when the chicks had been either supplied with the 2 or 4% protein diet in the preceding period. The three test diets, with the diet codes, were:

1. 10% gluten protein, T-X
2. 10% gluten protein supplemented with 0.21% lysine, 0.18% methionine and 0.21% arginine, T-Y
3. 12.5% gluten protein, T-Z

The protein supplements, casein or gluten, and amino acid supplements were added to basal diet D at the expense of starch. The level of supplements in the 2 or 4% protein diets and the test diets are given in Table I, Appendix 1.

The four treatment regimes were as follows:

Treatment regime	Period I (7 days)	Period II (7 days)	Period III (7 days)
X-1	4% protein diet	10% gluten protein	4% protein diet
X-2	2% protein diet	10% gluten protein	2% protein diet
Y	4% protein diet	10% gluten protein, supplemented.	4% protein diet
Z	4% protein diet	12.5% gluten protein	4% protein diet

There were two replicates each of 16 chicks (equal sexes) per treatment regime, and the 8 compartments were arranged as a randomised block over two tiers of a battery brooder. A schematic diagram of the treatment regimes and procedures is given in Figure 4.

Results

The mean weight change and food consumption per chick for each period of experiment 7 are given in Table XI. Feeding the low-protein diets in periods I and III resulted in some weight loss but the chicks remained healthy with only two deaths out of a total of 256 chickens, during the whole experiment. Supplementation of the wheat gluten with lysine, arginine and methionine increased the weight gain of the chicks in period II. The calculated metabolic excretion of amino acids is given in Table XII; this was very similar for both periods (I and III) that the low-protein diets were fed. However, the amino acid excretion for the combined periods I and III tended to be slightly lower with the 2% protein diet than when the 4% protein diet was supplied; lower amounts of threonine, valine and isoleucine were excreted ($P < 0.05$) but the difference was not significant for the other essential amino acids.

The values obtained for the amino acid excretion when the 2% protein diet was fed were used to correct the values obtained when the test diets of gluten were supplied to the chicks, and the calculated excretion and percentage apparent retention of dietary amino acids are shown in Tables XIII and XIV respectively. There was no difference in the excretion or apparent retention of amino acids with the 10% gluten protein diet when this was estimated after feeding either the 2 or 4% protein diets. Slightly lower excretion and apparent retention values were obtained when 12.5% gluten protein was present in the diet than with the 10% protein diet and supplementation of the latter diet with lysine, arginine and methionine further reduced the values for some amino acids but this

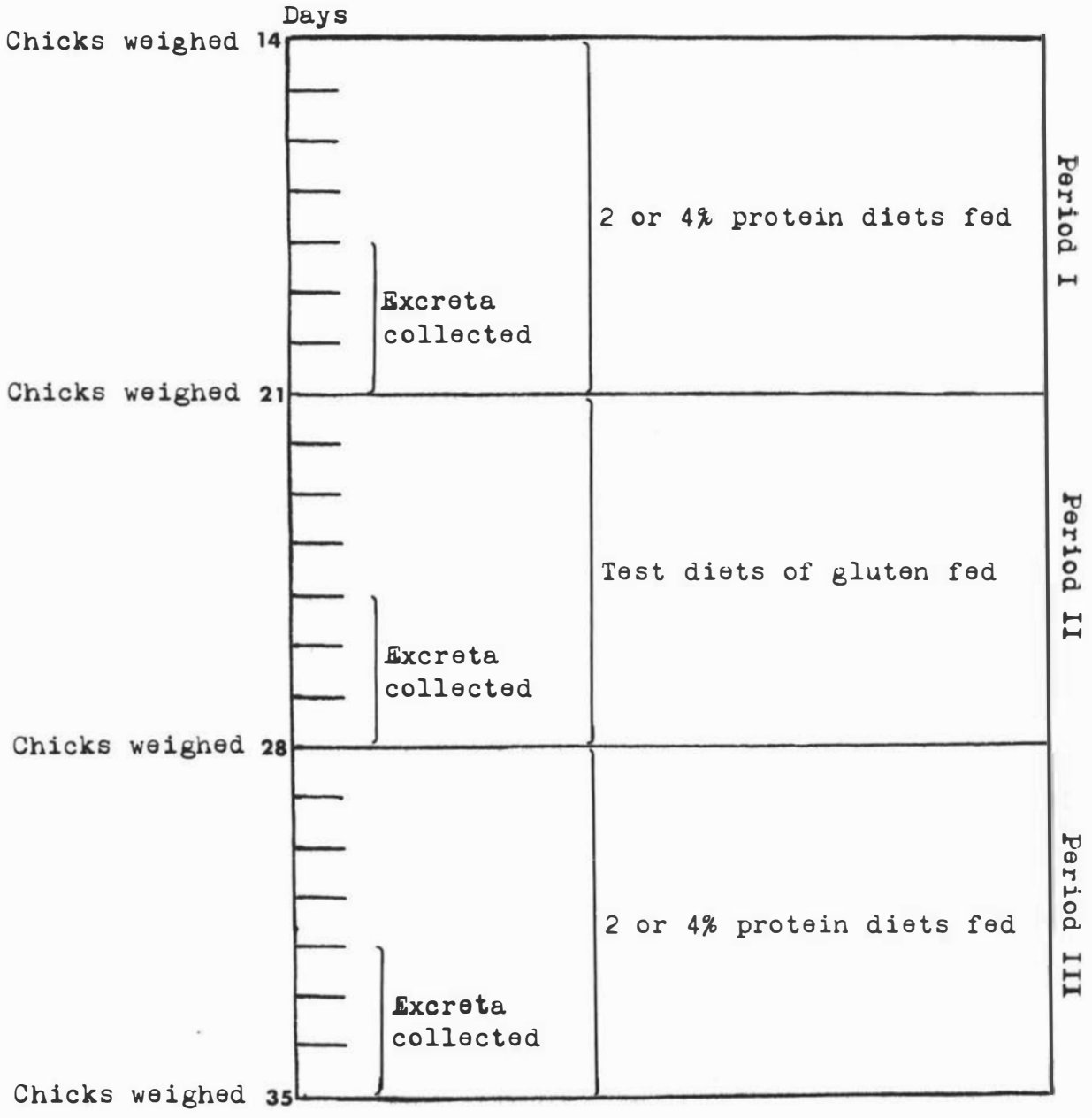


FIGURE 4. Schematic diagram of treatment regimes in experiment 7.

TABLE XI

Body weight change and food consumption (g/chick per seven day period) for each period of treatment regimes, experiment 7

		Body weight change			Food consumption		
Treatment regime	Period:	I	II	III	I	II	III
X-1		- 6.2	7.1	-3.5	81.9	59.4	60.3
X-2		-11.2	2.7	-3.1	72.4	49.6	48.8
Y		- 8.3	21.9	-4.1	78.5	87.4	68.4
Z		- 8.8	7.6	-2.4	75.0	63.0	66.7

TABLE XII

Amino acid excretion by chicks fed either

2 or 4% protein diets, experiment 7

	mg amino acid excreted / 100 g food eaten					
Diet:	4% protein diet			2% protein diet		
Period:	I *	III *	Mean values	I **	III **	Mean values (± S.E.)
Amino acid						
Lysine	52	49	50	43	47	45 ± 1.4
Histidine	22	24	23	22	22	22 ± 0.5
Arginine	22	32	27	20	21	20 ± 2.4
Threonine	90	77	83	57	54	55 ± 3.4
Valine	78	87	82	69	60	64 ± 4.2
Methionine	15	14	14	13	13	13 ± 1.7
Isoleucine	64	70	67	54	46	50 ± 3.6
Leucine	73	85	79	71	61	66 ± 3.8
Phenylalanine	34	43	38	46	31	38 ± 4.8
Tyrosine	34	36	35	32	26	29 ± 2.9
Aspartic acid	97	103	100	74	75	74 ± 4.4
Serine	113	116	114	95	70	82 ± 8.0
Glutamic acid	203	217	210	137	132	134 ± 2.0
Proline	79	85	82	80	56	68 ± 7.4
Glycine	245	198	221	184	134	159 ± 16.9
Alanine	72	82	77	68	59	63 ± 4.2
Half cystine	23	26	24	18	18	18 ± 1.4

* Mean of six replicates

** Mean of two replicates

TABLE XIII

Amino acid excretion* by chicks of dietary amino acids from wheat gluten, experiment 7

	mg amino acid excreted / 100 g food eaten			
Treatment regime:	X-1	X-2	Y	Z
% protein:	10.0	10.0	10.0**	12.5
Period I diet:	4% LPD	2% LPD	4% LPD	4% LPD
Amino acid				
Lysine	6	7	13	3
Histidine	5	6	3	3
Arginine	36	36	25	37
Threonine	38	34	9	25
Valine	34	26	3	13
Methionine	10	6	2	3
Isoleucine	16	15	-7	7
Leucine	44	40	6	27
Phenylalanine	38	22	7	22
Tyrosine	16	13	6	10
Aspartic acid	42	38	16	38
Serine	22	3	-13	10
Glutamic acid	121	142	35	116
Proline	51	45	2	55
Glycine	163	102	59	100
Alanine	36	37	2	20
Half cystine	27	14	6	10

* Mean of two replicates for each diet.

** Supplemented with lysine, arginine and methionine.

TABLE XIV

Apparent retention* (%) by chicks of dietary amino acids from wheat gluten, experiment 7

Treatment regime:	X-1	X-2	Y	Z	Mean values,
% protein:	10.0	10.0	10.0**	12.5	all diets
Period I diet:	4% LPD	2% LPD	4% LPD	4% LPD	(± S.E.)
Amino acid					
Lysine	96.4	95.8	91.1	98.7	95.5 ± 2.21
Histidine	98.7	97.2	99.2	99.0	98.5 ± 0.59
Arginine	89.9	90.0	93.1	91.7	91.1 ± 0.85
Threonine	83.7	85.3	91.3	91.2	89.1 ± 2.33
Valine	92.1	94.0	99.4	97.6	95.8 ± 1.40
Methionine	89.5	93.0	97.8	96.8	94.3 ± 1.81
Isoleucine	95.5	95.9	100+	98.5	97.4 ± 0.94
Leucine	93.7	94.0	99.1	96.9	96.0 ± 0.32
Phenylalanine	90.8	94.8	98.3	95.6	94.9 ± 0.40
Tyrosine	93.1	94.4	97.5	97.5	95.6 ± 0.95
Aspartic acid	87.1	87.6	94.7	90.2	89.9 ± 5.19
Serine	94.6	99.3	100+	98.0	98.0 ± 1.18
Glutamic acid	96.5	95.9	99.0	97.5	97.2 ± 0.59
Proline	96.0	96.5	99.1	96.5	97.2 ± 0.73
Glycine	53.0	70.9	84.9	77.2	71.5 ± 5.54
Alanine	86.7	86.5	98.9	94.0	91.5 ± 2.62
Half cystine	80.4	89.7	95.7	94.4	90.0 ± 3.23

* Mean of two replicates for each diet.

** Supplemented with lysine, arginine and methionine.

+ Subtraction of metabolic loss resulted in negative excretion.

decrease was not significant ($P < 0.05$) for any of the essential amino acids. Overall, the mean percentage excretion was less than 6% of the ingested essential amino acids of wheat gluten, with the exception of arginine and threonine which gave slightly lower retention values. The amino acids excreted in greatest quantity were glutamic acid and glycine, but as gluten contains a large amount of glutamic acid the percentage excreted was low; by contrast, for glycine, an amount nearly equivalent to one-third of that ingested, was excreted.

Experiment 8, the apparent retention of amino acids in meat meals

As the excretion of amino acids in experiment 7 was slightly lower with the 2% protein diet than with the 4% protein diet, the 2% protein diet was used in this experiment for the estimation of metabolic excretion, with a modification in one treatment. With this treatment, chicks were fed the 2% protein diet for only the first three days of periods I and III, instead of for all the seven days as in all other treatments. For the last four days of periods I and III, a protein-free diet was fed and the excreta samples collected on the last three days as usual.

The protein-free diet (coded PFD) was included in this experiment to verify that the amino acid excretion with the 2% protein diet was near to the minimal level, and was representative of the metabolic excretion. However, a four day period on this diet was instituted as it was expected that the complete seven day period would be too drastic for the chicks; in experiment 7 there was considerable weight loss over seven days with the 2 and 4% protein diets.

The apparent retention of amino acids in six meat meals by chicks was estimated in this experiment. These meat meals were the same as those assayed for lysine in the growth assay experiments; these are described in Table A, Appendix 1, and the proximate composition, FDNB-reactive lysine and calcium content are given in Table B, Appendix 1, and the amino acid

composition in Table C, Appendix 1. The meat meals were added to basal diet D at the expense of starch to supply 10% protein. The test diets were coded T with the code letter of the meat meal attached e.g. T-A for the diet containing Mm A. The amount of $Ca_3(PO_4)_2$ added to the diets was adjusted to that contributed by the meat meals so that all diets contained 1.6% calcium. Details of the supplements in these diets are given in Table J, Appendix 1. The test diet containing Mm A was included in two treatment regimes, A-1 in which the protein-free diet was supplied for part of period I, and treatment regime A-2 in which the 2% protein diet was supplied for all of period I. The treatment regimes were as follows:

Treatment regime	Period I (7 days)	Period II (7 days)	Period III (7 days)
A-1	2% LPD, 3 days PFD, 4 days	10% protein, T-A	2% LPD, 3 days PFD, 4 days
A-2	2% LPD, 7 days	10% protein, T-A	2% LPD, 7 days
B	2% LPD, 7 days	10% protein, T-B	2% LPD, 7 days
C	2% LPD, 7 days	10% protein, T-C	2% LPD, 7 days
D	2% LPD, 7 days	10% protein, T-D	2% LPD, 7 days
E	2% LPD, 7 days	10% protein, T-E	2% LPD, 7 days
F	2% LPD, 7 days	10% protein, T-F	2% LPD, 7 days

There were two replicates of 14 pullets per treatment regime and the 14 compartments were randomly assigned to two batteries. A schematic diagram of the treatment regimes and procedures used is given in Figure 5.

Results

The mean weight change and food consumption per chick for each period of each treatment regime in experiment 8 are given in Table XV. The mean weight loss when the chicks were fed the protein-free diet for the

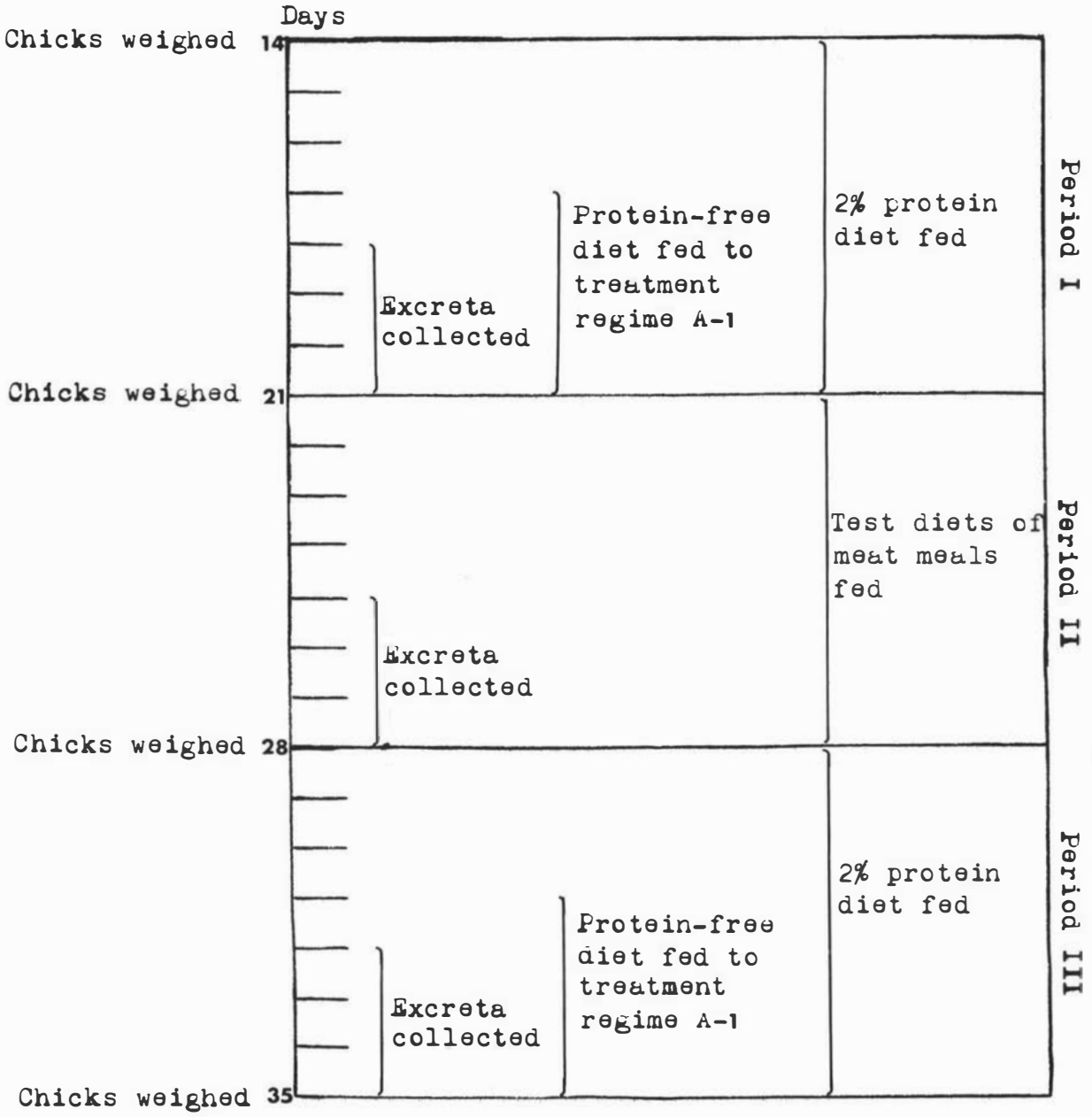


FIGURE 5. Schematic diagram of treatment regimes in experiment 8.

TABLE XV

Body weight change and food consumption (g/chick per seven day period) for each period of treatment regimes, experiment 8

	Body weight change			Food consumption		
Treatment regime Period:	I	II	III	I	II	III
A-1	-18.6	3.9	- 9.4	75.6	51.9	29.5
A-2	-17.5	12.4	-12.9	83.9	64.4	26.6
B	-18.1	30.1	-18.9	76.8	89.8	35.5
C	-19.7	21.3	-15.4	82.2	77.4	39.5
D	-14.6	20.0	-17.0	89.0	79.1	38.5
E	-18.3	13.3	-15.5	88.3	70.5	34.9
F	-15.5	54.0	-23.3	91.5	121.9	43.3

estimation of the metabolic amino acid excretion in periods I and III was no greater than for those fed the 2% protein diet. However, the chicks fed the protein-free diet showed a smaller weight gain when supplied Mm A (treatment regime A-1) in period II than those fed the same meat meal but which had been fed the 2% protein diet in period I (treatment regime A-2). Weight gains in period II when the test proteins were fed, varied from 3.9 to 54.0 g per chick probably reflecting the nutritional value of the meat meals. Three chickens were culled and there were four deaths out of the total of 196 chickens in the three weeks of the experiment.

The metabolic amino acid excretion for periods I and III are given in Table XVI. There was no difference in the amount of amino acids excreted between periods I and III when either the protein-free diet or that supplying 2% protein were fed to the chicks. The excretion values, when the protein-free diet or the 2% protein diet were fed, were very similar for all the essential amino acids except arginine. However, as the difference in excretion of arginine was only just significant ($P < 0.05$), the values for the excreted amino acids of all groups fed either the protein-free or the 2% protein diet, and for both periods I and III, were used to estimate a mean value for the metabolic excretion; these mean values are included in Table XVI.

The percentage apparent retention of amino acids (corrected for metabolic losses), and the amino acid excretion (mg per 100 g food eaten), when the meat meals were fed to chicks in period II, are given in Tables XVII and XVIII respectively. The agreement between replicates for the retention of amino acids was good except for those of treatment regime A-1, with Mm A, which had been fed the protein-free diet prior to the meat meal test diet being supplied. The standard deviation of the mean percentage apparent retention of each of the essential amino acids was much greater for this than in other treatments.

TABLE XVI

Amino acid excretion by chicks fed either a protein-free diet or a 2% protein diet, experiment 8

	mg amino acid excreted / 100 g food eaten				
	Protein-free diet		2% protein diet		
Period:	I*	III*	I*	III**	Mean values*** (± S.E.)
Amino acid					
Lysine	22	39	45	40	37 ± 3.3
Histidine	16	22	20	18	19 ± 1.0
Arginine	38	52	17	25	32 ± 5.2
Threonine	46	55	52	45	48 ± 2.5
Valine	52	57	51	47	51 ± 2.3
Methionine	10	17	11	13	13 ± 1.2
Isoleucine	36	45	47	42	42 ± 2.1
Leucine	60	71	59	56	61 ± 3.2
Phenylalanine	35	42	32	28	34 ± 2.5
Tyrosine	26	35	34	29	31 ± 1.8
Aspartic acid	57	88	68	78	73 ± 4.8
Serine	52	61	70	67	61 ± 4.3
Glutamic acid	91	131	132	145	123 ± 9.1
Proline	57	46	47	50	49 ± 2.9
Glycine	223	240	247	183	225 ± 10.2
Alanine	54	68	45	44	51 ± 4.2
Half cystine	28	26	19	17	22 ± 2.4

* Mean of two replicates.

** Mean of three replicates.

*** Standard error of mean excretion for both diets.

TABLE XVII

Apparent retention* (%) by chicks of dietary amino acids from meat meals (Mm A - Mm F), experiment 8 (With S.D. of replicates for essential amino acids)

Meat meal code: / Treatment regime:	A A-1	A A-2	B B	C C	D D	E E	F F	Mean values (all meals)
Lysine	$85.50^{a}\pm3.26$	$89.10^{b}\pm0.00$	$91.10^{b}\pm2.13$	$87.80^{ab}\pm0.15$	$91.25^{b}\pm0.64$	$89.20^{b}\pm0.43$	$99.70^{c}\pm0.43$	90.53
Histidine	$83.15^{ab}\pm5.17$	$79.45^{a}\pm1.50$	$86.15^{b}\pm4.6$	$86.40^{b}\pm0.43$	$89.30^{b}\pm0.00$	$88.45^{b}\pm0.00$	$99.45^{c}\pm0.75$	87.48
Arginine	$91.70^{a}\pm5.60$	$94.10^{ab}\pm0.00$	$95.05^{ab}\pm2.5$	$94.15^{ab}\pm1.70$	$96.80^{ab}\pm0.00$	$97.25^{ab}\pm1.10$	$100^{b}\pm0.64$	95.51
Threonine	$89.90^{abc}\pm9.63$	$87.10^{a}\pm0.57$	$89.90^{abc}\pm0.54$	$85.50^{a}\pm0.29$	$97.45^{bc}\pm2.90$	$88.20^{ab}\pm2.28$	$98.90^{c}\pm1.56$	90.99
Valine	$88.40^{a}\pm4.53$	$86.00^{a}\pm0.29$	$90.30^{ab}\pm4.25$	$87.75^{a}\pm0.50$	$95.00^{bc}\pm0.71$	$87.20^{a}\pm0.99$	$98.90^{c}\pm0.15$	90.51
Methionine	$85.60^{a}\pm9.76$	$81.25^{a}\pm1.77$	$90.25^{ab}\pm2.76$	$91.80^{ab}\pm3.82$	$92.40^{ab}\pm0.99$	$91.35^{ab}\pm4.45$	$97.35^{b}\pm1.91$	90.00
Isoleucine	$88.25^{a}\pm3.04$	$91.10^{ab}\pm1.56$	$90.20^{ab}\pm2.83$	$89.40^{ab}\pm0.42$	$92.85^{b}\pm1.06$	$88.60^{ab}\pm0.71$	$99.50^{c}\pm0.00$	91.41
Leucine	$89.75^{a}\pm1.48$	$89.85^{a}\pm0.35$	$91.50^{ab}\pm2.69$	$89.65^{a}\pm0.07$	$93.80^{b}\pm0.57$	$90.35^{a}\pm0.49$	$99.40^{c}\pm0.28$	**92.**04
Phenylalanine	$88.75^{a}\pm2.62$	$90.95^{a}\pm0.21$	$90.35^{a}\pm2.90$	$90.15^{a}\pm1.06$	$95.15^{b}\pm0.64$	$90.45^{a}\pm0.64$	$99.70^{c}\pm0.00$	92.21
Tyrosine	91.05 ± 10.39	93.95 ± 0.35	93.80 ± 1.13	89.50 ± 2.12	97.00 ± 2.83	91.95 ± 0.35	$100^{+}\pm0.71$	93.49
Aspartic acid	83.85 ± 7.14	85.00 ± 0.14	86.55 ± 4.45	82.80 ± 1.13	89.95 ± 0.92	83.10 ± 1.13	97.40 ± 1.98	86.95
Serine	92.35 ± 6.15	91.35 ± 0.21	94.00 ± 4.81	93.50 ± 1.13	$100^{+}\pm6.15$	94.70 ± 2.55	$100^{+}\pm0.00$	94.51
Glutamic acid	91.60 ± 4.81	91.20 ± 1.27	94.80 ± 1.84	88.45 ± 1.06	97.65 ± 0.35	93.75 ± 1.91	99.05 ± 1.34	93.79
Proline	93.30 ± 2.12	91.50 ± 0.14	93.40 ± 2.83	90.80 ± 0.85	96.90 ± 0.99	90.90 ± 0.00	97.85 ± 3.04	93.52
Glycine	82.85 ± 3.61	82.80 ± 0.71	90.20 ± 6.22	81.15 ± 0.22	93.30 ± 1.13	84.85 ± 5.59	97.40 ± 3.25	87.51
Alanine	90.75 ± 2.62	90.55 ± 0.07	90.05 ± 7.14	90.50 ± 0.57	95.35 ± 1.06	89.15 ± 0.49	96.70 ± 0.00	91.86
Half cystine	ND	65.00	ND	100^{+}	ND	92.00	100^{+}	89.25

* ^{a,b,c} Mean of two replicates for each meat meal, except values for cystine which were from a single replicate. Means on the same line bearing different superscripts differ significantly ($P<0.05$).

ND, Not determined. + Subtraction of metabolic loss resulted in negative excretion.

TABLE XVIII

Amino acid excretion* by chicks of dietary amino acids from meat meals (Mm A - Mm F), experiment 8

	mg amino acid excreted / 100 g food eaten						
Treatment regime:	A-1	A-2	B	C	D	E	F
Meat meal code:	A	A	B	C	D	E	F
Amino acid							
Lysine	85	64	62	63	51	61	2
Histidine	32	39	23	24	16	18	1
Arginine	45	32	30	46	15	18	-5
Threonine	36	46	50	43	10	40	3
Valine	58	70	64	52	28	55	5
Methionine	11	15	22	9	11	9	4
Isoleucine	27	20	49	36	23	36	2
Leucine	80	79	80	70	47	67	4
Phenylalanine	40	32	39	33	16	32	1
Tyrosine	17	11	23	21	6	18	-8
Aspartic acid	138	129	120	113	82	136	21
Serine	30	34	30	19	-2	18	-11
Glutamic acid	109	114	75	130	36	85	21
Proline	49	62	47	52	22	50	18
Glycine	252	252	65	239	96	160	30
Alanine	85	86	70	81	43	81	24
Half cystine	ND	14	ND	-19	ND	4	-9

* Mean of two replicates for each meat meal, except values for cystine which were from a single replicate.

ND, Not determined.

The apparent retention was 85% or more for all the essential amino acids in the meat meals except histidine and methionine which had a slightly greater range; only 79.45% histidine was retained with diet T-A in treatment regime A-2 and 81.25% methionine with diet T-A in treatment regime A-2. Of the essential amino acids, arginine was the most avidly retained from all the meat meals with an average retention of 95.51%. There was little difference in the percentage apparent retention of the essential amino acids from meat meals, Mm A - Mm E; the value for lysine was significantly lower with diet T-A in treatment regime A-1, compared to the same diet in treatment regime A-2, and it was also significantly lower than the values obtained for the other meals except for Mm C. The highest percentage apparent retention and the lowest excretion values for the essential amino acids was given by Mm F and the difference between the retention values for this and other meals was significant for lysine, histidine, isoleucine, leucine and phenylalanine.

The percentage apparent retention of the non-essential amino acids was similar to that of the essential amino acids and for most meals the retention was greater than 85% although the values for aspartic acid and glycine were occasionally lower than this. Glycine was excreted in large quantities when Mm A and Mm C were the test proteins and this may not have necessarily reflected the amount present in the meat meals as Mm D had a high content of glycine but the excretion was much lower than for Mm A and Mm C.

CHAPTER IV

PART I : DISCUSSION

A. Growth Assay for lysine

The assay procedure developed and used for the estimation of biologically available lysine in eight meat meals satisfied the normal statistical requirements. The range of response to the levels of lysine in the standard diets was large and the amounts of the meat meals included in the test diets resulted in responses of a similar magnitude. The dose - response relationship was linear with either weight gain or FCE as the response and % lysine in the supplement as the dose metameter. A valid analysis could be made by the slope-ratio technique with either response metameter for experiments 3 - 5; in experiment 6 the analysis based on weight gain was not statistically valid.

However, the potencies estimated when the meat meal replaced starch in the basal diet usually differed to those obtained when gluten was replaced isonitrogenously. This was particularly evident with weight gain as the response metameter, and for three of the four meat meals compared, the potency estimates based on weight gain differed by more than the approximate 95% fiducial limits. This difference in potency estimates by the two methods of meat meal addition indicates that the assays were affected by factors other than the quantity of lysine present in the supplements.

The addition of the meat meal at the expense of starch in the basal diet increased the crude protein content of the test diets by a minimum of nearly 2, 4 and 8% compared to that present in the respective standard diets, and for some meals the increments were greater (Tables G and H, Appendix 1). This increase in crude protein content would alter the amino acid pattern of the diets considerably, even although the basal diet

contained 20% protein. In addition, the calorie-protein ratio of the diets would be altered.

The addition of the meat meal to the basal diet by isonitrogenous substitution of the wheat gluten would minimise changes in amino acid pattern and the calorie-protein ratio, but the method required an estimate of a value for the availability of lysine in gluten. The assumption that lysine in wheat gluten is 93% available on the basis of the FDNB-reactive lysine value, may not be justified, although the percentage apparent retention obtained in this study was 96%. Calhoun et al (1960) reported that lysine in wheat gluten was 80% available by rat growth assay. The amounts of lysine required to be added to replace that in the removed gluten were 20 - 33% of the lysine expected to be contributed by the test proteins, so that an error in the calculation of the available lysine content of the gluten could have an appreciable effect on these isonitrogenous assays.

In addition, it has been suggested that free lysine added to diets may be metabolised to different effect to that of lysine present in proteins (Carpenter et al, 1963; Porter and Rolls, 1971). A difference in the metabolism of free and bound lysine would not only influence the results obtained in assays where the meat meal replaced gluten, but also the relationship of the response from the standard diets to that obtained with test diets. This might be a contributing factor in assays where the estimated potency of the meat meal was greater than the total lysine content of the meat meal.

Food consumption is influenced by many factors including the type of carbohydrate (Gupta et al, 1958), protein level, calorie-protein ratio and amino acid balance (De Muelenaere et al, 1967a), and it is probable that part at least of the variation in estimated potencies based on weight gain with different methods of meat meal inclusion in the test diets, may be

due to an effect on the appetite of the chicks. The potency estimates with FCE as the response metameter were not significantly different when the meat meals were assayed by either replacement of starch or isonitrogenous substitution of gluten, and for three of the four meals the two potency estimates agreed quite closely, including potencies for the same meal obtained in two different experiments. Calculations which take differences in food consumption into account, minimise any appetite-depressing or stimulating effect that the test material might have on the estimation of potency of an amino acid, and Carpenter et al (1963), and Miller et al (1965b), preferred the estimate based on FCE to that based on weight gain. The potency estimates with FCE as the response have been reported to be generally higher, by about 13%, than estimates based on weight gains (Uwaegbute and Lewis, 1966; Miller et al, 1965b; Carpenter, McDonald and Miller, 1972) but the values reported in this study do not show any consistent difference.

B. Apparent retention of dietary amino acids

As the excretion values obtained when the protein-free or 2% protein diets were fed were very similar, these values were probably representative of the combined metabolic excretion of amino acids by chicks. In contrast, the values obtained with the 4% protein diet were slightly higher, suggesting that some dietary amino acids were excreted. The estimates of the metabolic excretion of amino acids with the protein-free or 2% protein diets agreed closely for both experiments, and were unaffected by the small increase in body weight which occurred between periods I and III. However, when these values were used to correct the amino acid excretion with the test proteins, a negative value sometimes resulted. This suggests that the true metabolic amino acid excretion values may be lower than those obtained with the protein-free or 2%

protein diets. In addition, although the chicks fed the protein-free diet remained healthy, the severity of this treatment may have influenced results, as there was greater variation between replicates when the test protein was fed than for the other treatment regimes.

Large amounts of glycine and glutamic acid were excreted when the protein-free or low-protein diets were fed, and this may reflect the urinary excretion. Kiriyama (1970) reported that rats fed a protein-free diet had an increased urinary excretion of these amino acids and that they accounted for more than 50% of the total amino acid excreted.

The results obtained when test diets containing wheat gluten were fed indicated that the dietary protein level and amino acid balance of the protein component had little effect on the apparent retention of amino acids by chicks. The essential amino acids of wheat gluten were apparently highly retained, although the value for threonine was slightly less than 90%. Hepburn, Calhoun and Bradley (1966) also found that the digestibility of individual amino acids in wheat gluten was high.

The overall percentage apparent retention of the essential amino acids in the meat meals of about 85%, agrees with the nitrogen digestibility values of 81 - 87% reported by Atkinson and Carpenter (1970). There were however, some significant differences in the apparent retention of certain essential amino acids and in particular, the values for Mm F were generally greater than for the other meat meals. These differences in the apparent retention of amino acids would contribute to the variation in the availability of amino acids to animals.

Comparison of available lysine estimates

The percentage biologically available lysine of the meat meals assayed, using the potency estimates based on FCE (or mean of the estimates where there was more than one assay) are given in Table XIX, together with

TABLE XIX.

% lysine available in meat meals by chick growth assay (based on FCE), apparent retention and as FDNB-reactive lysine

Meat meal	Chick growth assay	Apparent retention	FDNB-reactive lysine
Mm A	61	87	76
Mm B	70	91	74
Mm C	69	88	73
Mm D	73	91	59
Mm E	93	89	79
Mm F	105	100	72
Mm G	83	-	74
Mm H	85	-	74

the percentage apparent retention values of the six meat meals investigated; the FDNB-reactive lysine values are also included. Comparison of these values indicates that there was little agreement between the three methods for the estimation of the biologically available lysine content of meat meals for chickens. The percentage apparent retention values for lysine were considerably higher and showed less variation than the values obtained by growth assay; this was in agreement with other studies in which the digestibility of amino acids were compared to results obtained by growth assay (Miller et al, 1965a; De Muelenaere et al, 1967a). The difference between these two biological estimates of lysine availability indicates that digestibility together with the possible absorption and urinary excretion of unavailable peptides, are not the only factors involved in determining the availability for growth of lysine in meat meals. It is probable that the rates of digestion and absorption influence the availability of amino acids to animals. The growth assay results do however indicate that a portion of the lysine present in meat meal proteins may not be available to chicks; for four of the eight meals more than 25% of the lysine was apparently unavailable.

Although for several meat meals, the FDNB-reactive lysine values were lower than the growth assay estimates for available lysine, these generally agreed more closely than did the estimates by the two biological methods. The FDNB-reactive lysine value would include the hydroxylysine contributed by the meat meals so that the value would tend to be over-estimated. By contrast, any free lysine would not be included in the estimate, and as meat meal proteins are poorly soluble, some lysine may have been unable to react with fluorodinitrobenzene, perhaps because of steric hindrance. Dinitrophenyl esters of aspartic and glutamic acids may also form which can act as acylating agents for

any lysine in that region of the protein (Asquith, Chan and Otterburn, 1969) and this would lower FDNB-reactive lysine estimates. However, the values obtained for the meat meals indicate that a portion at least of the ε-amino groups of lysine in some of these proteins, were blocked, and this would presumably contribute to the difference between the amount available for growth and the total lysine content of these meat meals.

Maillard-type reactions may have participated in the reduced availability of lysine in some of these meat meals but the apparent retention of lysine and other amino acids was quite high. In contrast, the digestibility of proteins heated in the presence of reducing sugars has generally been reported to be low (Miller et al, 1965a; Boctor and Harper, 1968). This suggests that the low availability of lysine in certain meat meals was partly the result of the chemical changes responsible for heat-damage in pure proteins.

CHAPTER V

PART II : MECHANISM OF HEAT-DAMAGE TO PURE PROTEINS

INTRODUCTION

The chemical changes which occur when pure proteins are heat-damaged, resulting in the decreased availability of certain amino acids, are not understood. Although many studies, already discussed in Part I, have shown that the ε-amino group of lysine is apparently bound when pure proteins are heated, the mechanism has not been defined. It is also not known if this binding is solely responsible for the reduced availability of lysine, and of other amino acids, with heat-damage of proteins.

Free amino groups were identified by Harris and Mattill (1940) as being the most likely groups involved in the formation of enzyme-resistant bonds in heat-damaged proteins. These workers suggested that under conditions favourable to dehydration the free amino groups might form anhydride linkages with carboxyl groups to create indigestible compounds such as diketo-piperazines. Mecham and Olcott (1947) found that when proteins were exposed to dry heat, the number of amino, acidic and basic groups decreased, and they interpreted this to indicate that new ester or amide linkages had been formed. Similarly, Evans and Butts (1949), on the basis of *in vitro* digestibility studies, suggested that the free amino groups of lysine and the free carboxyl groups of aspartic and glutamic acids combined to form enzyme-resistant linkages, when proteins were heat-damaged. This suggestion has remained the most quoted hypothesis to explain the mechanism of heat-damage to pure proteins.

The evidence obtained by *in vitro* digestibility studies for the involvement of aspartic and glutamic acids in new linkages with lysine may not be significant, as Ford and Salter (1966) pointed out that the rate of

release of these acids from cod meal was very slow even with unheated protein; this is also evident in the results of Evans and Butts(1949). Similarly, Hankes et al (1948) found that only about 11% of the total aspartic acid content of raw casein was released during *in vitro* digestion.

However, other studies have supported the possible formation of enzymatic-resistant linkages with lysine by aspartic and glutamic acids, when proteins were heat-damaged. For example, the results of Porter and Rolls (1971) on the digestion of heated cod meal by rats, showed that lysine and glutamic acid tended to be concentrated in peptides which were resistant to digestion. Similarly, Ford and Shorrock (1971) found that lysine, glutamic and aspartic acids together comprised 70% of the urinary peptide-bound amino acids when heated cod meal was fed to rats.

Bjarnason and Carpenter (1970) examined in detail the chemical changes which occurred when proteins were heated and they suggested that the main reaction of the ε-amino groups of lysine were with the amide groups of asparagine and glutamine, rather than with free carboxyl groups. These workers found some correlation of lysine binding with ammonia liberation and decrease in amide content of heat-damaged proteins. In addition, the presence of acetamide or N-acetyl-glutamine when proteins were heated, markedly decreased the FDNB-reactive lysine value, whereas the inclusion of ammonium acetate or N-acetyl-L-glutamic acid monosodium salt only slightly increased the binding. However, no product of such a reaction has been characterised.

The α-peptide linkages of lysine residues in which the ε-amino groups are blocked, can apparently be hydrolysed by digestive enzymes. Rats fed propionylated lactalbumen excreted in the urine a bound form of lysine, probably propionyl lysine (Bjarnason and Carpenter, 1969). Mauron (1972)

investigated the growth promoting property of ε-(γ -L-glutamyl)-L-lysine and found this peptide equivalent to lysine for rats, a finding confirmed by Waibel and Carpenter (1972) for both rats and chicks. These latter workers suggested that the peptide was probably hydrolysed in the intestinal wall as they found only traces of the compound in chick plasma. The ability of animals to utilise this peptide does not negate the hypothesis that heat-damage may be due to the formation of linkages between the ε-amino groups of lysine and aspartic or glutamic acids, or their amides. The presence of such linkages might hinder the digestion of the protein sufficiently to produce the decrease in nutritional value even although the bonds are eventually hydrolysed.

In the present study, an attempt has been made to determine if any covalent bonds involving lysine were formed during heat-damage of a protein. Samples of haemoglobin and globin were heated sufficiently to reduce the FDNB-reactive lysine value, and the products of digestion with enzymes, separated and compared to those formed from unheated protein. Initially, trypsin was used to digest the protein, since binding of the ε-amino groups of lysine would be expected to modify considerably the activity of trypsin on the protein. However, the products of tryptic digestion of the heated protein were large and difficult to investigate. Exhaustive enzyme digestion with a number of enzymes of varying specificity, was then used to try to detect fragments in which enzyme-resistant bonds were present. A peptide was isolated from exhaustive enzyme digests of heat-damaged globin which was composed of lysine and aspartic acid. This peptide was not detected in digests of unheated globin, and the nature of this compound was investigated.

MATERIALS AND METHODS

Chemicals

All solvents used for gel filtration, chromatography or electrophoresis (with the Pherograph), were refluxed with, and distilled from, ninhydrin. The following chemicals were used: 1 dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride), 2,4-dinitrofluorobenzene (FDNB), ε-(2,4-dinitrophenyl)-L-lysine hydrochloride, methyl chloroformate, hydrindantin (B.D.H. Ltd; England), L-1-tosylamide-2-phenylethyl-chloromethyl ketone (TPCK; Sigma Chemical Co; U.S.A.), phenylisothiocyanate (Pierce Chemical Co; U.S.A.), and trifluoroacetic acid (Koch-Light Laboratories Ltd; England).

Preparation of haemoglobin and globin samples

Samples of haemoglobin and globin were prepared from the same batch of ox blood, obtained from one animal. The fresh blood, with 0.2% potassium oxalate, was centrifuged at 4080 x g in a Sorvall refrigerated centrifuge (2°) to sediment the red blood cells. The cells were washed four times with 0.9% NaCl, lysed by freezing and thawing, and the cell debris removed by centrifugation at 23500 x g. Haemoglobin was precipitated by the addition of absolute ethanol at 0°, and after filtration, the precipitate washed with ice-cold ethanol. Another portion of the red blood cell contents was used for the preparation of globin; this was precipitated by the addition of 1.5% HCL in acetone at -20° (Anson and Mirsky, 1930), and the precipitate washed with ice-cold acetone. The haemoglobin and globin samples were dialysed against distilled water (2°) for 24 hours, lyophilised, and stored at 4°.

Heat-damaged haemoglobin or globin samples

The percentage moisture in the samples was estimated by drying in a vacuum oven at 60°, and sufficient water added to give a total moisture

content of 15 - 20%. Samples of haemoglobin, (about 2 g) and globin (1 g) were placed in thick walled Pyrex ampoules and, after the head space was reduced by the insertion of a solid glass tube, the ampoules were sealed and heated at 120° for 24 hours.

Enzymes

The following enzymes were used: trypsin, twice crystallised; α-chymotrypsin, four times crystallised (B.D.H. Ltd; England); pronase, carboxypeptidase A (Sigma Chemical Co; U.S.A.), aminopeptidase M (Rohm and Haas. GMBH. Darmstadt), and prolidase, extracted from swine kidneys by the method of Smith (1955). Pronase, prolidase, aminopeptidase M and α-chymotrypsin were dissolved in water before use. Carboxypeptidase A was suspended in water, centrifuged and the supernatant discarded; the precipitate was dissolved in 2 M NH_4HCO_3 and diluted ten times. A sample of trypsin treated with TPCK (Carpenter, 1967) was used for most enzyme digestions; the enzyme was dissolved in 0.001 M HCL before use. The TPCK-treated trypsin exhibited a low chymotryptic activity with N-benzyloxycarbonyl-L-tyrosine p-nitrophenyl ester equivalent to 1.3% of the total protein.

Tryptic digests of haemoglobin or globin

For tryptic digestions, a 1 - 2% solution or suspension of haemoglobin or globin was used. The control proteins, unheated, were dissolved in water and denatured in boiling water for five minutes. The heat-damaged samples were very insoluble and were ground in a Potter-Elvehjem homogeniser with 0.01 M NaOH until a fine suspension was obtained. The pH was adjusted to 8.0 and maintained in a Radiometer pH-stat, by the addition of 0.1 M NaOH. Sufficient trypsin was added to give a final enzyme-substrate concentration of 1% (w/w), and the digest was held at 37° with stirring (magnetic stirrer). The method of trypsin addition and the time of digestion were different for each of the digests.

Digestion was stopped in each case by the addition of glacial acetic acid to reduce the pH to about 2.0, and the solutions subsequently lyophilised.

Exhaustive enzyme digests of globin

Exhaustive enzyme digests of 160 mg of globin in a volume of 5 cm^3 were prepared. The unheated globin solution was denatured in boiling water for five minutes, cooled, and by the addition of 2 M NH_4HCO_3 brought to a final NH_4HCO_3 concentration of 0.2 M. The heated globin was ground in a Potter-Elvehjem homogeniser with 0.2 M NH_4HCO_3 until a fine suspension was obtained. The solutions or suspensions of globin were made 0.01 M to Ca^{++} and 0.002 M to Mn^{++}, and the pH adjusted to 8.0. The following enzymes were added, at 24 - 36 hour intervals, in at least two aliquots, to give for each enzyme, an enzyme/substrate ratio of 1%, w/w. Trypsin (TPCK-treated) was added first, followed by α-chymotrypsin, and the pH maintained at 8.0. The pH was then adjusted to 7.0 with acetic acid, and pronase added. This was followed by aminopeptidase M with prolidase. After adjustment of the pH to 8.0, carboxypeptidase A and another aliquot of prolidase were added together. Further aliquots of carboxypeptidase A were added with the pH at 5.0 and 9.2, to increase the rate of release of aspartic acid and lysine respectively (Ambler, 1972). The temperature of the digest was maintained at 37° and the solution agitated with a magnetic stirrer; toluene was added to all digests. At the completion of the digestion, the solution was acidified with glacial acetic acid to pH 2.0 and lyophilised.

Separation of peptides

The peptides from enzymatic digests of haemoglobin or globin were separated by one or several of the following methods: ion-exchange chromatography, gel filtration, paper or thin layer chromatography and/or electrophoresis.

Ion-exchange chromatography

(1) Dowex 50-X2. The procedure given by Schroeder (1967a) for chromatography with Dowex 50-X2, 200-400 mesh (Bio-Rad Laboratories, U.S.A.) was followed. The resin was cleaned, regenerated, degassed and repacked for each separation to avoid possible contamination with peptide material from previous separations. The column, 0.9 x 100 cm, was water jacketed and maintained at 37°. Three pyridine-acetic acid buffers were used for the development of the chromatogram; (a) pH 3.1 (0.2 M in pyridine; 64.5 cm^3 pyridine, 1114 cm^3 glacial acetic acid, diluted to 4l); (b) pH 5.0 (2.0 M in pyridine; 645 cm^3 of pyridine, 573 cm^3 glacial acetic acid, diluted to 4l); and (c) pH 5.6 (8.5 M in pyridine; 684 cm^3 of pyridine, 180 cm^3 glacial acetic acid, diluted to 1l). The initial gradient was linear, with 400 cm^3 each of buffers pH 3.1 and 5.0 supplied from a gradient vessel consisting of two identical, inter-connected cylinders. About 100 - 200 cm^3 buffer pH 5.6 was used to complete the development. The flow rate was maintained at 15 - 20 cm^3/hour and 4 - 5 cm^3 fractions were collected.

(2) Beckman UR-30 resin. The procedure used for chromatography with Beckman UR-30 resin was similar to that for Dowex 50-X2, except that the column was 0.9 x 69 cm, and the gradient differed. Initially, the column was developed with 50 cm^3 buffer pH 3.1 before the gradient was commenced. The gradient vessel consisted of three inter-connected, identical cylinders, and 200 cm^3 of buffer pH 3.1 was added to two of the cylinders and 200 cm^3 of buffer pH 5.0 to the third cylinder. The buffer, pH 5.6, was not used for development of the chromatogram.

(3) Dowex 1-X2. The procedure described by Schroeder (1967b) was used for chromatography with Dowex 1-X2, 200 - 400 mesh (Bio-Rad Laboratories, U.S.A.). The resin was cleaned, regenerated, degassed and repacked for each sample; the column, 0.9 x 100 cm, was water jacketed

and maintained at 37°. A complex gradient vessel, similar to that described by Schroeder (1967b), which permitted the automatic and successive entry of five buffers into the mixing vessel, was used for the development of the chromatograms. Four buffers, pH 9.4, 8.4, 7.4 and 6.5, were used; these were composed of 60 cm^3 N-ethylmorpholine, 80 cm^3 α-picoline, 40 cm^3 pyridine, with sufficient glacial acetic acid to adjust the pH to that required, and diluted to 41. Further development of the chromatogram was obtained with 0.5, 2.0 and 10 M acetic acid.

The mixing vessel was filled with 150 cm^3 buffer pH 9.4. Elution was commenced with 50 cm^3 of buffer pH 9.4 and this was followed by 50 cm^3 buffer pH 8.4, 100 cm^3 buffer pH 7.4 and 150 cm^3 buffer pH 6.5. The addition of the acetic acid solutions was commenced after the completion of the pH 6.5 buffer. Either 300 cm^3 2.0 M and 200 cm^3 10.0 M acetic acid, or 200 cm^3 each of 0.5, 2.0 and 10.0 M acetic acid, were used for the final development of the chromatograms.

Gel filtration. Sephadex G-25, G-50 and G-75 gels (Pharmacia Fine Chemicals AB, Sweden) were used for gel filtration studies. For preparative separations, a 1.9 x 150 cm column of Sephadex G-25 was used, and columns, 0.9 x 150 cm, of Sephadex G-50 and 0.9 x 100 cm of Sephadex G-75, were used for the purification of peptides. The gel was swollen in the solvent (either 10 or 30% acetic acid), at room temperature and degassed. The column was poured in sections and equilibrated before use by the passage of sufficient solvent to equal twice the volume of the gel. Downward flow of the solvent was used and the flow rates varied from 2 - 10 cm^3/hour, depending on the column size and type of gel. The void volume in each case was established with a solution of Dextran Blue (0.3%). The surface of the gel was gently stirred after each separation and, if any debris had collected, a portion was removed and replaced with fresh gel. The columns were used at room temperature and the size of fractions

collected varied from 5.0 cm^3 for preparative columns to 0.75 cm^3 when peptides were being purified.

High-voltage electrophoresis. A flat plate electrophoresis unit (Pherograph) was used for the investigation of peptides in the tryptic digests. Both paper (Whatman No 1 or 3MM), and thin layer plates of cellulose (MN-300 cellulose, Machery, Nagel and Co., Germany), washed with 1% acetic acid, were used. The paper, or plates, moistened with buffer, and the paper blotted to remove excess, were placed in position in the electrophoresis unit, and the sample(s) applied. A potential of about 1.5 KV was supplied for $1\frac{1}{2}$ - $2\frac{1}{2}$ hours. The buffers used were pH 1.9 (87 cm^3 glacial acetic acid, 25 cm^3 formic acid diluted to 1l), pH 3.5 (50 cm^3 glacial acetic acid, 5 cm^3 pyridine, diluted to 1l), and pH 6.5 (4 cm^3 glacial acetic acid, 100 cm^3 pyridine, diluted to 1l).

For the separation of peptides in the exhaustive digests, a water-cooled Michl solvent tank was available, with either pH 6.5 buffer, (described above) or pH 2.1 (80 cm^3 glacial acetic acid, 20 cm^3 formic acid, diluted to 1l). This apparatus gave a much better separation of peptides than obtained from the use of the Pherograph. Samples were applied to the dry paper, with, on either side, 1 - 2 cm bands of two marker solutions of free amino acids (5 mM in 10% isopropanol). Marker solution 1 contained lysine, arginine, valine, leucine, methionine, proline, phenylalanine and tyrosine; marker solution 2 included histidine, glycine, alanine, serine, isoleucine, threonine, glutamic and aspartic acids. In addition, a fluorescent marker was applied at intervals on the dried sample and marker bands. The fluorescent marker contained the dansyl derivatives of arginine and arginyl-arginine, and 1-dimethylaminonaphthalene 5-sulphonic acid (made by reacting 50 mg arginyl-arginine and 17 mg arginine in 3 cm^3 1.0 M $NaHCO_3$ with 80 mg dansyl chloride in 3 cm^3 acetone). The sample(s) was concentrated into a narrow

band by allowing the buffer to slowly wet the paper on either side of the sample zone. Excess buffer was blotted from the paper, and a potential of 3 KV applied for 45 minutes. After drying the paper, the position of the fluorescent markers were determined under ultraviolet light (366 or 254 nm).

Paper and thin layer chromatography. Either thin layer plates of cellulose or silica gel G (Merck, Germany), or paper (Whatman No 1 or 3MM), were used for the chromatographic separation of peptides. The solvent used was butanol-acetic acid-water, 3:1:1. The thin layer plates were washed with 1% acetic acid before use.

Two dimensional peptide maps were obtained by electrophoresis in one direction, using a full sheet of paper (32 x 38 cm for the Pherograph, and 46 x 57 cm for the Michl solvent tank), and chromatography in the second direction. A strip of paper, 10 cm wide, was sewn to the edge of the paper after electrophoresis, to act as a wick for the chromatography solvent.

Detection of peptides

Cadmium-ninhydrin reagent for thin layer plates or paper chromatograms. For qualitative purposes the whole paper was dipped in the cadmium-ninhydrin reagent, and thin layer plates were sprayed with the same reagent; this was prepared either by the method of Dreyer and Bynum (1967) or by mixing a solution of cadmium acetate (15 g cadmium acetate, 300 cm^3 glacial acetic acid and 600 cm^3 water) with a 1% ninhydrin solution in acetone, in the proportion of 3 to 17. Strips which were cut from preparative papers, on either side of and including about 1 cm of the sample and all the marker solutions, were developed, so that the position of the peptide band(s) could be identified. The areas containing peptide material were cut out and either eluted (with 1% acetic acid or 0.05 M NH_4OH) or sewn to another sheet of paper for further electrophoresis or chromatography.

Reaction of aliquots of fractions with ninhydrin. Aliquots, 0.05 - 0.2 cm^3, of fractions collected were reacted with 0.5 cm^3 freshly-made ninhydrin reagent (Hirs, 1967) in polypropylene tubes, 0.9 x 7.0 cm. The tubes were heated in boiling water for 15 minutes, cooled, and 2.5 cm^3 50% ethanol added. The absorption was read at 570 nm.

For the detection of large peptides the samples were first hydrolysed. Glass tubes, 0.9 x 6 cm, cleaned in 50% alcoholic KOH, were used, and aliquots (0.05 - 0.1 cm^3, of fractions collected) were hydrolysed with 0.15 cm^3 50% NaOH for 15 minutes in a pressure cooker under 15 lbs pressure. After cooling, the tube contents were neutralised with glacial acetic acid (about 0.25 cm^3) and reacted with ninhydrin as described.

Absorption at 280 nm. Column chromatography with Sephadex gels were monitored by recording the absorption at 280 nm with a LKB Uvicord or by measurement of individual fractions with a Hitachi 101 spectrophotometer.

Chemical analysis

Amino acid analysis. About 2 mg of the globin samples, heated and unheated, were hydrolysed with 1.0 cm^3 6 M HCL at 110° for 24 or 48 hours, in thick walled Pyrex tubes, 1.6 x 15 cm, sealed under vacuum. The dried peptide samples were hydrolysed with 50 - 100 µl of 6 M HCL at 110° for 20 - 24 hours in 0.9 x 7 cm Pyrex tubes. The peptide samples were bubbled with oxygen-free nitrogen, or evacuated, before the tubes were sealed. After hydrolysis, the sample was dried, either with a Buchi rotary evaporator, or *in vacuo* over NaOH pellets. The composition of the hydrolysate was determined using a Beckman 120C Amino Acid Analyser.

FDNB-reactive lysine. The FDNB-reactive lysine content of the unheated and heated globin samples was determined by the method of Carpenter (1960), with the application of the factor 1.09 to correct for the loss of dinitrophenyl lysine during hydrolysis.

Amino-terminal analysis. The dansyl chloride procedure of Gray (1972a) was followed for the determination of amino-terminal groups. Dansyl amino acids were separated by chromatography with polyamide sheets, 7.5 x 7.5 cm, the sample being applied to both sides of the sheet and a marker solution of dansyl derivatives (glycine, serine, glutamic acid, arginine, proline, isoleucine and phenylalanine) to one side only. The sheets were developed with solvent 1 (1.5% formic acid, v/v) for 15 minutes and after drying, were developed in the second direction, with the successive use of up to four solvents. Development in solvent 2 (toluene-acetic acid, 9:1, v/v) was for 15 minutes, solvent 3 (ethyl acetate-methanol-acetic acid, 20:1:1, v/v) and solvent 4 (75% of acetic acid-pyridine-water, 40:22.5:2500, v/v and 25% ethanol) for 10 minutes, and solvent 5 (0.1 M sodium phosphate-ethanol, 3:1, v/v) for 20 - 30 minutes. After development in each of these solvents, the presence and position of dansyl derivatives were detected by their fluorescence under ultraviolet light (366 nm).

Dansyl-Edman procedure for sequence analysis. The procedure of Gray (1972b) was slightly modified. Sufficient sample to provide about 10 nmoles per step, was dissolved in 150 µl water in a screw capped tube, and 150 µl of phenylisothiocyanate (5% in pyridine) added. The samples were mixed thoroughly, flushed with oxygen-free nitrogen, capped and incubated at 45° for 90 minutes. After drying *in vacuo*, over concentrated H_2SO_4 at 60°, the sample was dissolved in 250 µl trifluoroacetic acid, flushed with nitrogen and incubated at 45° for 30 minutes. The sample was then dried *in vacuo* over NaOH and re-dissolved in 200 µl water. The nonvolatile by-products were removed by extraction with 3 x 1.5 cm^3 butyl acetate or benzene, using centrifugation to break up the emulsion. A sample of the aqueous phase containing peptide was removed and the amino-terminal determined by dansylation. The remainder of the sample was

dried in vacuo and retained for the next degradation cycle.

Subtractive Edman degradation for sequence analysis. The Dansyl-Edman procedure already described was used for the formation of the 2-anilinothiazolinones. After removal of the trifluoroacetic acid the procedure of Konigsberg (1972) was followed. The residue was dissolved in 1.0 cm^3 0.2 M acetic acid and heated at 60° for 10 minutes, to form the phenylthiohydantoins which are more soluble in organic solvents. These compounds were extracted with 3 x 1.5 cm^3 of benzene, again with centrifugation to break up the emulsion, and a sample removed for acid hydrolysis and subsequent amino acid analysis; the remainder was dried in vacuo.

RESULTS AND DISCUSSION

I. Comparison of unheated and heated haemoglobin and globin samples

Amino acid composition. The results of the amino acid analysis of unheated and heated globin were calculated as molar ratios with leucine equal to 37, the number of leucine residues in the combined alpha and beta chains of bovine haemoglobin (Dayhoff, 1972); these values are given in Table XX. The ratios of the globin sample were slightly different to the expected values and a small amount of isoleucine was present, indicating that the sample was not completely pure. Heat-damage of the globin probably resulted in destruction of cystine as there was no trace of this in the hydrolysate of the heated protein, but the values for the other amino acids were similar for both the heated and unheated protein.

FDNB-reactive lysine. The FDNB-reactive lysine values (g/16g N) for the unheated haemoglobin or globin samples were 8.6 and 8.1 respectively, but for the heated samples, the values had decreased to 5.9 and 6.0, indicating that heat-damage had occurred.

TABLE XX

Amino acid molar ratios of unheated and heated globin, together with the combined residues of alpha and beta chains of bovine globin (Dayhoff, 1972)*

	Unheated globin	Heated globin	Bovine globin (α and β chains)
Lysine	20.8	20.6	22
Histidine	16.5	16.1	17
Arginine	6.6	6.7	7
Aspartic acid	27.3	27.1	28
Threonine	13.6	13.6	14
Serine	17.8	18.0	19
Glutamic acid	18.6	18.4	17
Proline	10.5	10.6	10
Glycine	18.5	18.7	19
Alanine	35.2	36.3	36
Half cystine	Trace	-	1
Valine	29.8	29.5	30
Methionine	3.2	3.1	4
Isoleucine	0.5	0.5	-
Leucine	37	37	37
Tyrosine	4.2	4.8	5
Phenylalanine	16.9	17.2	17
Tryptophan	ND	ND	3

ND, not determined

* Tryptophan not included

Amino-terminal analysis. The terminal amino acids of the unheated globin, determined by dansylation, were valine and methionine, in agreement with literature reports (Dayhoff, 1972); the sequence of the alpha and beta chains of bovine globin are given in Table A, Appendix 3. No other amino-terminals were detected. However, with the heated globin, in addition to valine and methionine, other N-terminal groups were detected, notably leucine, phenylalanine and alanine, with a trace of aspartic acid. The approximate intensities of each amino-terminal, by visual inspection were: 8 valine, 8 methionine, 4 leucine, 4 phenylalanine, 4 alanine and 1 aspartic acid. This cleavage of peptide bonds in the heated globin was investigated further by gel filtration of an extract.

Gel filtration of heated globin on Sephadex G-50. An extract of the heated globin was prepared by grinding 100 mg in 30% acetic acid in a Potter-Elvehjem homogeniser. Although most of the protein was insoluble a small amount dissolved; a 8 M urea solution did not improve the solubility of the protein. The extract was chromatographed on a column of Sephadex G-50 (0.9 x 100 cm) in 30% acetic acid and the elution profile (Figure 6) indicated that the extract was not homogeneous. The chromatogram was divided into four fractions, A - D, as shown in the figure and the amino acid composition of each fraction, and amino-terminal groups of fractions A and B, determined.

Amino acid analysis. The results for fractions A - C are given in Table XXI and are again calculated as molar ratios with leucine equal to 37. The ratios for the three fractions varied considerably; fraction B was particularly low in lysine, glutamic acid, glycine and alanine.

The results for fraction D are given separately in Table XXII, for both hydrolysed and unhydrolysed samples. A large amount of ammonia was present but also appreciable quantities of free amino acids, particularly aspartic acid, serine, glycine and alanine, and some peptide material.

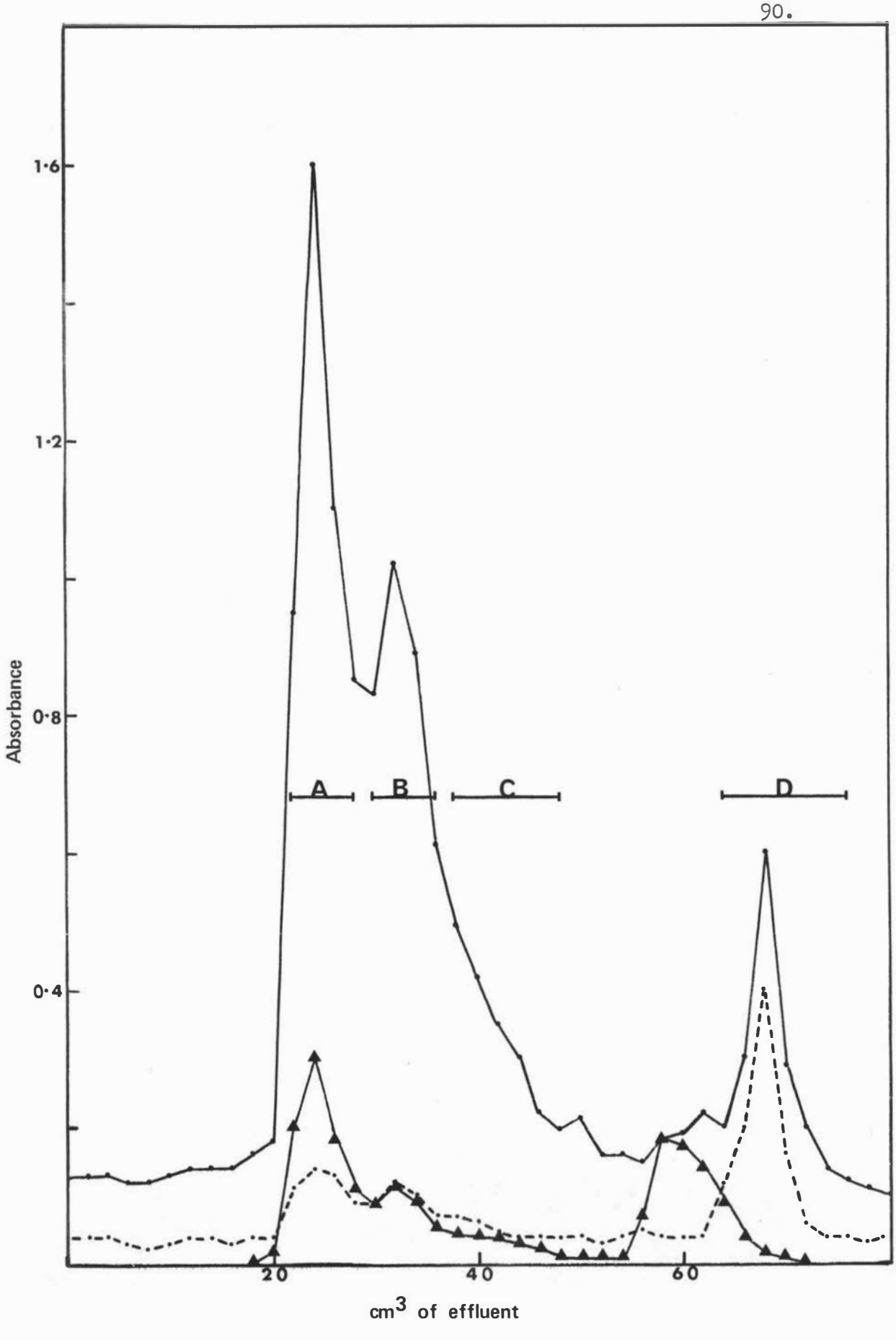


FIGURE 6. Gel filtration of an extract, with 30% acetic acid, of heated globin; 5.0 cm^3 fractions collected. ·--· absorbance at 570 nm after reaction with ninhydrin, 0.1 cm^3 aliquots; —— absorbance at 570 nm of alkali hydrolysed sample reacted with ninhydrin, 0.1 cm^3 aliquots; ▲—▲ absorbance at 280 nm.

TABLE XXI

Amino acid molar ratios of fractions A, B and C from heated globin, compared with reported values for alpha and beta chains of bovine globin (Dayhoff, 1972) *

	Fraction			Bovine globin
	A	B	C	(α and β chains)
Lysine	19-20	14	19-21	22
Histidine	19-20	13	13-14	17
Arginine	6-7	5-6	11-12	7
Aspartic acid	20-21	19	21-22	28
Threonine	14-15	16	17	14
Serine	20-21	24-25	24	19
Glutamic acid	13-14	4-5	9	17
Proline	11	11-12	8-9	10
Glycine	17-18	6-7	13-14	19
Alanine	36-37	23	31-32	36
Half cystine	-	-	-	1
Valine	21	21	25-26	30
Methionine	1	0.5	-	4
Isoleucine	-	-	-	-
Leucine	37	37	37	37
Tyrosine	2	2	2	5
Phenylalanine	13	13-14	11-12	17

* Tryptophan not included

TABLE XXII

Amino acid composition (μmols / 100 mg globin) of fraction D from heated globin; unhydrolysed and hydrolysed samples

	Unhydrolysed	Hydrolysed
Lysine	ND	0.07
Histidine	ND	0.09
Ammonia	ND	4.00
Arginine	ND	Trace
Aspartic acid	0.08	0.33
Threonine	Trace	0.05
Serine	0.10	0.25
Glutamic acid	Trace	0.35
Proline	Trace	0.42
Glycine	0.11	0.36
Alanine	0.09	0.50
Valine	-	0.14
Leucine	Trace	0.33
Phenylalanine	-	0.18

ND, not determined

Amino-terminal analysis. Valine was the only N-terminal group detected in fraction A, but in fraction B, leucine, phenylalanine and alanine were also present. Methionine was not detected in either fraction, which suggests that the β chain of globin had not been extracted.

These results show that some cleavage of peptide bonds had occurred when globin was heated, with the formation of various sized fragments, including free amino acids. The absence of free amino acids and small peptides in the original globin preparation was verified by gel filtration of a solution of unheated globin on the same column of Sephadex G-50, and only a single peak was eluted. Bjarnason and Carpenter (1970) also reported that a number of additional N-terminal groups were formed when bovine plasma albumin was heated, and they estimated that 0.3% of the peptide bonds had been hydrolysed.

Rate of digestion with trypsin

The rate of digestion of haemoglobin, unheated and heated, by trypsin, was compared, using a similar procedure to that described previously for the tryptic digests. Prior to the addition of trypsin, the prepared solution, 2.0 cm^3 of 1% protein, was maintained at 37°, with stirring, until the pH remained stable at pH 8.0. With the unheated globin, there was virtually no change from pH 8.0 after the initial adjustment but with the heated haemoglobin, increments of 0.01 M NaOH were required for 2 - 3 hours. Following the addition of trypsin (TPCK-treated, 1% w/w), the volume of 0.01 M NaOH required to maintain the pH at 8.0 was recorded at intervals for 60 minutes. The rate of digestion of the heated protein was much slower than for the unheated protein (Figure 7).

The initial decrease in pH of the heated haemoglobin suspension before the addition of trypsin, indicates that some titratable groups

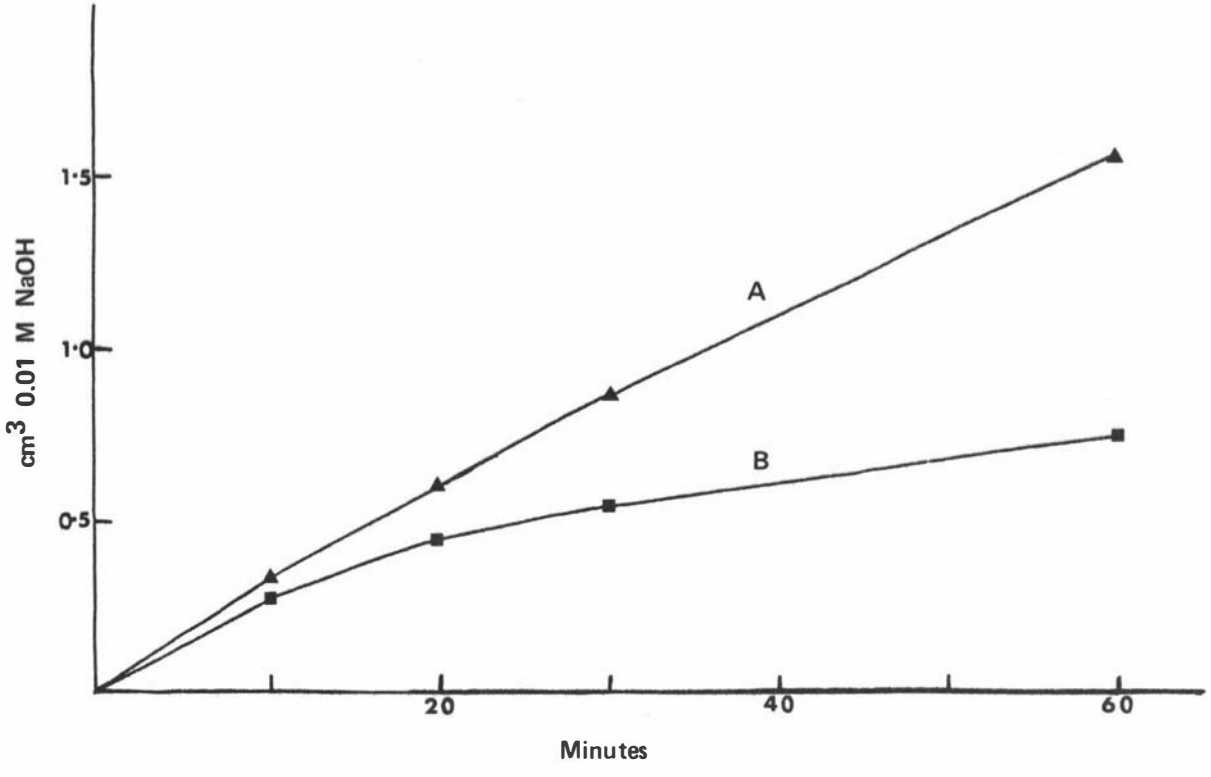


FIGURE 7. Rate of trypsin digestion of haemoglobin.
A, unheated; B, heated.

were slowly exposed in solution. The apparent masking of titratable groups, which would probably include the ε-amino groups of lysine and the guanidyl groups of arginine, in the heat-damaged protein would contribute to the slow rate of digestion by trypsin. In addition, the insolubility of the heated protein, which would also reduce the rate of digestion, would probably be due, partly at least, to the inaccessibility of some polar groups, presumably as the result of non-covalent interactions. Mecham and Olcott (1947) commented on the insolubility of proteins after exposure to dry heat, but they attributed this to a decrease in the number of polar groups with the formation of new ester or amide bonds.

II. Comparison of tryptic digests of unheated and heated haemoglobin or globin

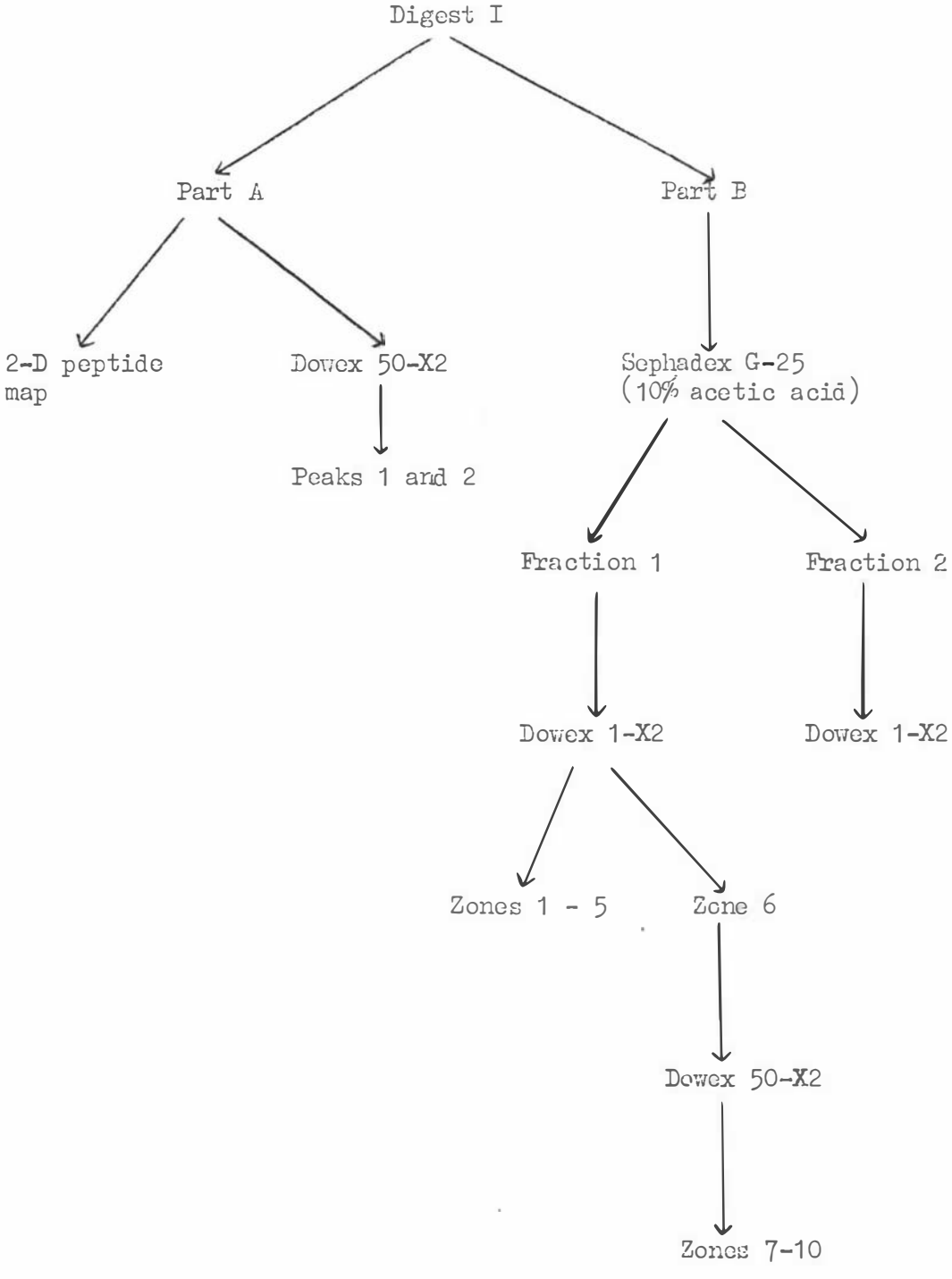
Samples of unheated and heated protein, either haemoglobin or globin, were digested with trypsin using the same conditions for each pair of samples. The peptide composition of each digest was compared, by a variety of methods, to determine whether different peptides had been released from heat-damaged protein by trypsin. Three pairs of tryptic digests of either haemoglobin or globin, were prepared, each digest by a slightly different method. The methods used to compare the peptide compositions were, as far as possible, identical for each pair of digests.

Digest I.

For Digest I, samples of haemoglobin (1 g), unheated and heated, were digested for 30 hours and aliquots of trypsin (untreated with TPCK) added at 0, 5, 10 and 20 hours; toluene was added to the digest. Each digest was divided into two parts, A and B, which were examined by different methods; an outline of the procedures used is given in Table XXIII.

TABLE XXIII

Outline of methods used with Digest I, heated and unheated haemoglobin



Part A. Part A was investigated with two-dimensional peptide maps and ion-exchange chromatography using Dowex 50-X2.

Peptide map. Samples of digests were separated by paper electrophoresis at pH 1.9 or 6.5 in the Pherograph, and for the second dimension, by chromatography. After staining with cadmium-ninhydrin solution, 29 - 30 peptides were visible with the unheated haemoglobin tryptic digest. However, with the heated protein, streaking occurred and only a few peptides were clearly defined. This streaking was probably caused by the presence of very large peptides.

Chromatography with Dowex 50-X2. Chromatography of part A of Digest I with Dowex 50-X2 showed that overall there was less ninhydrin positive material eluted from the heated haemoglobin digest than from that of the unheated protein (Figure 8). However, at the commencement of the gradient, two comparatively large peaks of ninhydrin positive material were eluted from the heated protein digest. Amino acid analysis of unhydrolysed samples of this material in peaks 1 and 2 (see Figure 8) for both the unheated and heated protein digests, showed that they were composed mainly of the free amino acids, valine, leucine and alanine. There were much greater quantities (8 - 10 times) of all three amino acids in the heated haemoglobin digest than from the corresponding areas of the unheated protein. Acid hydrolysis of samples followed by amino acid analysis, indicated that there was very little peptide material present.

As a considerable amount of the heated haemoglobin digest was not eluted from the resin (some could be seen still bound to the resin at the top of the column), Dowex 50-X2 was not used in future initial separations of tryptic digests.

Part B. Part B of Digest I was investigated by gel filtration and ion-exchange chromatography.

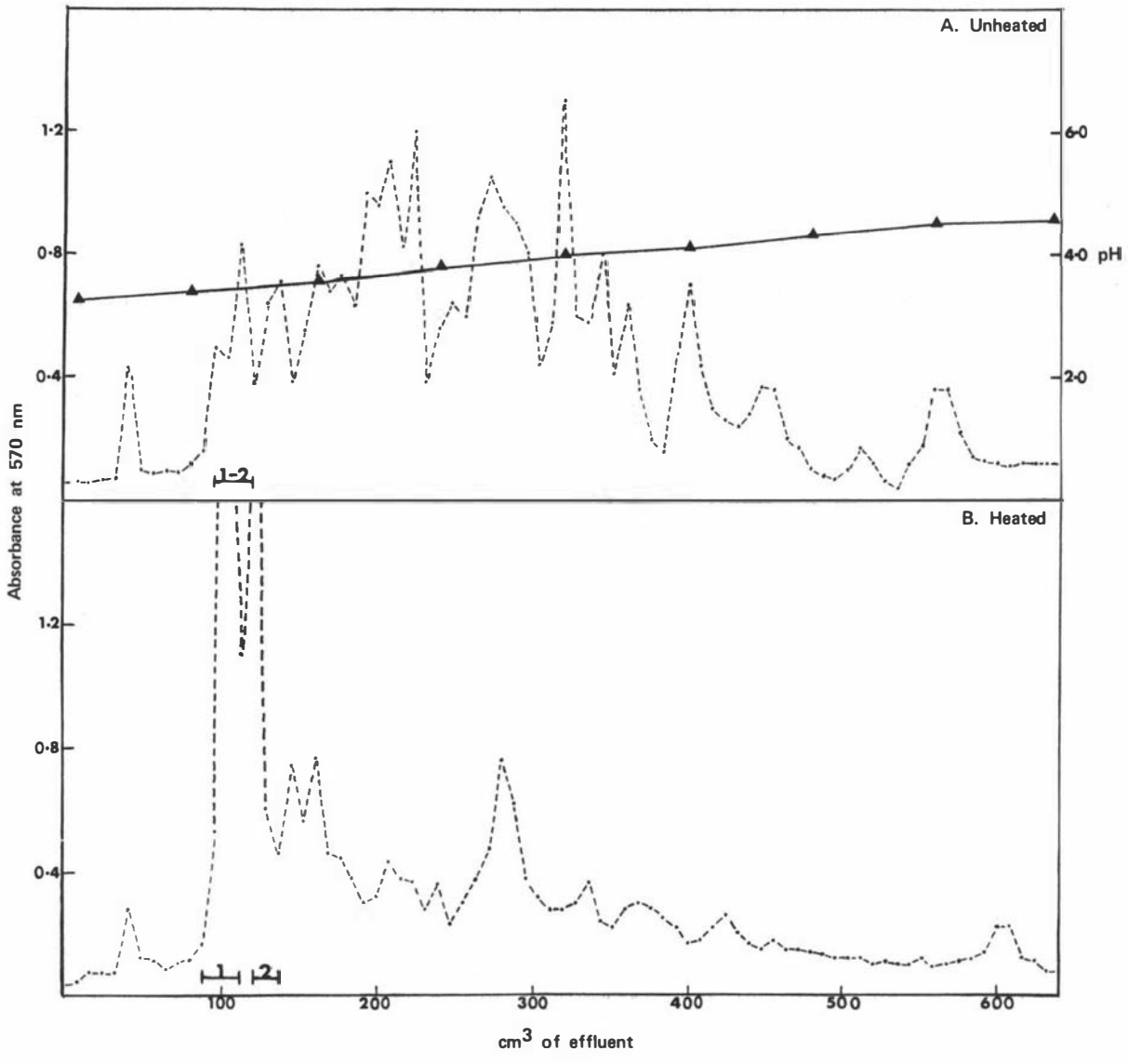


FIGURE 8. Digest I: Chromatography on Dowex 50–X2 of a tryptic digest of haemoglobin. A, unheated; B, heated. ---- absorbance after reaction with ninhydrin, 0.05 cm^3 aliquots; ▲—▲ pH gradient

Gel filtration. Part B was chromatographed on Sephadex G-25 (1.9 x 150 cm, 10% acetic acid) and the elution profiles are shown in Figure 9 for the unheated and heated haemoglobin digests. A considerable quantity of ninhydrin positive material, which also absorbed strongly at 280 nm, was eluted at the void volume of the gel with the heated protein digest. These were presumably large sized fragments; the exclusion limit for Sephadex G-25 is 5000 (for peptides). Since the largest tryptic peptide from bovine haemoglobin has a molecular weight of less than 3000, the heated haemoglobin could not have been completely digested. By contrast, there was very little material eluted at the void volume of the gel with the unheated protein digest. The material eluted from the gel was divided into two fractions, designated 1 and 2 in Figure 9, and these were chromatographed on Dowex 1-X2.

Fraction 1 (see Figure 9). Fraction 1 from the digests gave similar elution profiles on Dowex 1-X2 (Figure 10); there was rather more ninhydrin positive material in the fraction from the unheated protein. The compositions of the zones marked in Figure 10 were investigated.

Zones 1 - 5. Zones 1 - 5 were examined by thin layer chromatography and electrophoresis, and most contained several ninhydrin positive compounds; zone 1 from the heated protein consisted mainly of ammonia. Some components of the zones only occurred in the fraction from the heated haemoglobin digest, but in small amounts, and as amino acid analysis did not indicate the presence of appreciable quantities of lysine, the identification of these fragments was not pursued.

Zone 6. Considerable amounts of ninhydrin positive material was eluted at neutral pH, zone 6, for both the unheated and heated protein fractions. This zone was re-chromatographed on Dowex 50-X2 (Figure 11), and the amino acids, leucine, valine and alanine were again eluted at the beginning of the gradient, zone 7 in Figure 11. Examination of the other

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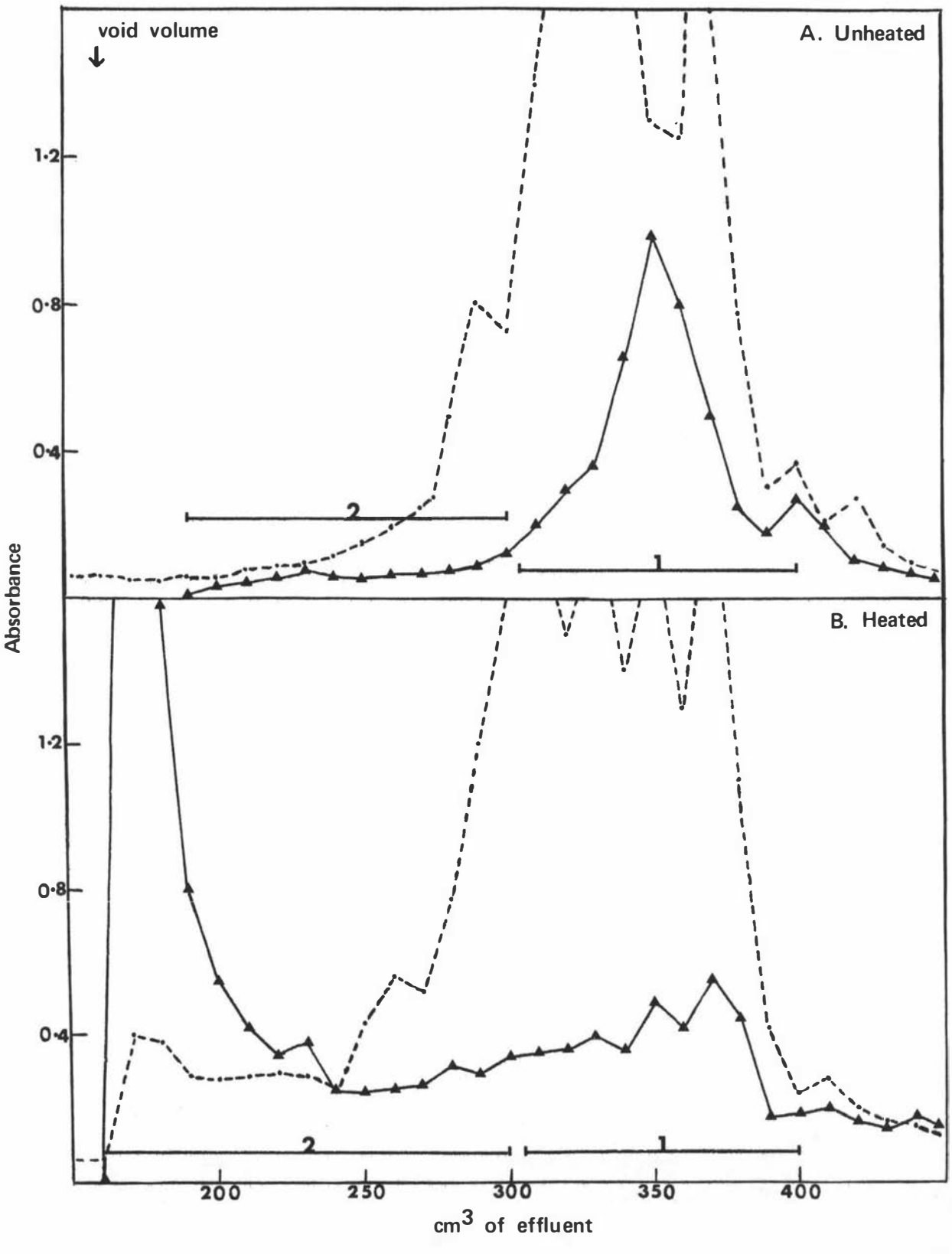


FIGURE 9. Digest I: Gel filtration of a tryptic digest of haemoglobin. A, unheated; B, heated; 4.5 cm^3 fractions collected. · - - - · absorbance at 570 nm after reaction with ninhydrin, 0.1 cm^3 aliquots; ▲——▲ absorbance at 280 nm.

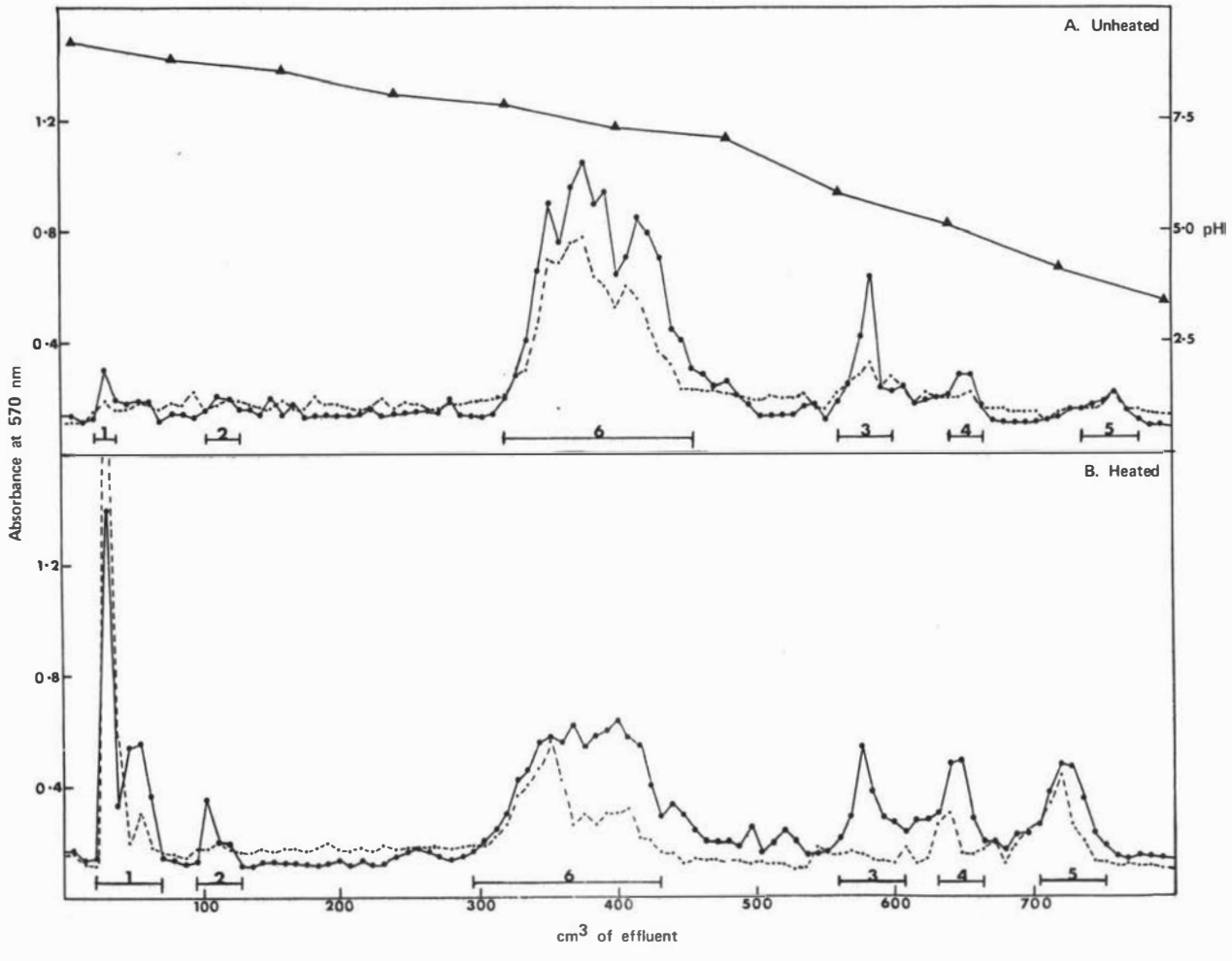


FIGURE 10. Digest I: Chromatography on Dowex I–X2 of fraction I from a tryptic digest of haemoglobin. A, unheated; B, heated. ·---· absorbance after reaction with ninhydrin, 0.05 cm^3 aliquots; ——— absorbance of alkali hydrolysed sample reacted with ninhydrin, 0.05 cm^3 aliquots; ▲——▲ pH gradient

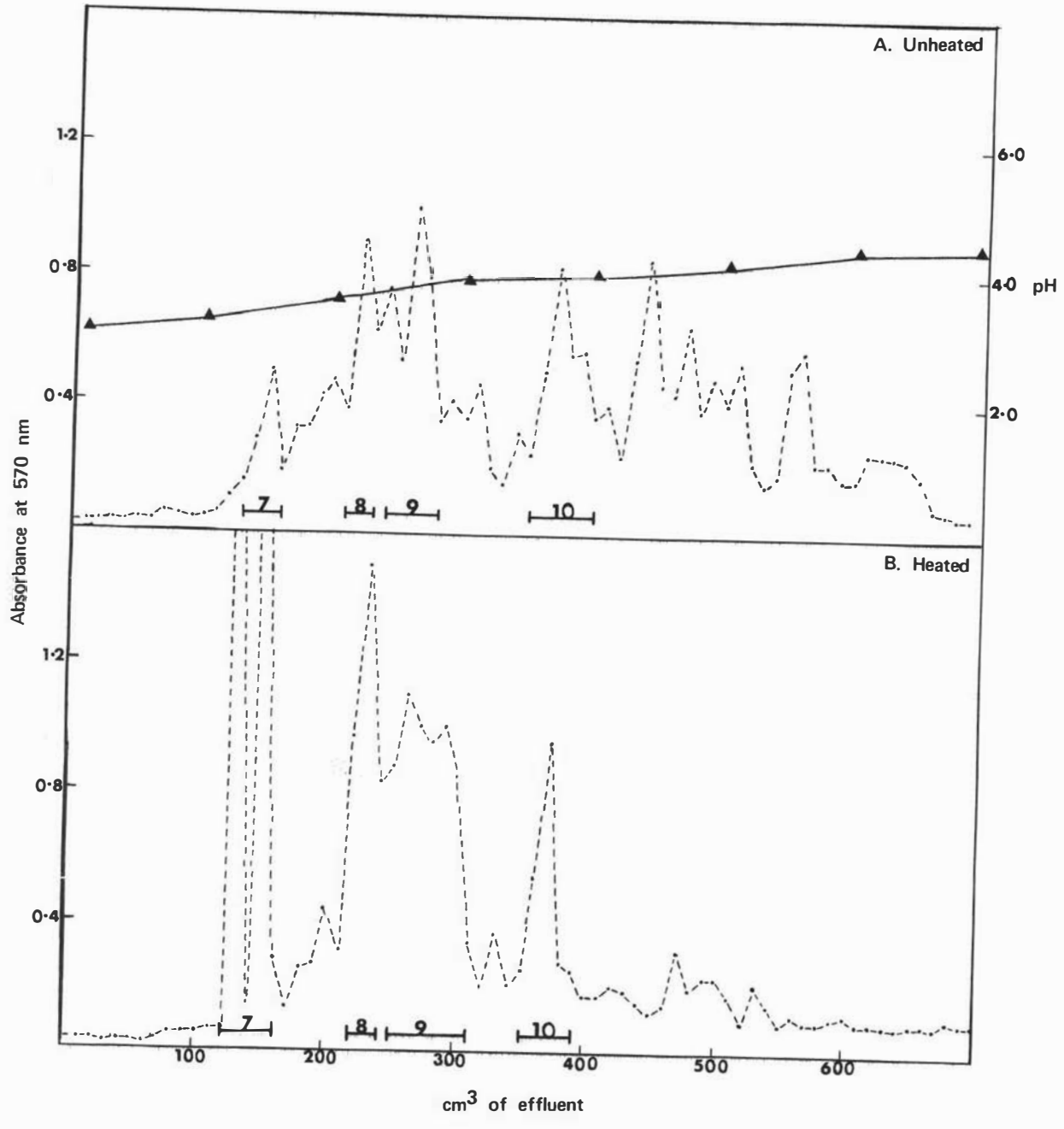


FIGURE 11. Digest I: Chromatography on Dowex 50–X2 of zone 6 from a tryptic digest of haemoglobin. A, unheated; B, heated. · - - - · absorbance after reaction with ninhydrin, 0.05 cm^3 aliquots; ▲—▲ pH gradient

zones (8 - 10) by thin layer chromatography or electrophoresis, did not reveal any difference in the peptides eluted from the unheated and heated protein fractions.

These results indicated that the compositions of fraction 1, derived from the unheated and heated haemoglobin digests, were similar, the main difference being that the heated protein was much less completely degraded.

Fraction 2 (see Figure 9). Chromatography of fraction 2 on Dowex 1-X2 (Figure 12) confirmed that there was more large sized peptide material in the fraction from the heated haemoglobin digest than in that from the unheated protein. These results showed that there may be different peptides present in this fraction from the heated protein digest to those in the unheated protein digest and these were investigated further with another pair of tryptic digests.

Digest II.

TPCK-treated trypsin was used for Digest II since it was possible that the free amino acids detected in Digest I were the result of contamination of the trypsin with chymotrypsin. In addition, the time of the digestion was reduced to minimise non-specific cleavage of peptide bonds. The pair of digests were prepared by digesting 250 mg of haemoglobin, with one addition of TPCK-treated trypsin, for four hours. The digests were fractionated into two parts by gel filtration as with part B of Digest I, the two fractions chromatographed on Dowex 1-X2, and the composition of zones from this separation, examined by gel filtration. Outlines of the methods used are given in Tables XXIV and XXV for the unheated and heated protein digests respectively.

Gel filtration. Gel filtration on a Sephadex G-25 column (1.9 x 150 cm) with 10% acetic acid, showed that there was considerable amounts of material eluted at the void volume of the gel, with both the digest of the unheated and heated haemoglobin (Figures 13A and 14A respectively).

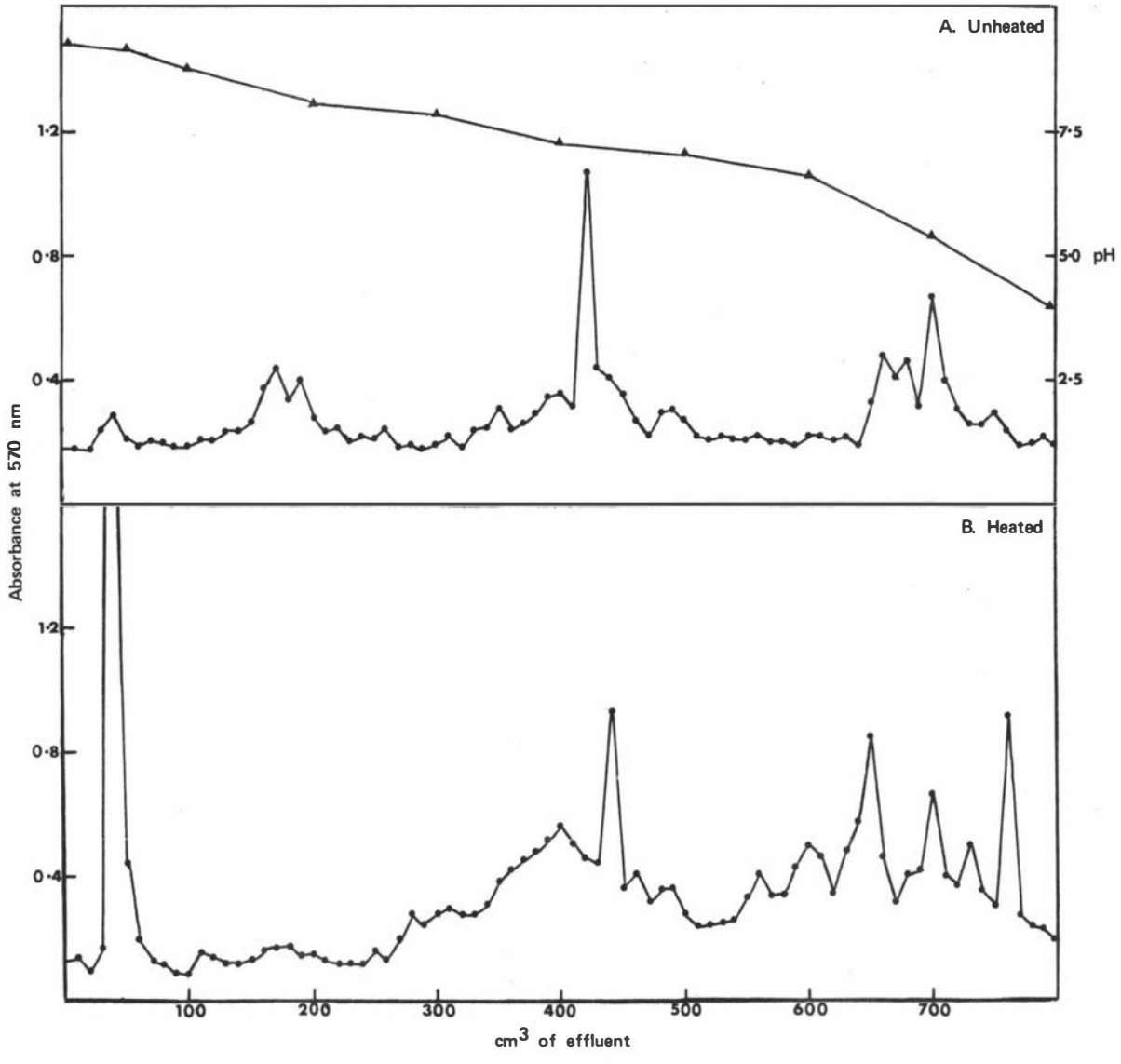


FIGURE 12. Digest I: Chromatography on Dowex 1–X2 of fraction 2 from a tryptic digest of haemoglobin. A, unheated; B, heated. ●—● absorbance of alkali hydrolysed sample reacted with ninhydrin, 0.25 cm^3 aliquots for A, 0.1 cm^3 for B; ▲—▲ pH gradient

TABLE XXIV

Outline of methods used with Digest II, unheated haemoglobin

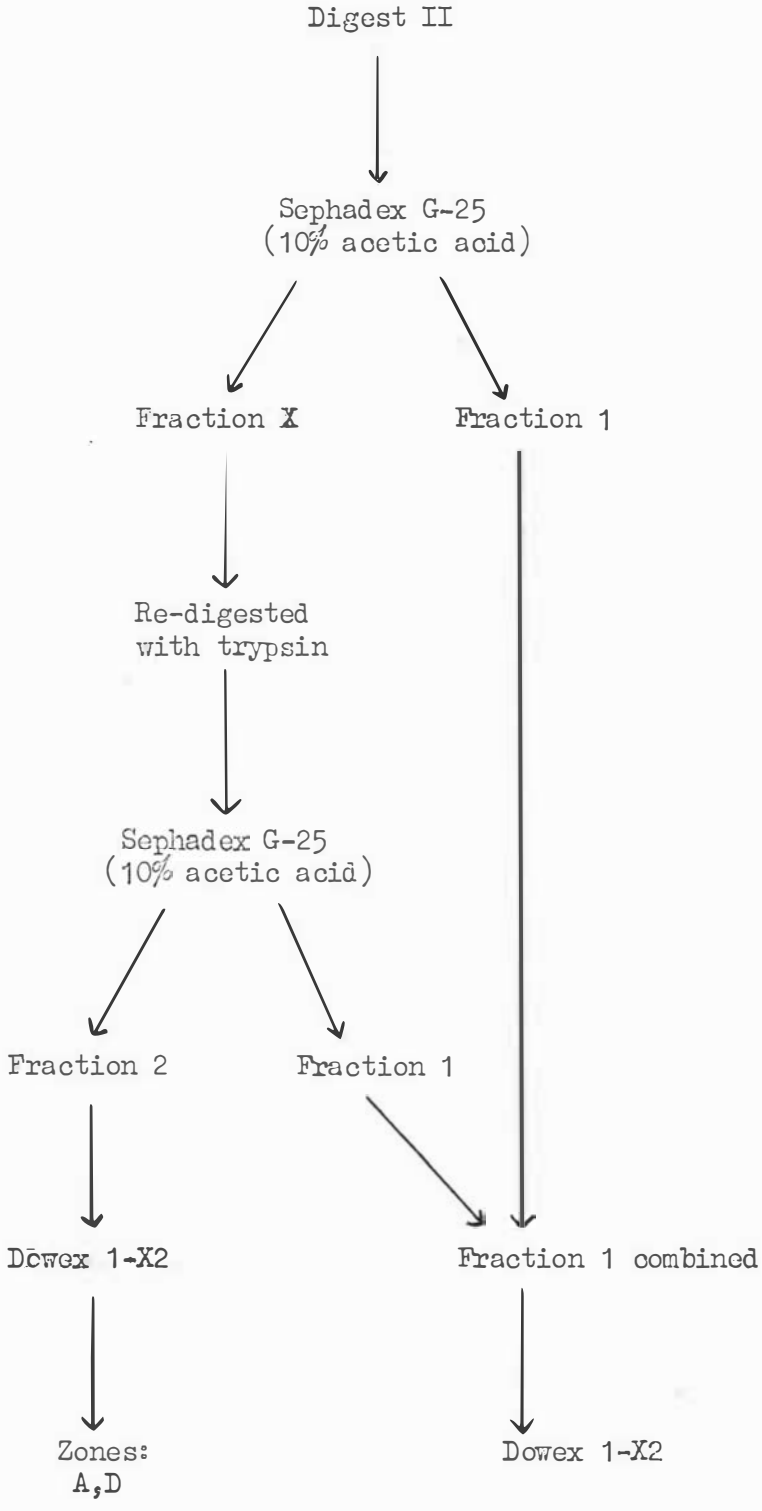
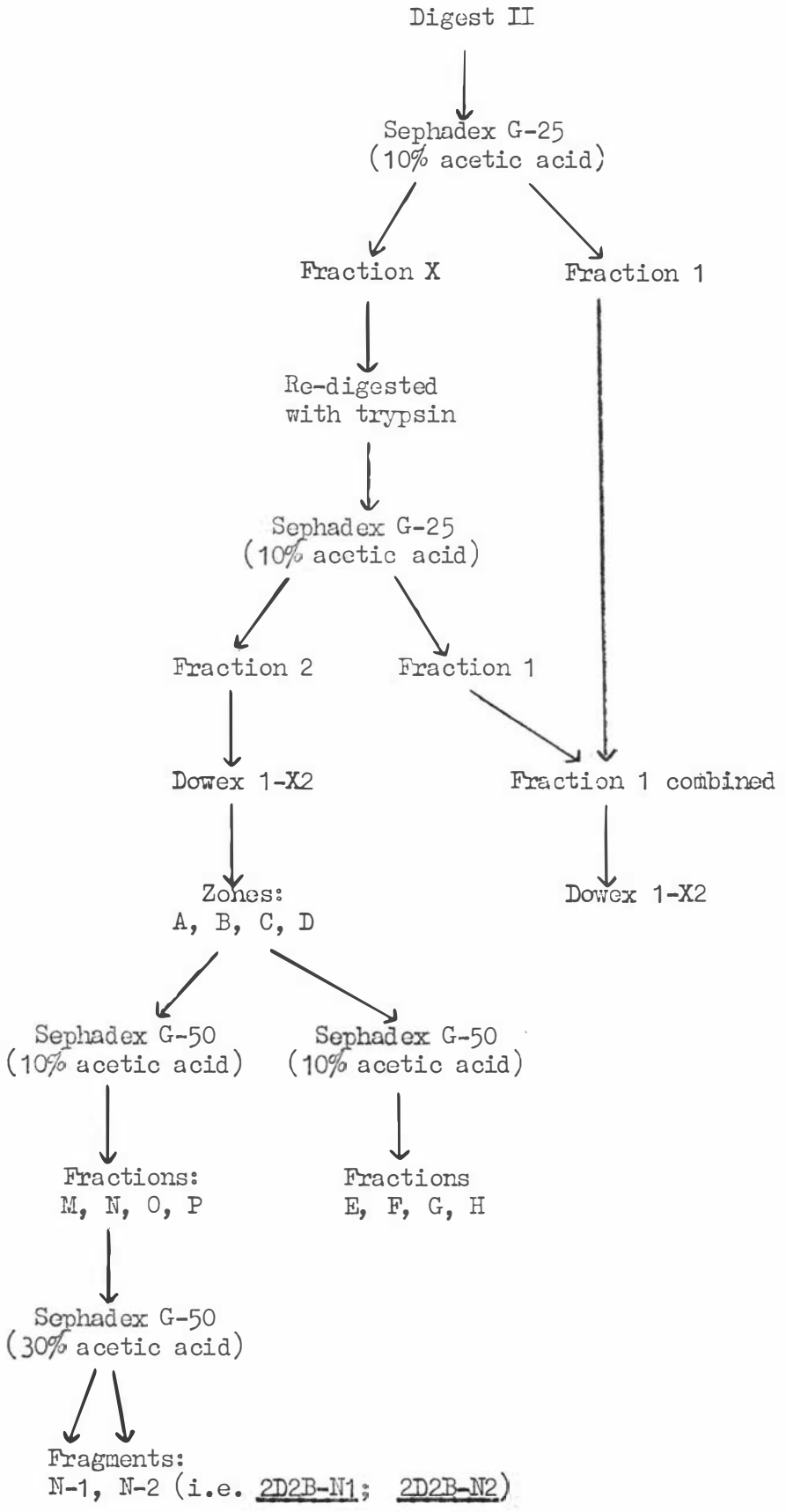


TABLE XXV

Outline of methods used with Digest II, heated haemoglobin



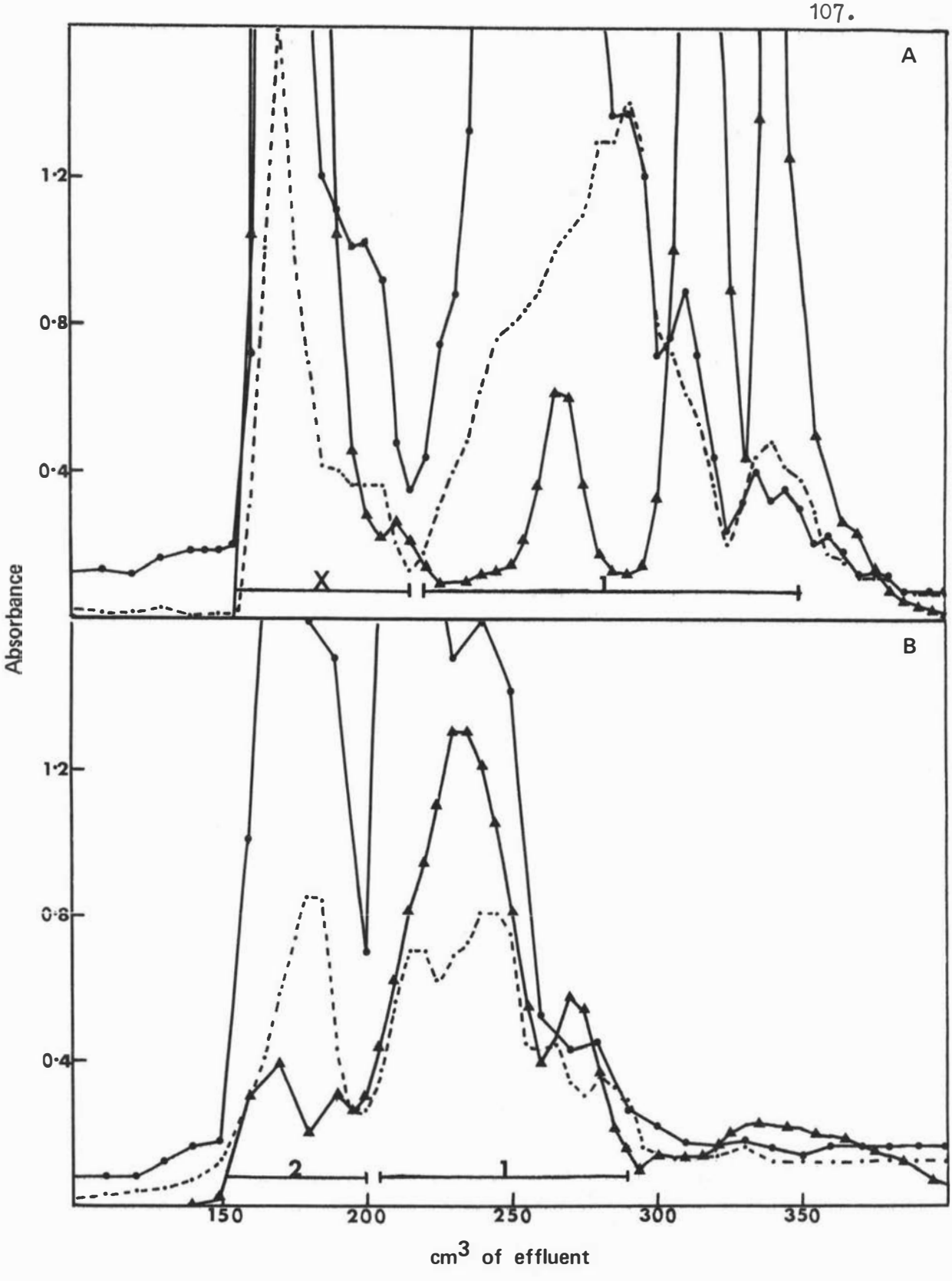


FIGURE 13. Digest II: Gel filtration of tryptic digests of unheated haemoglobin. A, 4 hour digest; B, fraction X re-digested for 2 hours; 4.0 cm^3 fractions collected. ·--· absorbance at 570 nm after reaction with ninhydrin, 0.1 cm^3 aliquots; ●—● absorbance at 570 nm of alkali hydrolysed sample reacted with ninhydrin, 0.05 cm^3 aliquots; ▲—▲ absorbance at 280 nm.

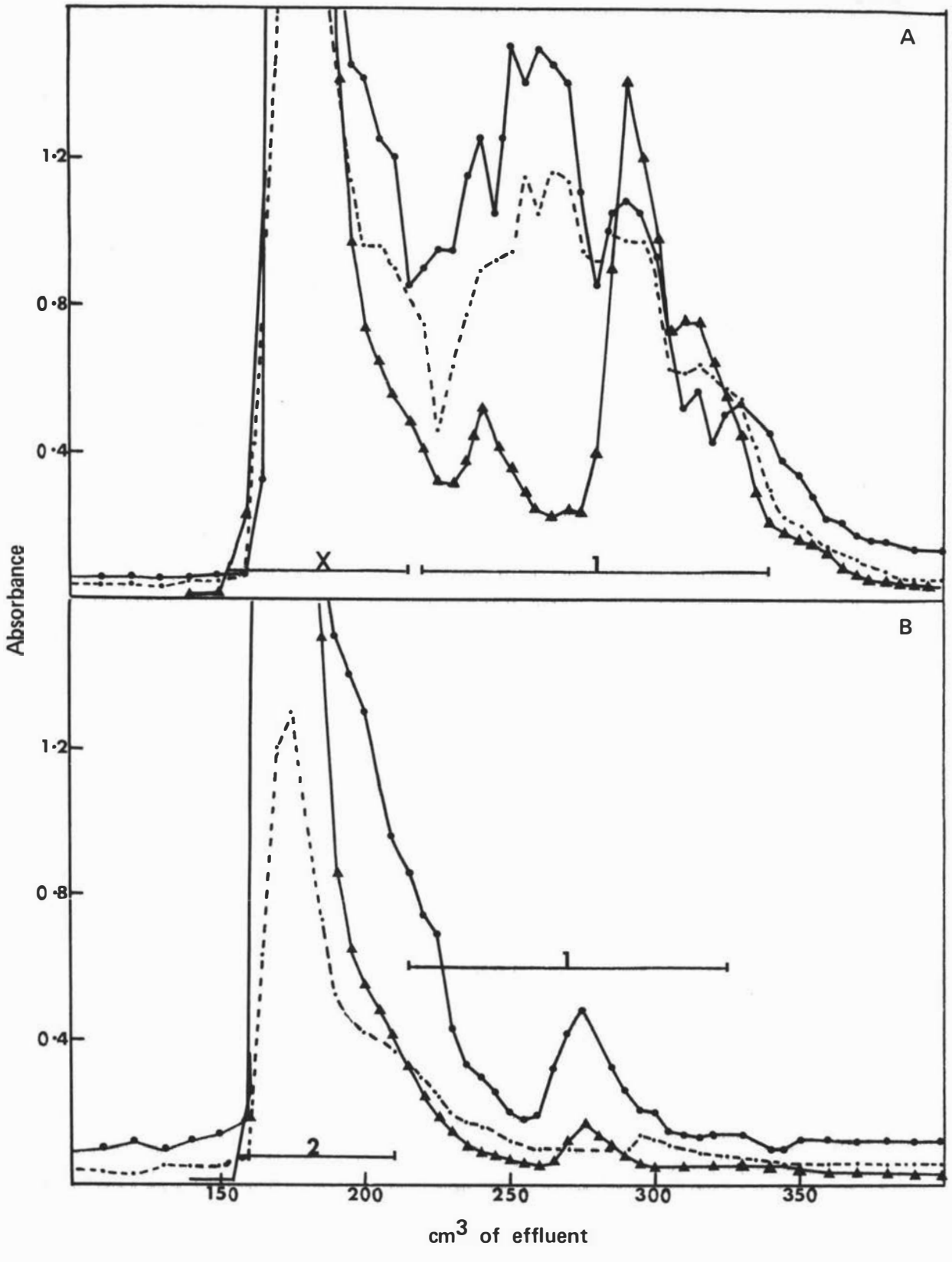


FIGURE 14. Digest II: Gel filtration of tryptic digests of heated haemoglobin. A, 4 hour digest; B, fraction X re-digested 2 hours; 4.0 cm^3 fractions collected. ·--· absorbance at 570 nm after reaction with ninhydrin, 0.1 cm^3 aliquots; ●—● absorbance at 570 nm of alkali hydrolysed sample reacted with ninhydrin, 0.05 cm^3 aliquots; ▲—▲ absorbance at 280 nm.

This fraction of large sized material, designated X in Figures 13A and 14A, was re-digested with TPCK-treated trypsin (giving a final trypsin-haemoglobin ratio of 2%, w/w) for 2 hours, and the digests re-chromatographed on Sephadex G-25. The further digestion with trypsin fragmented much of the large sized material in fraction X from the unheated protein (Figure 13B), but had little effect on that from the heated protein (Figure 14B). The peptide material from fraction X was divided into two fractions, 1 and 2, as shown in Figures 13B and 14B, and fraction 1 combined with the corresponding fraction 1 of the initial separation on Sephadex G-25, Figures 13A and 14A. These fractions 1 and 2 were further investigated by chromatography on Dowex 1-X2.

Fraction 1 (see Figures 13 and 14). Chromatography on Dowex 1-X2 showed that there was much more ninhydrin positive material in fraction 1 from the unheated haemoglobin than from the heated protein digest (Figure 15). Small amounts of free amino acids, mainly leucine and alanine, were eluted at the beginning of the gradient with the heated protein fraction, but these were not detected in the unheated protein fraction. Since only a low level of chymotryptic activity was associated with the TPCK-treated trypsin, the presence of these free amino acids must reflect some change in the heated protein. In particular, free leucine had not been detected in the extract of the heated globin (undigested) so that this must have been produced as the result of trypsin activity. However, the amounts of free amino acids found were much less than in Digest I (about one-twentieth) so that presumably in Digest I chymotryptic contamination of trypsin was mainly responsible for their release.

Other areas of ninhydrin positive material in fraction 1 of the heated protein digest were not investigated as the amounts were small and corresponding but much larger quantities of peptides were present

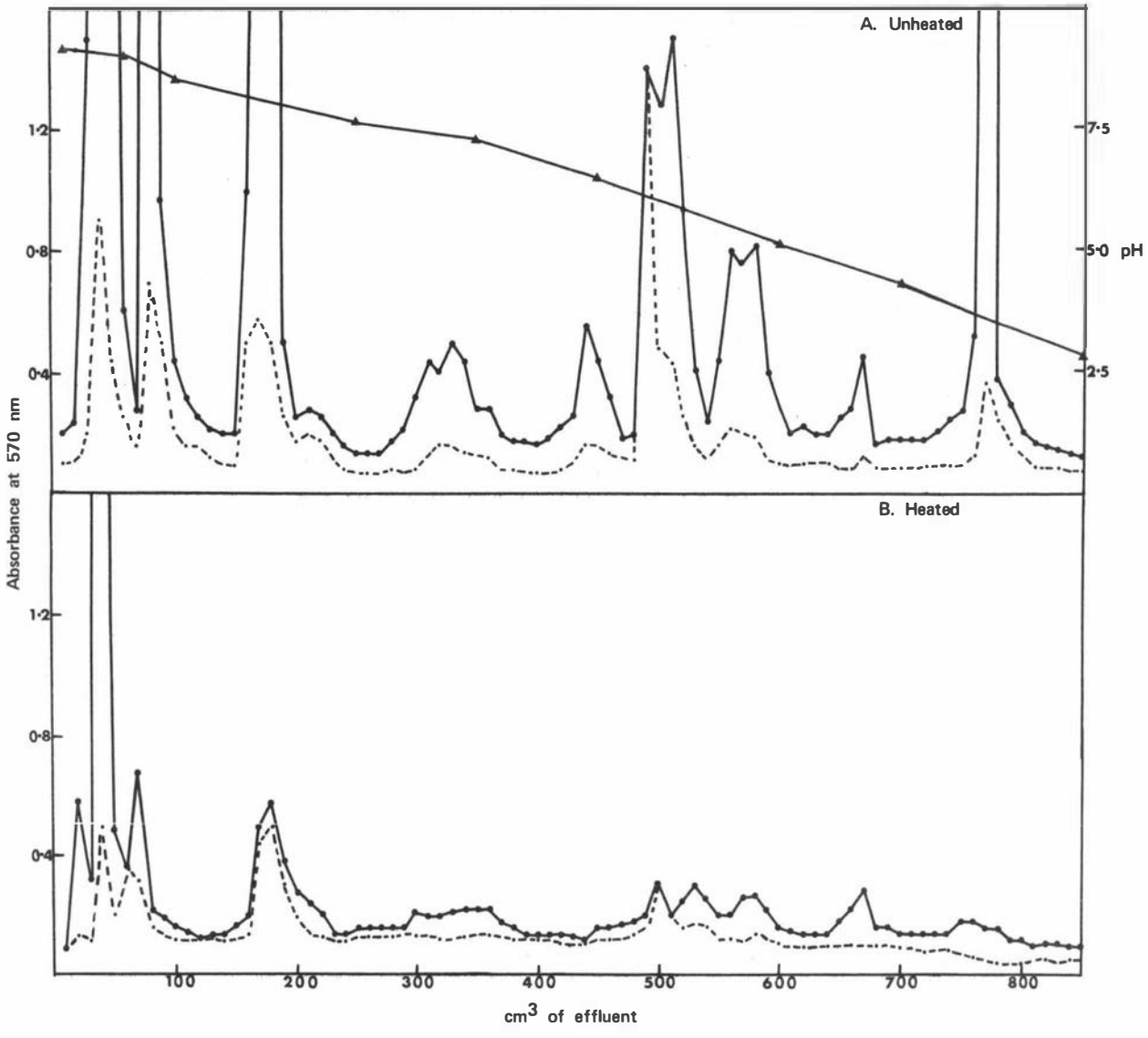


FIGURE 15. Digest II: Chromatography on Dowex I–X2 of fraction I from a tryptic digest of haemoglobin, A, unheated; B, heated. ·--· absorbance after reaction with ninhydrin, 0.1 cm^3 aliquots; —— absorbance of alkali hydrolysed sample reacted with ninhydrin, 0.05 cm^3 aliquots; ▲—▲ pH gradient

in the unheated haemoglobin fraction.

Fraction 2 (see Figures 13 and 14). There was rather more ninhydrin positive material eluted from Dowex 1-X2 with fraction 2 from the heated haemoglobin digest than from the unheated protein (Figure 16). This peptide material was divided into four zones, A - D, as shown in Figure 16. Zones A and D were not investigated as considerable amounts of ninhydrin positive material were also eluted in the corresponding regions of fraction 2 from the unheated protein digest. However, zones B and C clearly contained peptide material not present in the unheated protein fraction. These zones, B and C, seemed to contain comparatively large peptides since there was little colour development with ninhydrin unless the reaction was preceded by alkaline hydrolysis. Separation of the fragments present was attempted by gel filtration. Zone B was designated 2D2B since it was obtained from Digest II of heated haemoglobin (coded D) and was from fraction 2, and similarly, zone C was designated 2D2C.

Gel filtration of zones B and C (see Figure 16) from heated haemoglobin. A Sephadex G-50 column (1.9 x 150 cm) with 10% acetic acid, was used for the gel filtration of 2D2B and 2D2C. The elution profiles (Figures 17A and 17B) showed that both zones were composed of a mixture of many different sized peptides, and both contained material which eluted before the void volume of the gel. The eluted peptide material from each zone was divided into four fractions as indicated in Figures 17A and 17B; 2D2B into fractions M, N, O and P, and 2D2C into fractions E, F, G and H.

Attempted purification of fractions from zones B and C (Figures 17A, 17B). The fractions from 2D2B and 2D2C were very insoluble; glacial acetic acid was found to be the best solvent, and once dissolved the fragments remained in solution on dilution to a concentration of 10% acetic acid. The fractions were not soluble in mixtures of phenol and

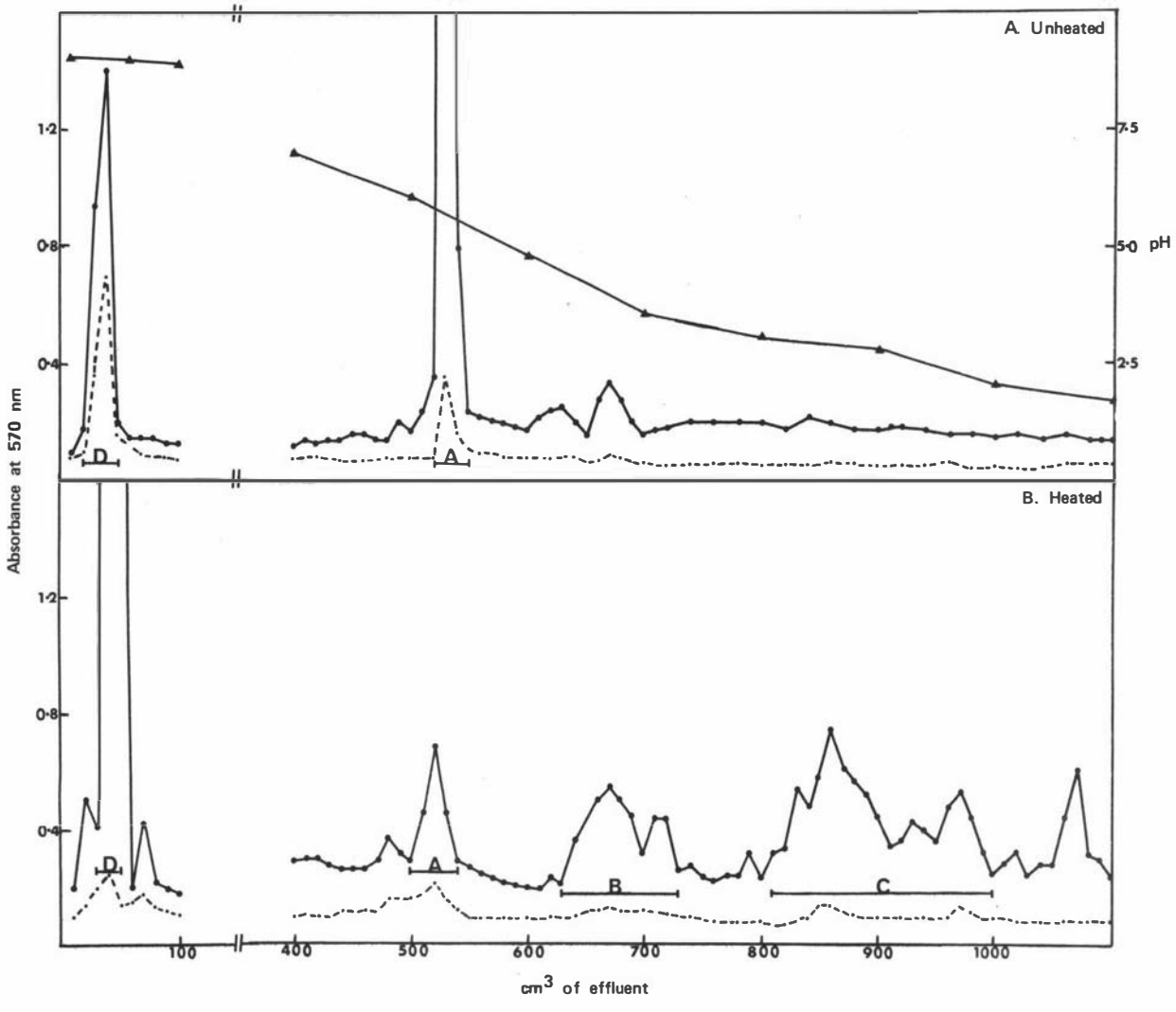


FIGURE 16. Digest II: Chromatography on Dowex I–X2 of fraction 2 from a tryptic digest of haemoglobin. A, unheated; B, heated. ·---· absorbance after reaction with ninhydrin, 0.1 cm^3 aliquots; ●—● absorbance of alkali hydrolysed sample reacted with ninhydrin, 0.1 cm^3 aliquots; ▲—▲ pH gradient

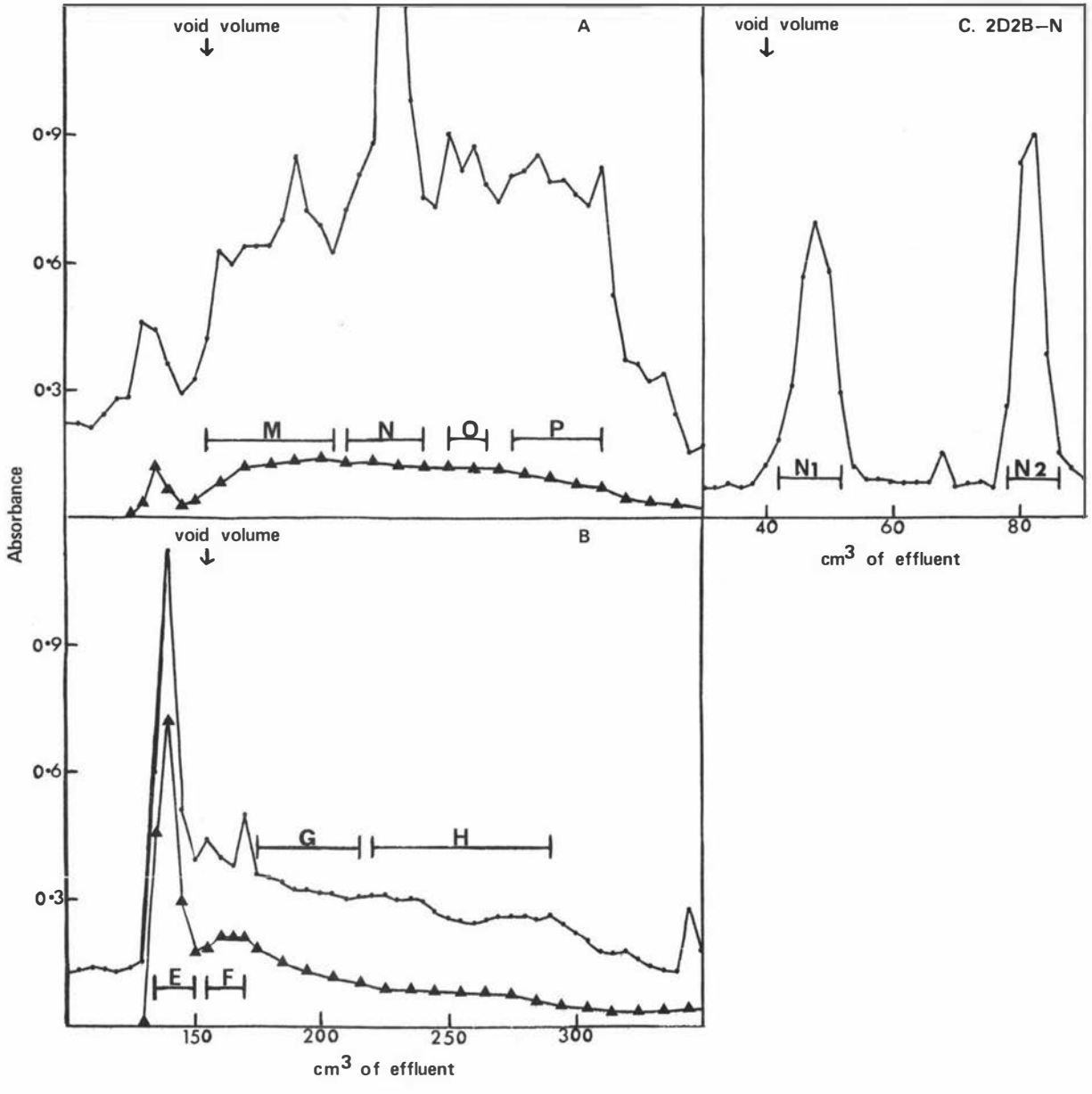


FIGURE 17. Gel filtration of zones B and C from a tryptic digest of heated haemoglobin. A, zone B; B, zone C; 5.0 cm^3 fractions collected; C, fraction N from zone B; 2.0 cm^3 fractions collected.
—— absorbance at 570 nm of alkali hydrolysed sample reacted with ninhydrin, 0.2 cm^3 aliquots for A and B, 0.1 cm^3 for C.
▲—▲ absorbance at 280 nm.

acetic acid, in formamide, or in 8 M urea. Insolubility of the components limited the methods which could be used for the resolution of the fractions. Chromatography on CM-cellulose, in the presence or absence of urea, or with phosphocellulose, using various buffer systems (mainly acetic acid - pyridine buffers), or with Dowex 1-X2 or Dowex 50-X2, were unsuccessful, as a major portion of the sample remained on the column; even 0.1 - 1.0 M NaOH or HCL (depending on the ion-exchange material) failed to elute the bound samples. As these fractions had been obtained by chromatography on Dowex 1-X2, it was unexpected that this resin could not be used for their purification. It is possible that the groups responsible for binding to the resin were masked in the complex mixture, fraction 2, initially chromatographed on Dowex 1-X2.

As a result of these purification attempts, the supplies of fractions obtained from gel filtration of 2D2B and 2D2C were exhausted except for fraction 2D2B-N (see Figure 17A). This was re-chromatographed on Sephadex G-50 (0.9 x 150 cm) with 30% acetic acid, instead of the 10% acetic acid previously used, in an attempt to minimise association of peptides. Two peaks were obtained, 2D2B-N1 and 2D2B-N2 (Figure 17C). The amino acid composition of these two peaks was determined, using a sample from both sides of peak 1, 2D2B-N1, and a single sample of 2D2B-N2. The smaller fragment, 2D2B-N2, was much richer in arginine, aspartic and glutamic acids, than 2D2B-N1. Amino-terminal analysis of 2D2B-N1 indicated that there were four N-terminal groups present, leucine, valine, phenylalanine and alanine, so the fragment apparently contained more than one peptide. The N-terminal of 2D2B-N2 was not determined. The results for the amino acid composition and amino-terminal analysis of fractions 2D2B-N1 and 2D2B-N2 are given later in Table XXIX (page 129) together with results for fragments obtained from Digest III.

Digest III.

A third digest was prepared so that the fragments of the heated protein detected in Digest II could be further investigated. In contrast to the other two digests, globin rather than haemoglobin, was used for Digest III. About 150 mg globin was digested for 6 hours with aliquots of trypsin (TPCK-treated) added at 0 and 2 hours. The digest was fractionated by the same methods used for Digest II, except that there was no further digestion with trypsin of the material eluted at the void volume when the digest was chromatographed on Sephadex G-25. An outline of the methods used is given in Tables XXVI and XXVII for the unheated and heated globin digests respectively.

Gel filtration. The elution profiles for gel filtration of the Digest III on Sephadex G-25 are given in Figure 18. The profiles were similar to those obtained with Digest II (see Figures 13 and 14) and the material was again divided into two fractions, and chromatographed on Dowex 1-X2.

Fraction 1 (see Figure 18). The results obtained when fraction 1, from Digest III, was chromatographed on Dowex 1-X2 (Figure 19) were similar to those obtained from the same fraction from Digest II (Figure 15) and the peptide material was not further investigated.

Fraction 2 (see Figure 18). The chromatograms for fraction 2 chromatographed on Dowex 1-X2 are shown in Figure 20. Compared to fraction 2 from Digest II (see Figure 16), more peptide material was eluted at zone C with the fraction from Digest III of the heated globin. In addition, fraction 2 of the unheated protein, contained material which eluted in the regions corresponding to zones B and C of the heated protein digest. These zones B and C, from both the unheated globin (coded M and the zones designated as 3M2B and 3M2C) and heated globin (3D2B and 3D2C) were partially resolved by gel filtration.

TABLE XXVI

Outline of methods used with Digest III, unheated globin

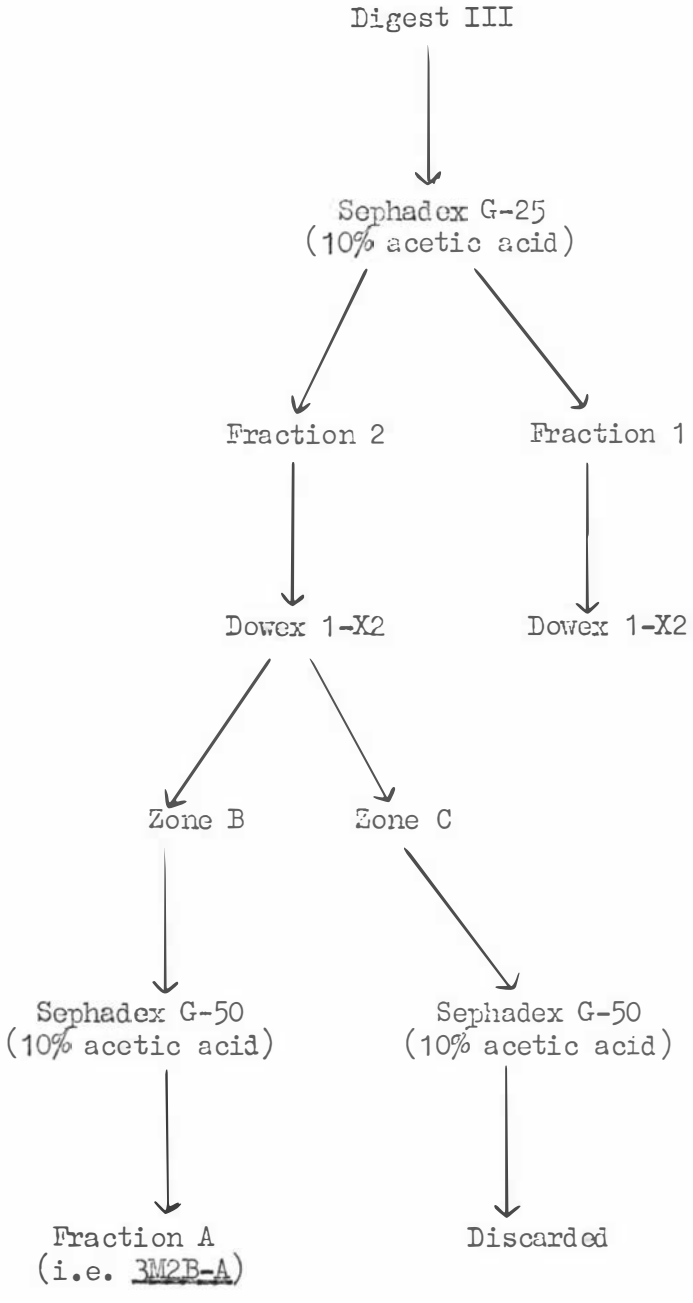
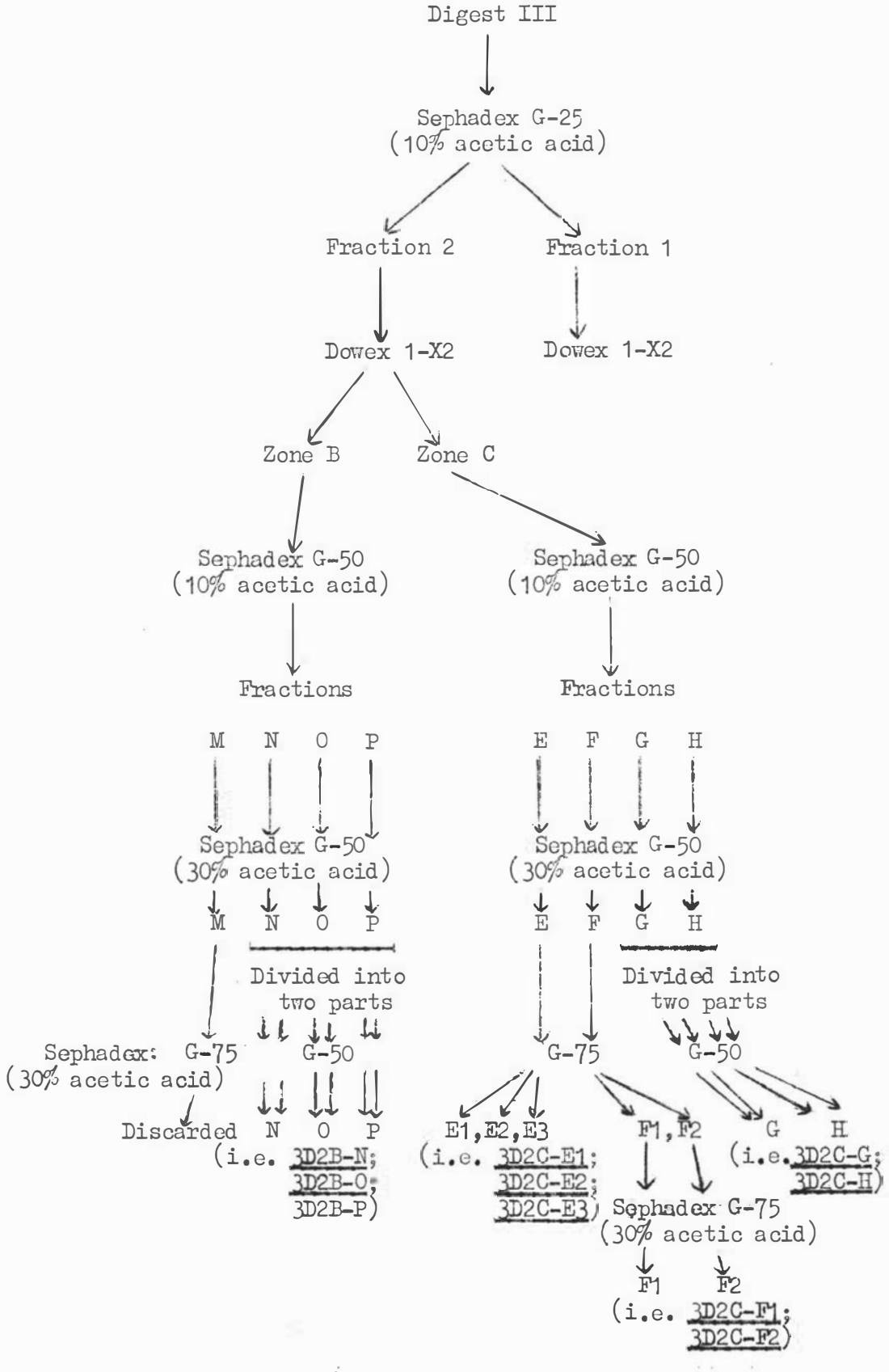


TABLE XXVII

Outline of methods used with Digest III, heated globin



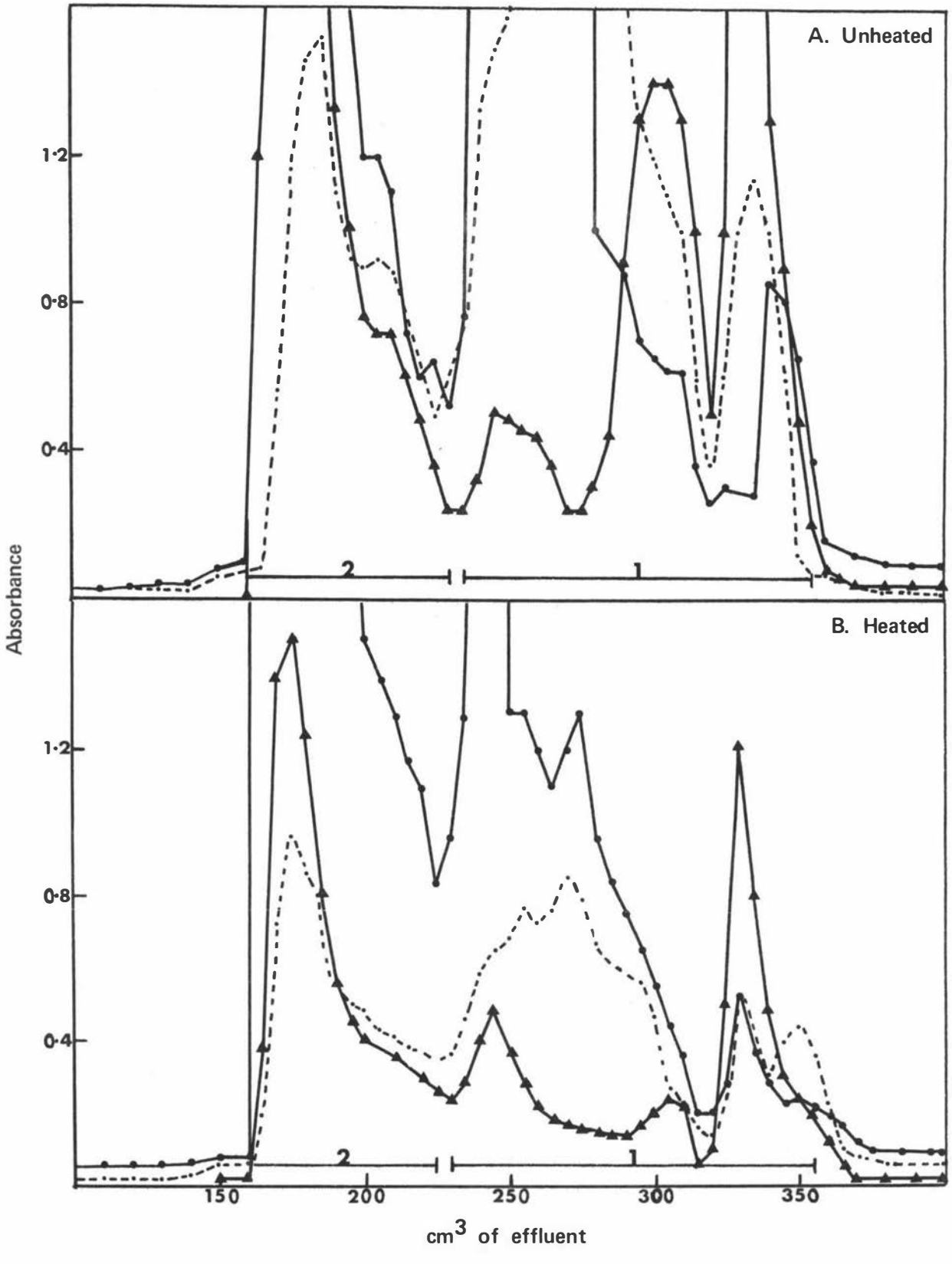


FIGURE 18. Digest III: Gel filtration of a tryptic digest of globin. A, unheated; B, heated; 4.0 cm^3 fractions collected. ·– – · absorbance at 570 nm after reaction with ninhydrin, 0.1 cm^3 aliquots; ●—● absorbance at 570 nm of alkali hydrolysed sample reacted with ninhydrin, 0.05 cm^3 aliquots; ▲—▲ absorbance at 280 nm.

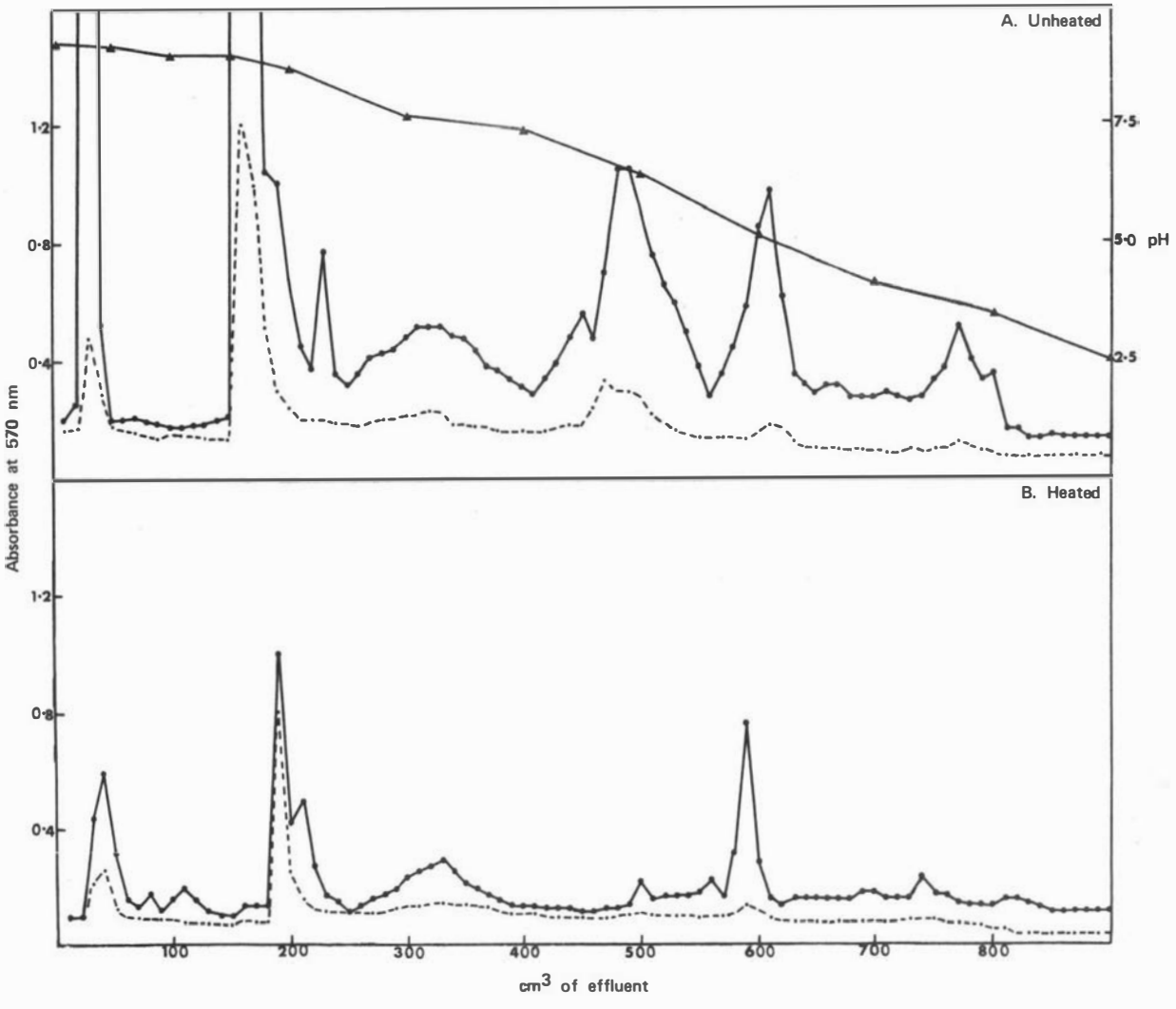


FIGURE 19. Digest III: Chromatography on Dowex I–X2 of fraction I from a tryptic digest of globin. A, unheated; B, heated. · - - - · absorbance after reaction with ninhydrin, 0.1 cm^3 aliquots; ●——● absorbance of alkali hydrolysed sample reacted with ninhydrin, 0.05 cm^3 aliquots; ▲——▲ pH gradient

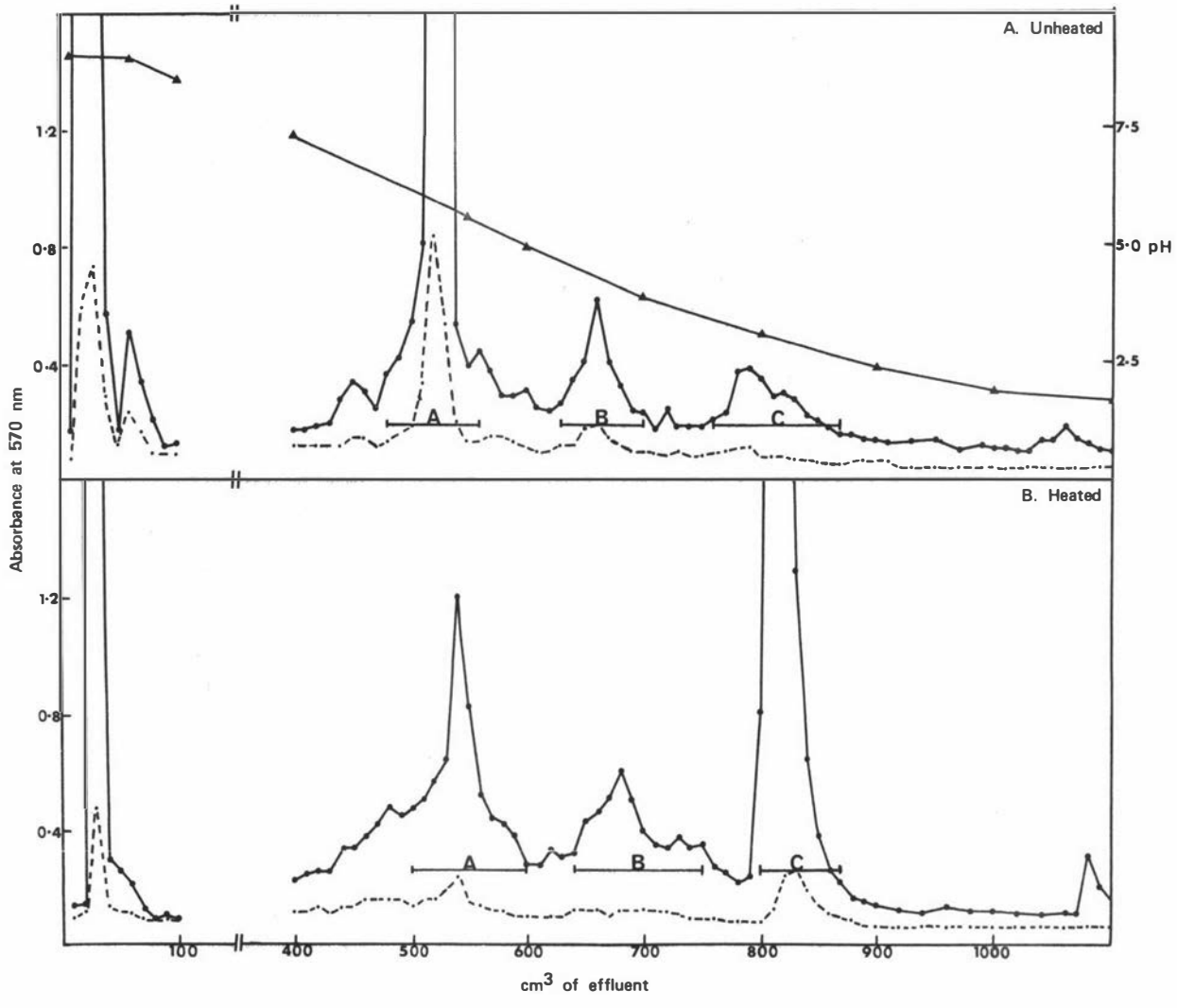


FIGURE 20. Digest III: Chromatography on Dowex I--X2 of fraction 2 from a tryptic digest of globin. A, unheated; B, heated. - - - - absorbance after reaction with ninhydrin, 0.2 cm^3 aliquots; ——● absorbance of alkali hydrolysed sample reacted with ninhydrin, 0.1 cm^3 aliquots; ▲—▲ pH gradient

Gel filtration of zones B and C from unheated and heated globin. Gel filtration with Sephadex G-50 (1.9 x 150 cm, 10% acetic acid) of zones B and C (see Figure 20) resulted in quite different elution profiles for the unheated and heated protein fractions (Figures 21 and 22). For both the unheated and heated protein, zones B and C were a mixture of different sized fragments and these eluted over nearly the entire range of the gel. The chromatograms for zones B and C from the heated protein were qualitatively similar to those obtained with Digest II (Figure 17), although zone C from Digest III contained less material which absorbed at 280 nm. Zones B and C of the heated globin also contained smaller amounts of peptides which eluted before the void volume of the gel, than occurred with the corresponding zones of Digest II (Figure 17).

The material from zones B and C of the heated globin was divided into four fractions; 3D2B into fractions M, N, O and P, and 3D2C into E, F, G and H; these fractions are indicated in Figures 21 and 22. One well defined peak was obtained from zone B of the unheated globin, 3M2B-A (Figure 21) and this was further investigated, but as the chromatogram of zone C of the unheated protein indicated only very small amounts of many peptides, these fractions were discarded.

Attempted purification of fractions from zones B and C from unheated and heated globin. The fractions from zones B and C of the heated globin, and 3M2B-A from the unheated globin (see Figures 21 and 22), were re-chromatographed on a column of Sephadex G-50 (0.9 x 150 cm) with 30% acetic acid. For each fraction, single and usually quite symmetrical peaks were obtained (Figures 23 and 24). However, determination of the amino-terminal groups, by dansylation, showed that each fraction from the heated globin, contained more than one N-terminal, indicating that more than one peptide was present. The fraction, 3M2B-A, from the unheated globin digest, gave only one amino-terminal, phenylalanine, and the

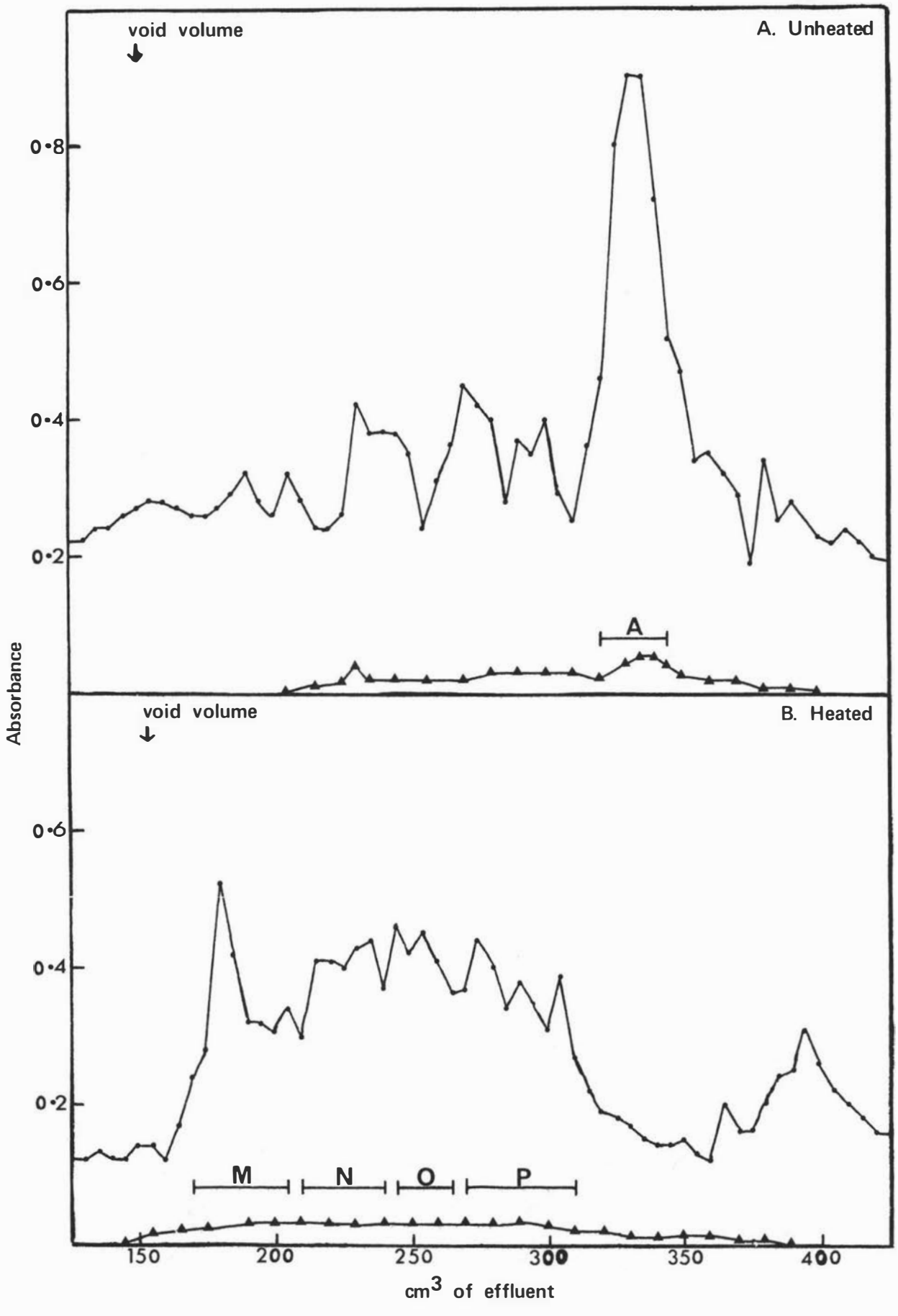


FIGURE 21. Digest III: Gel filtration of zone B from a tryptic digest of globin. A, unheated; B, heated; 4.5 cm^3 fractions collected. —— absorbance at 570 nm of alkali hydrolysed sample reacted with ninhydrin, 0.2 cm^3 aliquots; ▲—▲ absorbance at 280 nm.

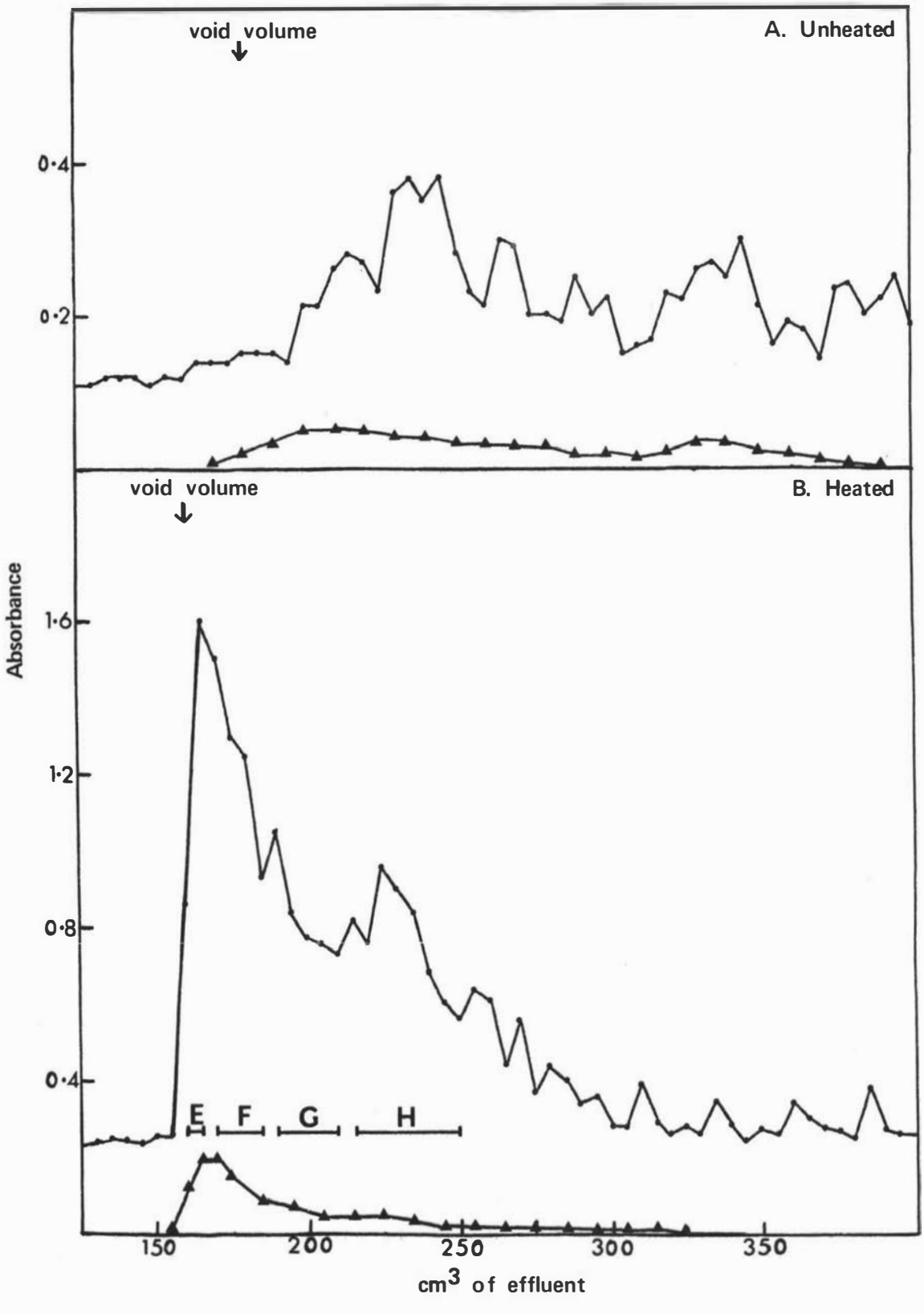


FIGURE 22. Digest III: Gel filtration of zone C from a tryptic digest of globin. A, unheated; B, heated; 4.5 cm^3 fractions collected.
——— absorbance at 570 nm of alkali hydrolysed sample reacted with ninhydrin, 0.2 cm^3 aliquots; ▲——▲ absorbance at 280 nm. Note change in absorbance scale.

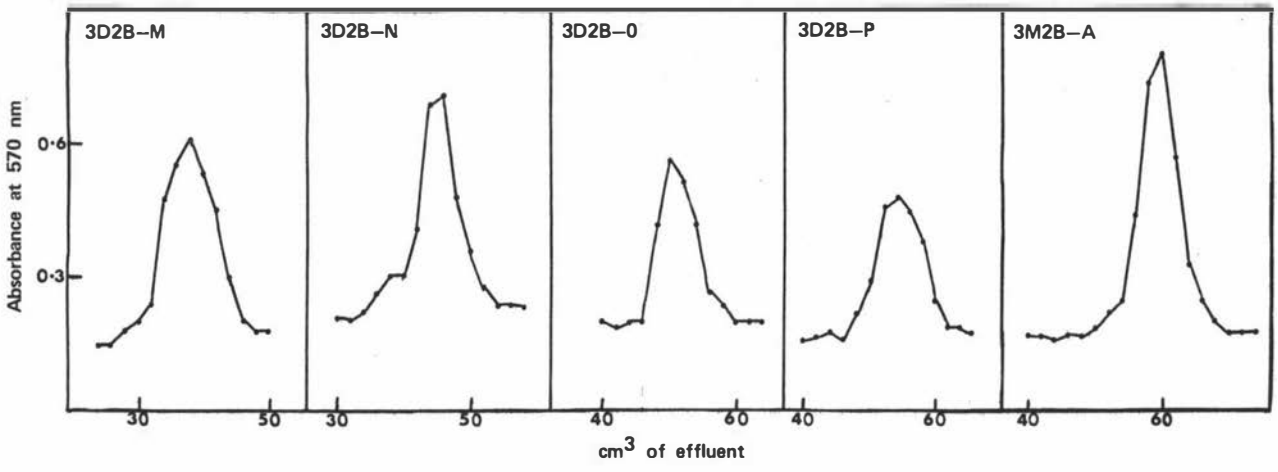


FIGURE 23. Digest III: Initial purification of fragments from zone B of heated (3D2B–M to P) and unheated globin (3M2B–A). The void volume was 34.0 cm^3 and 2.0 cm^3 fractions were collected; 0.1 cm^3 aliquots reacted with ninhydrin after alkaline hydrolysis.

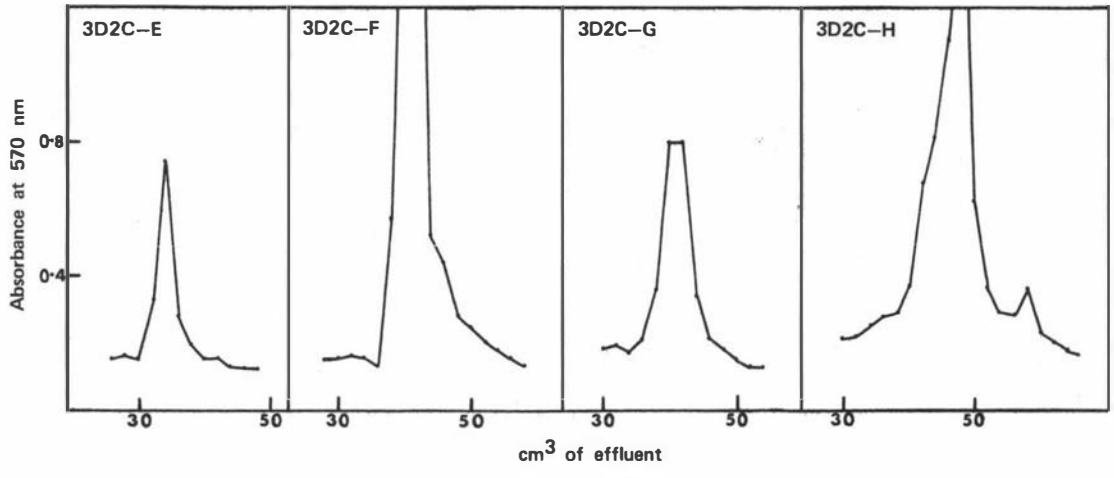


FIGURE 24. Digest III: Initial purification of fragments from zone C of heated globin (3D2C–E to H). The void volume was 34.0 cm^3 and 2.0 cm^3 fractions were collected; 0.1 cm^3 aliquots reacted with ninhydrin after alkaline hydrolysis.

fraction was not further purified. The amino acid composition (of samples from each side of the peak eluted) and the N-terminal group of 3M2B-A are given in Table XXVIII.

Further purification of fractions from zones B and C of the heated globin. Gel filtration in 30% acetic acid was again used for the attempted purification of fractions from zones B and C of the heated globin. Fragments which eluted at the void volume of Sephadex G-50, 3D2B-M, 3D2C-E and 3D2C-F (see Figures 23 and 24) were re-chromatographed on a column of Sephadex G-75 (0.9 x 100 cm). The other fractions were divided into two parts, the two sides of the peak eluted from Sephadex G-50, and each half re-chromatographed on that gel using the same column (0.9 x 150 cm). The volumes collected were reduced to 0.75 - 1.0 cm^3 to minimise possible overlaps of similar sized peptides. The following results were obtained:-

Fractions from zone B: 3D2B-M was eluted as a broad zone and, as there was little ninhydrin positive material after alkaline hydrolysis, the fraction was discarded. 3D2B-N, 3D2B-O and 3D2B-P were eluted as single peaks with like elution volumes for both halves chromatographed; the elution profiles were qualitatively similar to those shown in Figure 23.

Fractions from zone C: 3D2C-E was resolved into three peaks, 1 - 3, peak 1 eluting at the void volume of Sephadex G-75, and peaks 2 and 3, eluting much later and over-lapping each other (Figure 25). 3D2C-F eluted as two over-lapping peaks, 1 and 2, (Figure 25); these two peaks were re-chromatographed separately on Sephadex G-75, and each resulted in a broad peak (Figure 25), the elution volumes of the two peaks were slightly different. 3D2C-G and 3D2C-H both eluted as single peaks with like elution volumes for both parts chromatographed; the elution profiles were qualitatively similar to those shown in Figure 24.

TABLE XXVIII

Amino acid composition and amino-terminal of fraction 3M2B-A (see Figure 21) from a tryptic digest of unheated globin, together with the composition of two tryptic peptides (α-13 and β-5) from bovine globin. The samples a and b were from either side of the peak eluted from Sephadex G-50.

	3M2B-A		Tryptic peptides	
	a	b	β-5	α-13
Lysine	1.4	1.2	1	1
Histidine	-	-		
Arginine	-	-		
Aspartic acid	4.5	4.4	4	1
Threonine	1.1	1.1	1	2
Serine	1.8	1.8	2	2
Glutamic acid	2.1	1.6	1	
Proline	1.1	1.0	1	
Glycine	2.0	1.5	1	
Alanine	3.2	2.5	2	1
Valine	1.3	1.3	1	2
Methionine	0.2	0.2	1	
Leucine	1.2	1.4	1	2
Tyrosine	-	-		
Phenylalanine	3.0	3.0	3	1
Amino-terminal:	phenylalanine		phenylalanine	

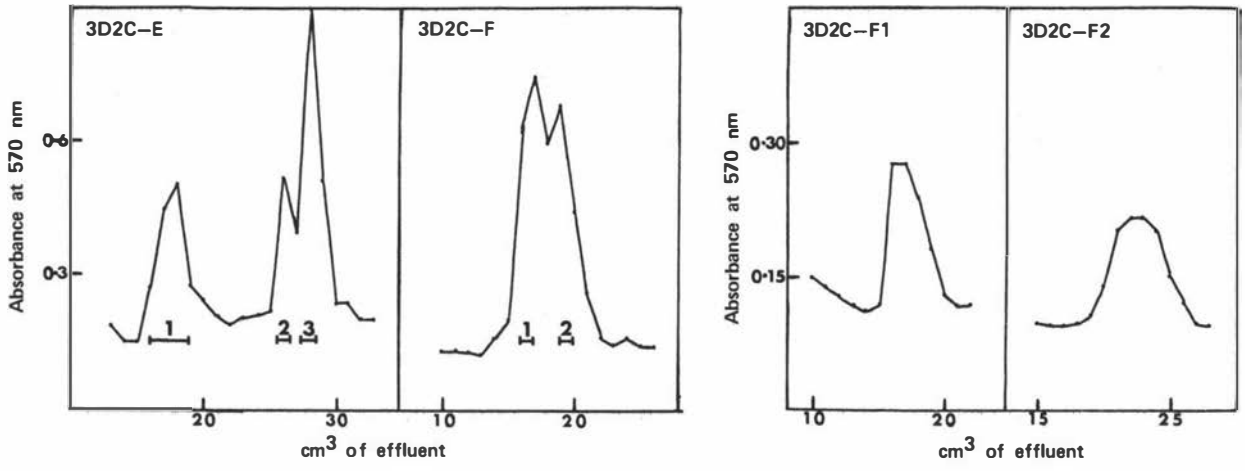


FIGURE 25. Digest III: Final purification of fragments from zone C of heated globin (3D2C–E, 3D2C–F and two fractions from 3D2C–F).
The void volume was 20.0 cm^3 and 0.75–1.0 cm^3 fractions were collected; 0.1 cm^3 aliquots reacted with ninhydrin after alkaline hydrolysis.
Note change in absorbance scale.

For all fractions, there was very little ninhydrin positive material eluted other than the peaks described. The amino acid composition and amino-terminals of these fragments were determined.

Analysis of fractions from zones B and C from heated globin.

Amino acid analysis. The amino acid composition of each sub-fraction chromatographed on Sephadex G-50 or G-75 (see Figures 23, 24 and 25) from the heated globin, was determined. A sample from both sides of peak 1 from 3D2C-E (3D2C-E1), was analysed, and the entire samples corresponding to 3D2C-E2 and 3D2C-E3 (Figure 25) were used for analysis. The results are given in Tables XXIX and XXX; the composition of all samples have been calculated as molar ratios with leucine equal to 20, the number of leucine residues in the alpha chain of bovine globin. The composition of the alpha and beta chains of bovine globin are included in Table XXIX. The analyses for the two sides of peaks were generally quite similar, that is, fractions 3D2B-N, 3D2B-O, 3D2B-P (Figure 23), 3D2C-E1 (Figure 25), 3D2C-G and 3D2C-H (Figure 24) were probably homogeneous. The two sub-fractions from 3D2C-F, 3D2C-F1 and 3D2C-F2 (Figure 25), also had nearly identical amino acid compositions. The samples from either side of peak 1 from 3D2C-E (3D2C-E1) had similar amino acid ratios but the analyses for peaks 2 and 3 from 3D2C-E were quite different. These fractions, 3D2C-E2 and 3D2C-E3, were particularly rich in lysine, arginine, aspartic acid and glutamic acid, compared to 3D2C-E1 or other fractions analysed, or to either chain of bovine haemoglobin.

Amino-terminal analysis. The amino-terminals for only one of the sub-fractions chromatographed on Sephadex G-50 or G-75 (see Figures 23, 24 and 25), was determined by dansylation. The results are included in Tables XXIX and XXX and show that there was more than one N-terminal for each fraction from the heated globin digest; the approximate intensities of each N-terminal amino acid, by visual inspection, is given in

TABLE XXIX

Amino acid molar ratios and amino-terminals, of fractions from zone B of tryptic digests of heated globin or haemoglobin. The samples a and b were from either side of the peak eluted from Sephadex gel. The composition of alpha and beta chains of bovine globin are included (Dayhoff, 1972).

	2D2B-N1		2D2B-N2	3D2B-N		3D2B-O		3D2B-P		Bovine globin	
	a	b		a	b	a	b	a	b	α	β
Lysine	10.0	9.9	4.7	7.1	6.3	6.9	ND	8.3	6.6	11	11
Histidine	7.7	7.9		6.3	8.3	9.3	ND	ND	7.3	10	7
Arginine	2.2	2.4	14.2	2.9	1.6	1.8	ND	2.4	1.6	3	4
Aspartic acid	16.6	17.8	47.9	12.1	11.7	11.0	11.2	14.5	16.3	11	17
Threonine	6.5	6.4	13.3	6.7	6.4	4.8	5.9	4.4	4.1	8	6
Serine	8.4	9.5	16.0	9.9	10.1	10.0	10.3	9.2	8.7	13	6
Glutamic acid	12.3	11.8	52.8	5.3	5.0	5.3	5.7	12.3	13.4	6	11
Proline	4.3	4.3		6.0	5.6	6.2	6.9	4.5	5.4	6	4
Glycine	11.3	11.8	55.2	6.5	6.1	6.1	8.8	11.4	12.1	9	10
Alanine	19.8	20.0	28.5	14.6	14.5	14.8	14.5	19.0	18.8	20	16
Valine	14.0	13.8	16.0	12.1	11.0	9.6	9.4	10.5	11.0	12	18
Leucine	20	20	20	20	20	20	20	20	20	20	17
Phenylalanine	9.2	8.2	12.0	6.4	5.7	4.7	3.8	4.8	4.4	7	10
Amino-terminals:*	val (1)			val (1)		val (1)		val (1)			
	leu (1)			leu (1)		leu (3)		leu (1)			
	phe (1)			phe (1)							
	ala (1)			ala (1)							

* Abbreviations given in Table B, Appendix 3.

( ), approximate intensity ND, not determined.

TABLE XXX

Amino acid molar ratios and amino-terminals, of fractions from zone C of a tryptic digest of heated globin. The samples a and b were from either side of the peak eluted from Sephadex gel.

	3D2C-E1		3D2C-E2	3D2C-E3	3D2C-F1	3D2C-F2	3D2C-G		3D2C-H	
	a	b					a	b	a	b
Lysine	9.3	8.2	16.6	22.8	8.1	7.8	7.5	6.6	6.4	5.2
Histidine	ND	9.1	6.4	12.1	7.3	9.1	8.3	9.1	7.7	6.0
Arginine	2.6	3.2	7.9	8.0	2.0	ND	1.8	1.0		
Aspartic acid	12.4	12.7	17.8	18.6	11.9	11.9	11.3	11.4	9.8	10.7
Threonine	7.1	7.8	10.6	11.0	6.7	7.2	6.4	6.4	7.1	6.4
Serine	11.1	12.9	11.9	11.7	11.4	11.8	9.4	9.6	12.4	12.9
Glutamic acid	5.8	5.9	24.2	27.3	5.9	5.3	4.4	4.0	1.9	1.5
Proline	7.1	7.4	6.7	10.0	5.1	7.0	7.9	7.0	9.5	8.1
Glycine	7.6	8.6	9.2	8.9	6.7	5.8	5.3	5.3	3.5	4.1
Alanine	14.7	15.6	16.4	15.7	13.5	13.7	12.6	12.7	9.6	9.5
Valine	12.5	12.0	11.3	12.5	9.4	11.2	11.6	11.3	10.6	11.3
Leucine	20	20	20	20	20	20	20	20	20	20
Tyrosine							Tr	Tr		
Phenylalanine	7.1	7.1	7.3	8.9	6.8	5.9	6.6	5.8	4.7	5.3
Amino-terminals:*	val (2) leu (1)		ND	ND	val (3) leu (1) phe (tr)		val (1) leu (1) phe (1)		val (2) leu (1)	

* Abbreviations given in Table B, Appendix 3.

ND, not determined

( ), approximate intensity

parenthesis. All fragments had both valine and leucine as amino-terminals, and phenylalanine was also present as an N-terminal in several fragments; alanine was the fourth amino-terminal of fraction 3D2B-N.

Origin of fragments from zones B and C of tryptic digests of globin.

Unheated globin. The fragment 3M2B-A (Figures 21 and 23) from unheated globin was apparently a single peptide as only one amino-terminal group was detected, phenylalanine. There are two tryptic peptides from bovine haemoglobin with phenylalanine as the N-terminal, α-13 and β-5 (Table A, Appendix 3) and the amino acid composition of these are included in Table XXVIII. The amino acid ratios of 3M2B-A were similar to those of tryptic peptide β-5 except that the methionine content was very low (methionine was determined mainly as the sulphone and sulphoxides). Fragment 3M2B-A was relatively small in size as it had a large elution volume when chromatographed on Sephadex G-50, and this agrees with the moderate size of the possible tryptic peptides from bovine haemoglobin, α-13 has 12 residues and β-5 has 19 residues. The peptide, 3M2B-A, was probably on the border of the initial fractionation on Sephadex G-25 of the tryptic digest of globin.

Heated globin. It is noteworthy that all the fragments investigated from the heated globin tryptic digest had valine as an N-terminal group. The alpha chain of bovine globin also has valine as the amino-terminal, and as the fragments generally showed a greater resemblance to the amino acid ratios of the alpha chain rather than to the ratios of the beta chain, this may indicate that the fragments were derived mainly from the alpha chain.

The larger fragments from zones B and C had quite similar amino acid ratios but the ratios of the smaller fragments, 3D2B-P (Figure 23) and 3D2C-H (Figure 24) were unlike that of other fragments from the same zone. The fragments, 2D2B-N1 (Figure 17C) and 3D2B-N (Figure 23) from Digests II

and III respectively, which had similar elution volumes when zone B was chromatographed on Sephadex G-50 (Figures 17 and 21), had the same N-terminal groups but the amino acid compositions were different.

Some of these fragments, particularly 3D2C-E and 3D2C-F (Figure 25), were very large since they eluted at the void volume of Sephadex G-75; this gel has an exclusion limit of 70,000 for peptides and proteins, and 50,000 for dextrans. However, when a solution of myoglobin (Mol. Wt of 17,000), previously denatured by heating in boiling water for 15 minutes, was chromatographed on the same Sephadex G-75 column, this protein also was eluted at the void volume. It would seem therefore that these fractions from heated globin had expanded shapes (Andrews, 1964, 1965), and were not necessarily larger than either the alpha or beta chain of bovine globin.

The presence of more than one N-terminal in these fragments suggests that they may either:

1) be composed of several peptides so intricately entwined that the methods used in attempts to dissociate such complexes were ineffective, or

2) contain cross-linked fragments which may have originated from one polypeptide or may have formed by reaction between groups on different polypeptides.

Unfortunately only small amounts of these fragments were isolated so that it was not possible to examine their structures more fully. The fragments were evidently very resistant to cleavage by trypsin, since material was eluted in corresponding regions to those isolated, from the 30 hour digest, Digest I. However, although these trypsin-resistant fragments apparently occurred to only a limited extent in the heated globin, their presence would undoubtedly contribute to the reduced rate and extent of digestion by trypsin, and probably by other enzymes. The

presence of these fragments, together with the insolubility of the protein and the masking of titratable groups, provide an explanation for the observed reduced *in vitro* digestibility of heat-damaged proteins.

From these studies it was not possible to determine if new covalent bonds involving lysine had been formed with heat-damage of globin. As the fragments isolated from tryptic digests were large and difficult to characterise, this approach was not pursued, and exhaustive enzyme digests of heated globin were examined for the presence of peptides with enzyme-resistant bonds.

III. Comparison of exhaustive enzyme digests of unheated and heated globin

Exhaustive enzyme digests of unheated and heated globin samples were prepared in pairs using the same conditions, as far as this was possible. The lyophilised digests were dissolved in 2.0 cm^3 of 10% acetic acid and centrifuged. The insoluble material was discarded; this was equivalent to 6 - 7% of the weight of globin, unheated or heated, used for the digests and would probably contain part, at least, of the enzymes used for the digestion.

Exhaustive enzyme digests I

The products present in the first pair of digests, unheated and heated globin, were investigated by amino acid analysis, ion-exchange chromatography and electrophoresis.

Amino acid analysis. The amino acid molar ratios of unhydrolysed and hydrolysed samples of the exhaustive enzyme digests are given in Table XXXI; the ratios have been calculated with leucine equal to 37, the number of residues in the combined alpha and beta chains of bovine globin (Dayhoff, 1972). The samples used for analysis would contain a portion of the enzymes, or their breakdown products, used in the digests. The values for the hydrolysates would also not be accurate as the

TABLE XXXI

Amino acid molar ratios of unhydrolysed (U) and hydrolysed (H) samples of exhaustive enzyme digests of unheated and heated globin. The combined amino acid residues of alpha and beta chains of bovine globin (Dayhoff, 1972) are included.*

	Unheated		Heated		Bovine globin
	U	H	U	H	(α and β chains)
Lysine	13.8	21.4	14.3	20.3	22
Histidine	9.7	12.5	10.6	12.4	17
Arginine	6.3	7.1	6.1	7.4	7
Aspartic acid	5.6	30.8	5.9	34.9	28
Threonine	13.6	14.7	14.4	13.3	14
Serine	16.3	20.0	12.4	16.0	19
Glutamic acid	4.0	19.2	4.5	19.6	17
Proline	4.2	7.5	4.8	7.5	10
Glycine	11.0	19.5	8.6	18.7	19
Alanine	31.7	35.5	34.6	33.5	36
Half cystine	NR	Tr	NR	Tr	1
Valine	25.2	29.2	33.9	28.8	30
Methionine	NR	3.6	NR	3.2	4
Isoleucine	-	1.9	-	1.6	-
Leucine	37	37	37	37	37
Tyrosine	NR	Tr	NR	Tr	5
Phenylalanine	26.6	16.2	20.1	16.5	17

NR, not resolved

Tr, trace

*, tryptophan not included

hydrolysis tubes were not sealed under vacuum and some threonine, serine , cystine and tyrosine would have been destroyed during acid hydrolysis. In addition, the resolution of the unhydrolysed samples was poor due to the presence of peptides so that many of the values are approximate. The values for serine would also be distorted as asparagine and glutamine probably elute in this position. Despite these variables, the values obtained for the unhydrolysed samples from the unheated and heated digests, agreed quite closely. In addition, the composition of the peptide material in the two digests was probably similar as the ratios for the hydrolysed samples also showed reasonable agreement.

Chromatography with Beckman UR-30 resin. The elution profiles obtained by chromatography on Beckman UR-30 resin were very similar for the unheated and heated globin digests (Figure 26). Aliquots of these fractions were examined by electrophoresis.

High-voltage electrophoresis. About 20 µl of each fraction was applied as a 1 cm band to the paper and these were examined by electrophoresis at pH 2.1. A map showing the ninhydrin positive compounds in the two digests is shown in Figure 27. A large number of compounds were present in both digests, most of which could be identified as free amino acids when compared with the applied marker solutions of known amino acids. With the heated globin digest, there was an additional ninhydrin positive compound, staining a pink-orange colour, which had eluted at pH 3.6 from the ion-exchange column, and had an electrophoretic mobility of 0.96 relative to dansyl-arginine (DNS-arginine); this compound is shown darkened in Figure 27. A compound of similar characteristics could not be identified in the corresponding fractions of the unheated globin digest, even when loadings for electrophoresis were increased to 100 µl.

A sample of this unusual component of the heated protein digest,

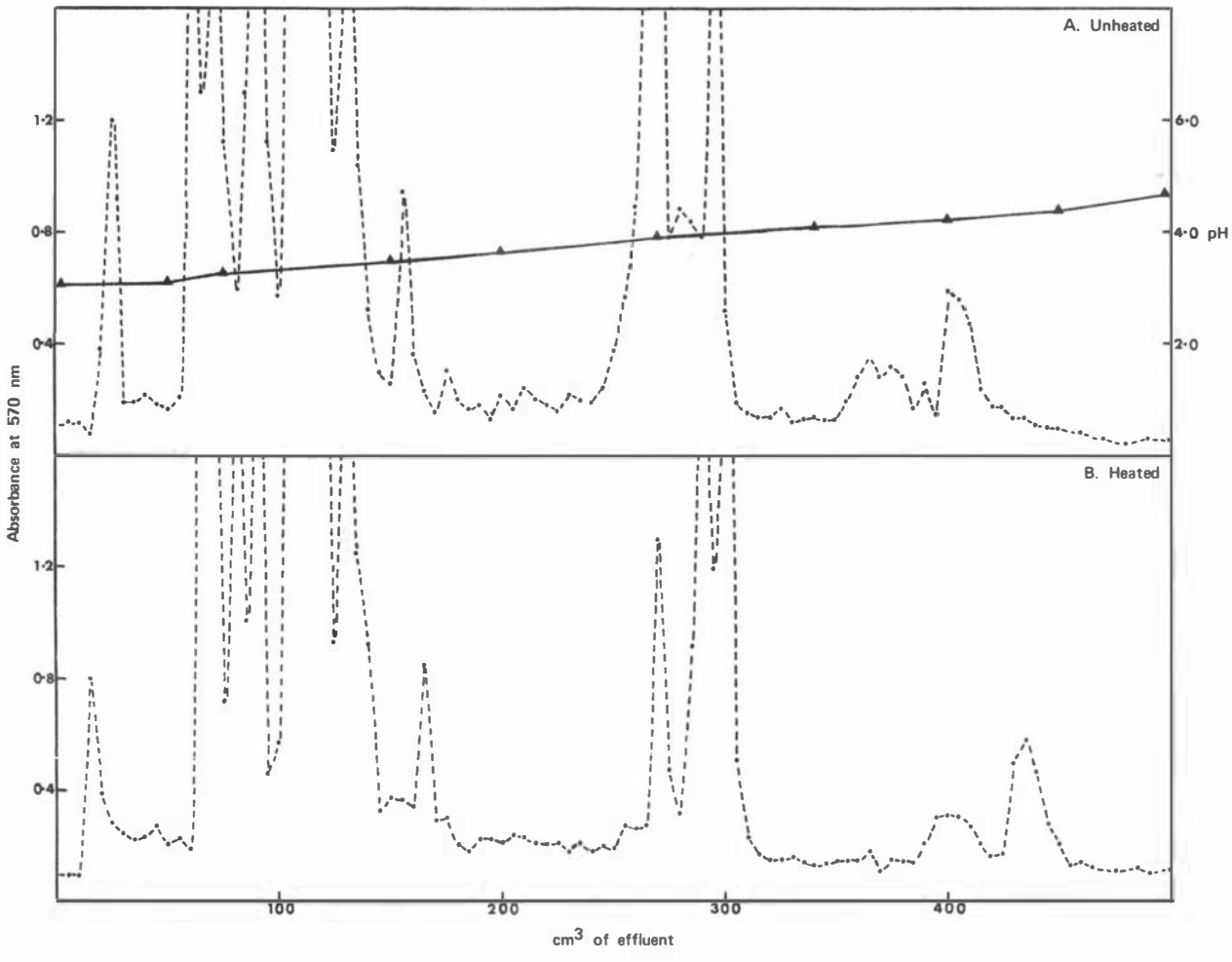


FIGURE 26. Chromatography on Beckman UR–30 resin of an exhaustive enzyme digest of globin. A, unheated; B, heated.
- - - - absorbance after reaction with ninhydrin, 0.1 cm^3 aliquots;
▲—▲ pH gradient

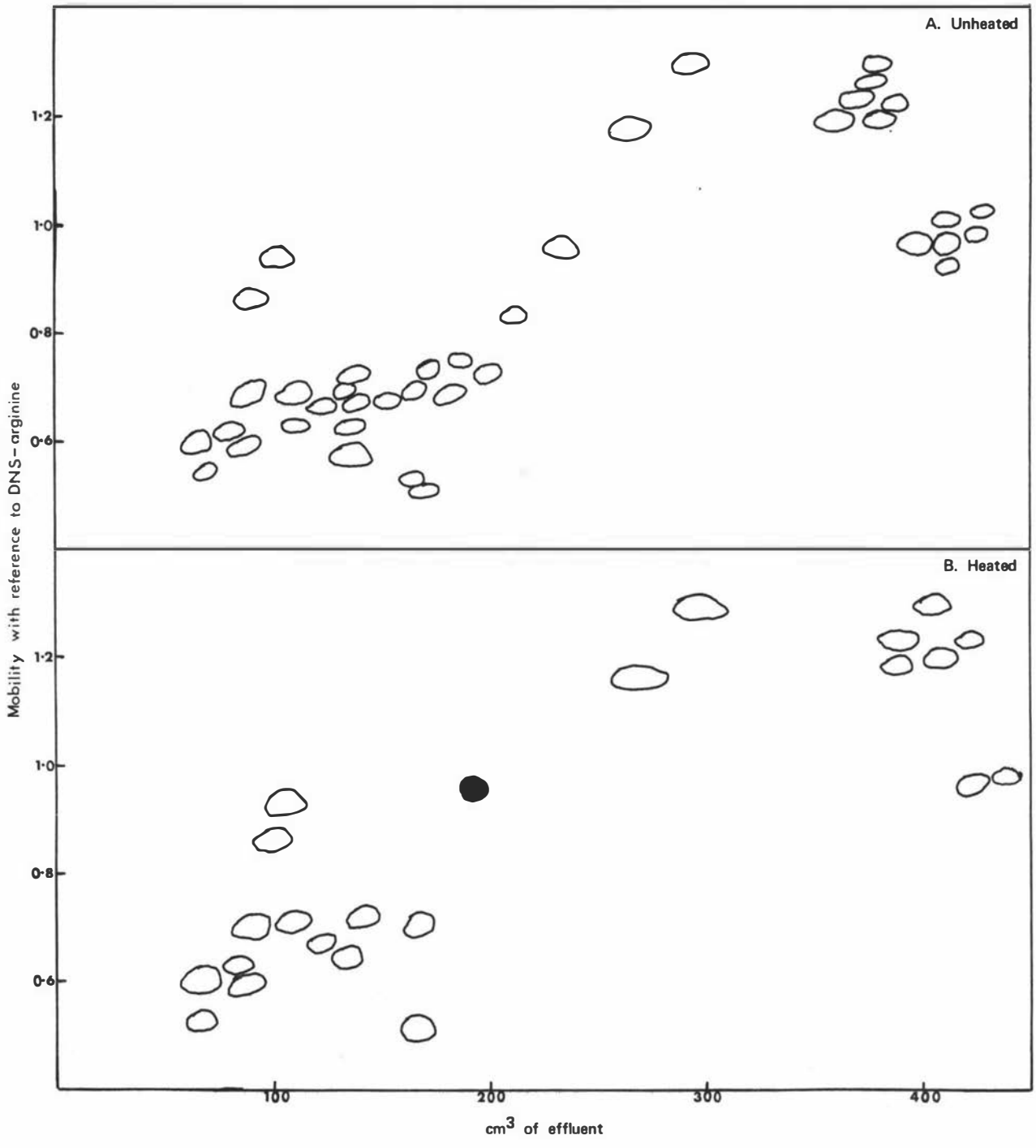


FIGURE 27. Map, by electrophoresis, of ninhydrin positive compounds in an exhaustive enzyme digest of globin. A, unheated; B, heated.

coded X, was hydrolysed and the amino acid composition determined (Table XXXII). The compound was composed mainly of lysine and aspartic acid in similar quantities, but significant amounts of other amino acids were also present. Since this compound was apparently only present in the digest of the heat-damaged globin, it was possible that enzyme-resistant linkages were present. To verify that the presence of this compound was not an artifact of the digestion procedure, another pair of digests were prepared, and separated and compared by the same methods used for the first digests (I).

Exhaustive enzyme digests II

A compound with the same characteristics as compound X was present in the exhaustive enzyme digest II of the heated globin only; i.e. the component eluted at pH 3.6, stained pink-orange with ninhydrin, and had an electrophoretic mobility of 0.96 relative to DNS-arginine. The amino acid composition of this compound, coded Y, was similar to, but not quite identical with that of compound X (Table XXXII). Since the yield of these compounds was low, about 0.4 mg from each digest, or 0.25% of the weight of globin, the two fractions, X and Y, were combined, and treated as one fraction, XY, in further studies.

Purification of fraction XY

Enzymatic digestion. The combined fraction XY was re-digested with pronase, aminopeptidase M and carboxypeptidase A, in that order, in an attempt not only to remove contaminating peptide material which appeared to be present from the amino acid analysis, but also to check the stability of the compound to enzyme digestion. Aliquots of the enzymes were added to provide about 0.01 mg of each enzyme, and the mixture digested for 4 - 6 hours after each addition. After lyophilisation of the mixture, the products were separated by preparative paper electrophoresis.

TABLE XXXII

Amino acid molar ratios of compounds X, Y and XY-1, isolated from exhaustive digests of heated globin

	X	Y	XY-1
Lysine	5.2	4.2	9.0
Aspartic acid	5.6	5.5	10.0
Threonine	1.0	0.5	0.8
Serine	1.1	1.0	1.5
Glutamic acid	1.1	0.8	1.1
Glycine	2.9	2.0	1.8
Alanine	1.0	1.0	1.0

High-voltage electrophoresis. High-voltage electrophoresis at pH 2.1 of the digestion mixture showed that a compound of similar mobility to that of compounds X and Y was present. The amino acid composition and sequence of this compound, coded XY-1, was investigated.

Composition and size of compound XY-1

Amino acid analysis. The amino acid composition of compound XY-1 is given in Table XXXII, and is similar to the other fractions analysed. Slightly smaller amounts of amino acids other than lysine and aspartic acid were present suggesting that the further enzyme digestion had removed some peptide material. A single peak was obtained on analysis of an unhydrolysed sample of XY-1, and there were no free amino acids present apart from the trace amounts of ninhydrin positive compounds eluted from the paper. This single peak presumably represented the major peptide component of compound XY-1; this eluted just after phenylalanine using a single column programme for amino acid analysis with a Locarte automatic loading system.

Electrophoretic mobility at pH 6.5. The compound XY-1 was electrically neutral at pH 6.5, indicating an equal number of amino and carboxyl groups, and suggesting that aspartic acid rather than asparagine was present.

Gel filtration. A sample of compound XY-1 was chromatographed on a column of Bio-Gel P-2 (0.9 x 20 cm, 10% acetic acid), and 1.0 cm^3 fractions were collected. The gel (obtained from Bio-Rad Laboratories, U.S.A.) and the column were prepared by the same methods used for Sephadex gels. The elution volume for compound XY-1 (8.0 cm^3, $\pm$ 0.5 cm^3) was the same as that for a tetrapeptide, leucyl-asparaginyl-lysyl-glycine, Mol. Wt. 434 (synthesised by Dr W. Hancock). Although the column was small, the elution volumes for these compounds were quite different to that for bacitracin (5.5 cm^3, Mol. Wt. 1450), and this in turn, was distinguishable

from that for cytochrome c (4.5 cm^3, Mol. Wt. 12,800). The latter protein would be completely excluded from the gel, which has an operating range of 200 - 2600.

The results suggested that the compound XY-1 has a molecular weight considerably less than 1450, and probably about 300 - 600. This indicated that not all the amino acids present in compound XY-1 formed part of the peptide since such a compound would have a molecular weight greater than 2000. A tetrapeptide of lysine and aspartic acid in equal quantity, as suggested by the amino acid composition, would have a molecular weight of 506, and it seemed likely that this would be the major component of compound XY-1. However, the electrophoretic mobility of XY-1 corresponded to a peptide of molecular weight of 500 with two positive charges at pH 2.1 (Offord, 1966), whereas a tetrapeptide of the suggested composition should have three positive charges. A possible explanation for this discrepancy is that the ε-amino groups of lysine are bonded in the tetrapeptide; this would be expected if the peptide was the result of cross-linkages having formed during heat-damage of globin. The structure of this tetrapeptide was further studied by sequence techniques.

Sequence studies with compound XY-1

Amino-terminal analysis. The amino-terminal of compound XY-1 was shown to be aspartic acid by dansylation. The dansyl derivative of either the α- or ε-amino groups, of lysine, was also present, but these derivatives of lysine are not able to be distinguished by the chromatographic procedures used to separate dansyl derivatives. The intensities of these aspartate and lysine derivatives were approximately equal by visual inspection.

Dansyl-Edman procedure for sequence analysis. After the first cycle of degradation of the compound XY-1 by the Dansyl-Edman procedure,

dansylation produced the derivative of aspartic acid and the bis derivative of lysine. In addition, some α- and/or ε-amino derivatives of lysine were detected but these may have formed from the bis derivative during hydrolysis (Narita, 1970). The same dansyl derivatives of aspartic acid and lysine were formed after two subsequent degradation cycles. These results indicated that the initial degradation had exposed the α-amino groups of both amino acids, instead of forming only one new N-terminal as with normal peptides. However, the repetition of these two amino acids as amino-terminals with further degradation is probably due to the incomplete removal of these groups, rather than their repeated occurrence in the peptide, especially as this was expected to have only four residues.

The recurrence of aspartic acid as the N-terminal group has been reported to occur in sequence studies due to the formation of β-aspartyl linkages, which are not degraded by the Edman procedure (Konigsberg, 1972). These linkages have also been reported to be produced in enzymatic digestions (Haley and Corcoran, 1967), and to occur particularly between asparagine and glycine residues (Haley and Corcoran, 1967; Konigsberg, 1972). The possible presence of such a linkage in compound XY-1 was investigated using hydroxylamine which specifically cleaves aspartyl-glycine bonds (Bornstein and Balian, 1970), but this treatment had no effect on the products formed with subsequent dansylation.

Subtractive Edman degradation for sequence determination. The results of the subtractive Edman procedure for compound XY-1 are given in Table XXXIII. With the first cycle of degradation, both lysine and aspartic acid appeared to have been removed, as the ratio of these amino acids was unchanged, but their ratios to other amino acids such as alanine or threonine were reduced. A second cycle of degradation also seemed to remove both amino acids, but in the third cycle lysine only

TABLE XXXIII

Amino acid* molar ratios with subtractive Edman degradation of compound XY-1

Stage of degradation	Asp/Lys	Ratio to threonine						
		Asp	Lys	Thr	Ser	Glu	Gly	Ala
None	1.1	12.5	11.3	1.0	1.9	1.4	2.3	1.3
One cycle	1.2	7.7	6.3	1.0	1.5	2.0	4.0	1.5
Two cycles	1.2	Not determined						
Three cycles	1.7	9.4	5.5	1.0	1.6	1.6	4.8	1.4

* Abbreviations of amino acids are given in Table B, Appendix 3.

appeared to have been removed as the aspartic acid/lysine ratio increased. There was little change in the ratios of the other amino acid with degradation although there was a slight increase in the amount of glycine. This increase is evidently due to the production of ninhydrin positive material during the Edman degradation procedure which elutes at the same position as glycine; an increase in the apparent serine content with the procedure has also been reported to occur (Konigsberg, 1972).

These results seem to agree with those obtained by the Dansyl-Edman technique in suggesting that both lysine and aspartic acid are removed with degradation. However, the interpretation of these results is again complicated by the possible formation of β-aspartyl linkages, and in addition, by the incomplete removal of the phenylthiohydantoin derivative of aspartic acid by benzene extraction, as a small amount of aspartic acid would be regenerated from the derivative on acid hydrolysis (Konigsberg, 1972).

In an attempt to clarify the nature of the remaining peptide after one cycle of degradation, a portion of the product was examined by electrophoresis at pH 2.1. However, no stain was obtained when the paper was developed with ninhydrin-cadmium solution even although the amount applied (about 20 nmols if XY-1 is a tetrapeptide) was twice that normally used. This was unexpected since dansyl derivatives were formed readily with this product, but evidently the amino groups were not able to react with ninhydrin.

The results obtained by Edman degradation, by either technique, support the hypothesis that the major component of compound XY-1 is a peptide of aspartic acid (or its amide) and lysine. Trace amounts only of amino acids other than lysine and aspartic acid, were detected by dansylation at any stage of degradation. Similarly, the subtractive procedure did not indicate the loss of any other amino acids, apart from

lysine and aspartic acid, with any cycle of degradation. It seemed anomalous that these other components of compound XY-1 were not degraded and consequently removed from the aqueous phase; however, the phenylthiohydantoin derivatives of several amino acids are not completely extracted with benzene (Konigsberg, 1972).

Partial acid hydrolysis. A sample of compound XY-1 (about 100 nmols if a tetrapeptide) was partially hydrolysed with 50 µl of 6 M HCL for 35 minutes at 110°. After removal of the acid, the sample was subjected to electrophoresis at pH 2.1. On staining with ninhydrin-cadmium solution, eight bands were apparent, and only a faint trace of the compound XY-1 remained. These bands were eluted and the amino acid composition with unhydrolysed and hydrolysed samples determined. The results are given in Table XXXIV and show the amount of peptide-bound or free amino acids present in each band; these values have been corrected for the small amounts of ninhydrin positive material eluted from the paper which are determined as amino acids on analysis.

Bands B, C, D and E: Bands B, C, D and E each contained small amounts of peptide material, and in bands C and E some free amino acids were also present. These peptides and amino acids must have been associated with compound XY-1 prior to partial acid hydrolysis.

Bands A, G and H: Considerable amounts of free aspartic acid (band A) and lysine (band H) were produced by partial acid hydrolysis of compound XY-1. Band G also contained a compound which eluted at the position of lysine on amino acid analysis, but which moved differently electrophoretically. This compound was dansylated and the derivative produced, behaved the same as the bis derivative of lysine. The origin of this compound is unknown but presumably it was a consequence of heat-damage to the protein. The presence of such a compound in heated proteins would be significant nutritionally since it behaves as lysine on

TABLE XXXIV

Amino acid composition (nmols) and electrophoretic mobility of bands formed on electrophoresis of a partial acid hydrolysate of compound XY-1.

(P), peptide-bound amino acids; (F), free amino acids.

	A	B	C		D	E		F		G	H	Amount in
	(F)	(P)	(P)	(F)	(P)	(P)	(F)	(P)	(F)	(F)	(F)	hydrolysate
Lysine						2		23		15	26	180
Aspartic acid	51	1	1	1		1		23				200
Threonine				1					3			16
Serine		1	2	2					9			30
Glutamic acid		4	2						10			22
Glycine			3		1	4	4		12			36
Alanine			2		2				8			20
Electrophoretic mobility (relative to DNS-arginine)	0.55	0.61	0.72		0.87	0.97		1.08		1.16	1.25	

ion-exchange chromatography and would be included in estimates of lysine.

Band F: Band F was the major product of partial acid hydrolysis of compound XY-1, and was composed mainly of aspartic acid and lysine in equal amounts; asparagine rather than aspartic acid may have been present as this was indicated by the yellow stain with ninhydrin (Shotton and Hartley, 1973), but with the acid conditions the amide group would have been expected to be lost. Small amounts of other amino acids, in the free state, were also present. The main product of dansylation was the bis derivative of lysine together with the α- and ε-amino derivatives, indicating that lysine was the amino-terminal. Small amounts of the dansyl derivatives of aspartic acid and glycine were also formed, with traces of threonine, alanine, serine and glutamic acid derivatives. After one cycle of the Edman degradation procedure, only a trace of lysine and aspartic acid were detected by dansylation. These results suggest that the main component of band F was the dipeptide, lysyl-aspartic acid (or asparagine).

Since the main product of partial acid hydrolysis was a dipeptide of lysine and aspartic acid (or asparagine), and free lysine and aspartic acid were released, these results support the hypothesis that the major component of compound XY-1 is a tetrapeptide of these two amino acids in equal quantities. However, the electrophoretic behaviour of the dipeptide formed by partial acid hydrolysis was anomalous; the mobility indicated that a peptide with two positive charges, which lysyl-aspartic acid would have at pH 2.1, should have a molecular weight of about 350 (Offord, 1966), whereas the proposed dipeptide would have a molecular weight of 261. This relationship between mobility, molecular weight and charge, is however, based on peptides with α-peptide bonds, so that the difference in mobility may reflect the presence of a bond involving the ε- rather than the α-amino group of lysine. The behaviour would also be

altered from that of normal peptides if part or all of the lysine estimated to be present, by ion-exchange chromatography, was the unknown compound found in band G. In addition, the presence of the free amino acids associated with the dipeptide may affect the electrophoretic mobility.

The association of these amino acids with the dipeptide is difficult to explain. The conditions used for partial acid hydrolysis would not be expected to hydrolyse peptide bonds apart from those involving aspartic acid residues, so that the free amino acids were evidently present in compound XY-1. Similarly, the small peptides released with partial acid hydrolysis and shown to be present in bands B, C, D and E, must also have been present in compound XY-1. This close association of peptides and amino acids with the major component of compound XY-1 must reflect some particular characteristic of the tetrapeptide.

Mass spectrometry. A sample of compound XY-1 was permethylated (by Dr D. Harding) and the structure investigated by mass spectrometry (by Professor R. Hodges). However, the spectra was complex and no definitive information on the structure of the compound could be obtained.

Structure and origin of tetrapeptide

A tetrapeptide of aspartic acid and lysine in equal amounts does not occur in the sequence of either the alpha or beta chains of bovine globin (see Table A, Appendix 3), so that the tetrapeptide isolated must have formed either as a result of heat-damage to the protein, or have been synthesised during the digestion process. The latter possibility seems unlikely as the peptide was isolated from two separate digests and was not detected in digests of the unheated protein, which had been prepared in a similar manner to those of the heated protein. It therefore seems likely that the peptide resulted from the formation of covalent linkages between

aspartic acid (or asparagine) and lysine residues during heat-damage of the protein.

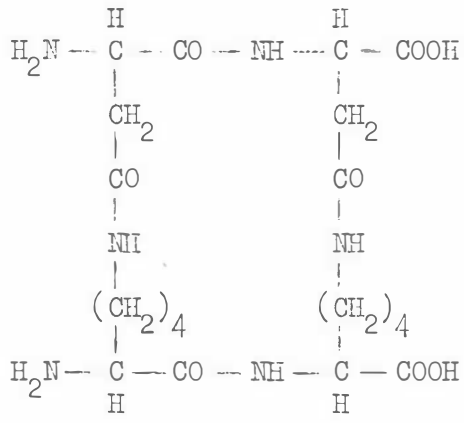
Since some cleavage of peptide bonds occurred with heat-damage, these new bonds could be formed by reaction of the α- as well as ε-amino groups of lysine, and similarly, the α-amino groups of aspartic acid (or its amide) could be involved. However, the presence of the additional charged groups in such fragments would probably prevent such reactions. Heating a synthetic tetrapeptide containing asparagine and lysine residues (previously described on page 140) under a range of conditions, in terms of moisture content and pH, did not result in the formation of any new bonds. It is probably more likely that cross-linking occurred between side chains of two appropriate sequences favourably positioned in the protein.

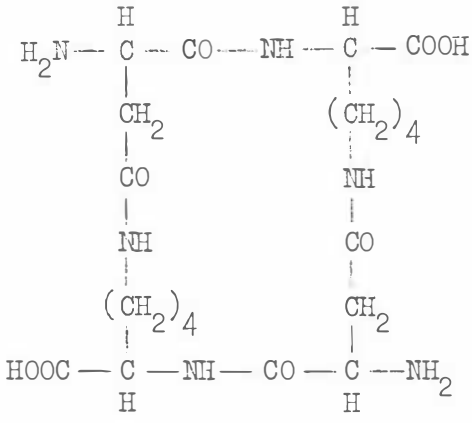
In the alpha and beta chains of bovine globin, there are three sequences of aspartyl-lysyl, two of aspartyl-aspartyl, and one each of aspartyl-asparaginyl and lysyl-lysyl. Cross-linking of either an aspartyl-aspartyl sequence (or aspartyl-asparaginyl) with a lysyl-lysyl sequence (see Structure 1, Table XXXV), or of two aspartyl-lysyl sequences (see Structure 2, Table XXXV), could lead to the formation of a tetrapeptide which would have only two positive charges at pH 2.1 and so would agree with the electrophoretic mobility of the isolated peptide. However, the dansyl derivatives of both aspartic acid and of the α-amino group of lysine would only be obtained with Structure 1; these derivatives were found on N-terminal analysis of compound XY-1.

In Structure 1, both the α-amino groups of lysine and aspartic acid are free and so might be removed by the Edman degradation procedure, whereas this would seem less likely with Structure 2. It is however, difficult to explain how one degradation cycle results in a product unable to react with ninhydrin although able to form dansyl derivatives. This

TABLE XXXV

Possible structures for the tetrapeptide isolated from heated globin.

Structure 1	Sequence
	Aspartyl - aspartic acid (or asparagine)
	Lysyl - lysine

Structure 2	Sequence
	Aspartyl - lysine
	Aspartyl - lysine

may be due to the difference in sensitivity of the two methods, but it is also possible that the product forms a cyclic compound: the conditions would be acidic and this favours the formation of diketopiperazines (Kasper, 1970). Such a compound would be unable to react with ninhydrin but in the alkaline conditions used for dansylation, the cyclic structure may open sufficiently to allow the reaction to occur. Diketopiperazines are reported to be sensitive to alkali, with conversion to the dipeptide (Karrer, 1950).

A similar cyclic intermediate may also account for the formation, on partial acid hydrolysis of the tetrapeptide, of a dipeptide which on dansylation formed the bis derivative of lysine and which was presumed to be lysyl-aspartic acid. If Structure 1 represented the tetrapeptide, the dipeptide produced would be expected to form the dansyl derivatives of aspartic acid and of the α-amino group of lysine. This peptide, if produced initially, might, under the acid conditions, have formed a bond between the free α-**COOH** group of lysine and the α-NH_2 group of aspartic acid with rupture of the ε-peptide bond.

However, the ε-peptide bond is reported to be very stable to acid hydrolysis (Schröder and Lübke, 1965). In addition, the results obtained by Swallow and Abraham (1958) do not support the formation of a cyclic intermediate of the type suggested. These workers found that ε-(α-L-aspartyl)-L-lysine or ε-(β-L-aspartyl)-L-lysine, in the presence of strong acid (1 M HCL, 80°, for 30 minutes) formed the amino-succinyl derivative. This compound was quite stable and only slowly hydrolysed to the free amino acids; it was also still reactive to ninhydrin since free amino groups were present. Treatment of the amino-succinyl derivative with mild alkali (0.15 M $Ba(OH)_2$, 20°, for 30 minutes) opened the ring structure with the formation of both isomers, but there was no indication of a rearrangement to form an α-peptide bond.

Although the structure of the tetrapeptide cannot be defined from these results, it seems that the free carboxyl groups of aspartic acid residues must take part in the formation of covalent bonds with the ε-amino groups of lysine. Asparagine may contribute one of the two aspartyl residues, but it seems unlikely that the amide only is involved in such linkages as suggested by Bjarnason and Carpenter (1970).

General comment

The isolation of the tetrapeptide suggests that the reduced nutritional value of heat-damaged proteins may be due to the formation of cross-linkages between the ε-amino groups of lysine and the free carboxyl groups of aspartic acid (or possibly with its amide). This was originally suggested by Evans and Butts (1949) on the basis of *in vitro* digestibility studies but no direct chemical evidence was presented. These authors also suggested that glutamic acid could form similar linkages with lysine, but this was not observed in the present study.

The tetrapeptide is apparently not readily attacked, if at all, by trypsin, chymotrypsin, carboxypeptidase A, aminopeptidase M, prolidase or pronase, and it is likely that it would not be hydrolysed in the digestive tract of animals. The lysine present in the peptide would therefore be unavailable to animals. However, less than 2% of the total lysine present in globin was present in this peptide so that the loss of this amount would certainly not account for the decrease in available lysine content reported for proteins heat-damaged under similar conditions to those used in this study. In addition, the FDNB-reactive lysine value of globin was reduced by about 25% with heat-damage. Since this value usually shows reasonable agreement with values obtained by growth assays, this also indicates a considerable difference between the amount of lysine found bound and the amount which would be biologically unavailable.

It is of course possible that other covalent bonds involving the ε-amino group of lysine were formed during heat-damage of globin. Other peptides, apart from the tetrapeptide isolated, with such bonds may have been present in the heated globin digests and may not have been detected. However, the peptide material remaining in both the unheated and heated digests had a similar composition (see Table XXXI). The increase in lysine content with acid hydrolysis was similar for both digests, so that there does not seem to be any concentration of lysine in enzyme-resistant peptides in the heated protein digest. Alternatively, some bonds involving the ε-amino groups of lysine in the heat-damaged globin, may have been hydrolysed during the digestion procedure. However, all of the enzymes, except pronase, used for the exhaustive enzyme digests, would be available for the digestion of dietary protein by animals, together with enzymes of other specificities, so that a similar extent of digestion would be probably achieved in vivo to that obtained with these in vitro digests.

It seems therefore that only a relatively small proportion of the ε-amino groups of lysine were involved in enzyme-resistant linkages in the heat-damaged globin. A greater number of cross-linkages may possibly form with more severe heat treatment of proteins, but the present results suggest that the blocking effect of these bonds is unlikely to be the only factor involved in the reduced availability of lysine with heat-damage of proteins. The unidentified compound in the partial acid hydrolysate of compound XY-1 (in band G) which behaved like lysine with ion-exchange chromatography, may also be involved, as this could contribute to the biologically unavailable lysine content, but only a small amount was apparently present in the heat-damaged globin. It is obviously important that the nature and occurrence of this compound in heat-damaged proteins be established.

It is probable however, that the change in structure with the formation of cross-linkages could also decrease the availability of lysine, and other amino acids in heat-damaged proteins. Cross-linking, by either inter- or intra-molecular reactions, would reduce the extent to which a protein could unfold. This could account for the insolubility of heat-damaged proteins since some polar groups would not be exposed. Similarly, some ε-amino groups of lysine may be unable to react with fluorodinitrobenzene because of steric hindrance. The reduced solubility and accessibility of groups, which would include peptide bonds, could also explain the reduced rate of digestion of heat-damaged proteins by proteolytic enzymes. It is likely that cross-linkages were present in the large fragments isolated from tryptic digests of heated globin, and that these bonds were responsible for the complex nature of these fragments.

A decreased rate of digestion could probably explain the reduced nutritional value, and lowered availability of amino acids, of heat-damaged proteins. This was suggested originally by Melnick et al (1946) and recent studies have reaffirmed the likelihood of such an effect (Ford, 1965; Ford and Salter, 1966), although the mechanism is not understood. The overall digestibilities of the proteins would not necessarily be decreased, since in this study, degradation was apparently as complete with the heated globin (except for the tetrapeptide) as with the unheated protein. The possibility still remains however, that the extent, as well as the rate, of digestion of heat-damaged proteins is reduced, and that in animals this is masked by the activities of the intestinal micro-organisms.

APPENDIX 1

TABLE A

Sources of dietary ingredients and purity of amino acid supplements

Meat meals. All the meat meals were products of dry-rendering plants except Mm H which was the hash from a wet renderer. Soft materials (paunches, trimmings etc) were included only in Mm B, Mm F and Mm H. The other meals were a mixture of both soft and hard waste products.

Amino acid supplements. The purity of the amino acid supplements were estimated to be 78% lysine, 83% arginine and 100% for threonine and methionine (supplied by Gollin and Co. Ltd).

Wheat gluten and starch. Obtained from N.Z. Starch Products Ltd.

Cellulose. Sheets of Kraft wood pulp were supplied by Tasman Pulp and Paper Co. Ltd, and these were cut, ground and sieved before use in experiments 1 and 2. In other experiments, cellulose flock was obtained from Mickro Technik (per Gollin and Co. Ltd).

Vitamins and minerals. These were obtained from either Gollin and Co. Ltd or W.H. Terry.

Casein. The sample was obtained from the Dairy Research Institute and contained 82.0% protein.

Cr_2O_3. The Cr_2O_3 was purified by shaking 200 g with 800 cm^3 I M HCL. The supernatant was discarded and the process repeated until the supernatant was clear. After washing with distilled water and drying at 120^o for 24 hours, the Cr_2O_3 content was estimated to be near 100%.

Appendix 1: TABLE B

Composition (%) of meat meals and wheat gluten, including FDNB-reactive lysine (%) values

Concentrate	Water	Crude protein (N x 6.25)	Fat	Ash	Calcium	FDNB-reactive lysine
Mm A	6.7	52.4	10.4	26.3	7.6	2.38
Mm B	1.1	70.0	10.6	5.3	1.1	3.62
Mm C	4.6	55.9	12.0	21.7	7.2	2.11
Mm D	6.2	57.8	15.6	15.0	3.1	2.02
Mm E	8.0	55.8	14.1	19.1	7.5	2.50
Mm F	10.5	60.1	10.9	5.4	0.7	2.85
Mm G	10.0	59.5	12.0	15.7	5.2	2.40
Mm H	6.8	65.5	13.1	3.1	0.7	3.46
Gluten, batch 1	7.8	74.1				0.98
Gluten, batch 2	6.0	73.8				0.98
Gluten, batch 3	8.8	67.8				1.01

Appendix 1: TABLE C

Amino acid* composition of meat meals and wheat gluten (batch 1)

(g/16 g N)

Amino acid	Meat meals								Wheat gluten
	A	B	C	D	E	F	G	H	
Lysine	5.9	7.0	5.2	5.9	5.7	6.5	5.0	7.2	1.8
Histidine	1.9	1.7	1.8	1.5	1.6	1.9	1.5	2.0	2.3
Arginine	5.5	6.0	7.9	4.8	6.5	5.8	7.2	6.7	3.6
Threonine	3.6	5.0	2.9	4.0	3.4	3.7	3.7	4.9	2.3
Valine	5.0	6.6	4.3	5.6	4.3	5.0	5.2	7.6	4.3
Methionine	0.8	2.3	1.1	1.5	1.1	1.5	0.8	1.7	0.9
Isoleucine	2.3	5.0	3.4	3.3	3.2	3.7	3.4	5.3	3.6
Leucine	7.8	9.4	6.8	7.6	7.0	7.5	7.2	9.9	7.0
Phenylalanine	3.6	4.1	3.4	3.4	3.4	3.7	3.9	5.0	4.1
Tyrosine	1.9	3.7	2.0	2.0	2.3	3.0	2.4	3.5	2.4
Aspartic acid	8.6	8.9	6.6	8.2	8.1	8.2	7.9	9.8	3.1
Serine	4.0	5.1	3.0	4.6	3.4	3.8	4.0	4.4	4.2
Glutamic acid	13.0	14.4	11.3	15.7	13.6	12.8	14.5	13.7	34.6
Proline	7.3	5.7	5.7	7.1	5.6	6.7	6.6	4.7	12.6
Glycine	14.7	6.7	12.7	14.4	10.6	11.7	12.9	8.3	3.5
Alanine	9.2	7.0	8.6	9.3	7.5	7.3	8.1	8.1	2.7
Half cystine	0.4	0.6	0.4	ND	0.5	0.9	0.8	1.2	1.4

ND, Not determined

* Tryptophan not determined

Appendix 1:

TABLE D

Composition (%) of basal diet A, basal diet B, diet C and of basal diet D

	Basal diet A	Basal diet B	Diet C	Basal diet D
Wheat gluten, batch 1 or 2*	27.0	27.0	27.0	-
Corn or soybean oil	2.0	2.0	2.0	2.0
Cellulose	3.0	3.0	3.0	3.0
Vitamin premix	2.0	2.0	2.0	2.0
Mineral premix	4.0	4.0	4.0	4.0
Starch	62.0	61.03	57.47	89.0
Methionine	-	0.50	0.50	-
Arginine	-	0.42	0.42	-
Threonine	-	0.05	0.05	-
Lysine	-	-	0.56	-
$Ca_3 (PO_4)_2$	-	-	3.00	-

* For batch 3, 29.5 wheat gluten was used and the amount of starch reduced correspondingly.

Appendix 1: TABLE E

Composition of vitamin and mineral premixes

	g/2 lb vitamin premix		g/4 lb mineral premix
Vitamin A (325,000 IU/g)	1.5	$CaCO_3$	227.0
Vitamin D_3 (200,000 IU/g)	0.3	Na_2HPO_4	455.0
Vitamin E (250 IU/g)	16.0	NaCl	182.0
Vitamin K (Hetrazeen)	0.25	KCl	318.0
Thiamine	0.4	$ZnCO_3$	6.0
Riboflavin	0.5	$CuSO_4.5\ H_2O$	0.8
Niacin	5.0	$FeSO_4.7\ H_2O$	8.6
Biotin (1 mg/g)	15.0	$MgSO_4.7\ H_2O$	225.0
Calcium pantothenate (45%)	2.0	$MnSO_4$	11.0
Pyridoxine	0.4	KIO_3	0.45
Folic acid	0.1	$Na_2MoO_4\ 2H_2O$	0.2
Vitamin B_{12} (90 mg/lb)	4.0	$Al_2(SO_4)_3(NH_4)_2SO_4.24H_2O$	8.3
Choline chloride (50%)	180.0	H_3BO_3	0.004
Santoquin No. 6	18.0	$CoCl_2.6H_2O$	1.2
Wheat starch	+	Selenium premix (300 ppm)	60.0
		Wheat starch	+
Total	2.0 lb	Total	4.0 lb

Appendix 1: TABLE F

Supplements (%) added to basal diet B for diets in experiment 2, including standard and test diets for the bioassay of Mm B

Diet code	lysine	arginine	threonine	tryptophan	Mm B
1	-	-	-	-	-
2	0.56	0.05	-	-	-
3	0.56	-	0.05	-	-
4	0.56	-	-	0.05	-
5	0.14	-	-	-	-
6	0.28	-	-	-	-
7	0.56	-	-	-	-
8	-	-	-	-	1.50
9	-	-	-	-	3.01
10	-	-	-	-	6.02

Appendix 1: TABLE G

Supplements (%) in test diets of experiments 3 and 4, together with the amount of gluten remaining in the basal diet and the calculated protein (N x 6.25) content of the diets

Meat meal	Substituted material	Diet code	%				
			Meat meal	$Ca_3(PO_4)_2$	Gluten*	Lysine	Protein
Experiment 3:							
Mm B	starch	5	2.94	3.70	27.0	-	22.07
		6	5.88	3.60	27.0	-	24.12
		7	11.76	3.50	27.0	-	28.23
	gluten	8	2.94	3.70	24.20	0.028	20.00
		9	5.88	3.60	21.43	0.056	20.00
		10	11.76	3.50	15.86	0.111	20.00
Mm A	gluten	11	4.55	2.90	23.78	0.032	20.00
		12	9.10	2.00	20.57	0.064	20.00
		13	18.20	0.25	14.13	0.128	20.00
Mm C	gluten	14	5.13	2.90	23.12	0.038	20.00
		15	10.26	1.90	19.24	0.076	20.00
		16	20.52	0.00	11.51	0.152	20.00
Experiment 4:							
Mm H	starch	5	2.89	3.06	27.00	-	21.89
		6	5.78	3.00	27.00	-	23.79
		7	11.56	2.93	27.00	-	27.57
	gluten	8	2.89	3.06	24.45	0.026	20.00
		9	5.78	3.00	21.88	0.051	20.00
		10	11.56	2.93	16.76	0.102	20.00

* Batch 1 or 2.

Appendix 1: TABLE H

Supplements (%) in test diets of experiments 5 and 6, together with the amount of gluten remaining in the basal diet and the calculated protein (N x 6.25) content of the diets

Meat meal	Substituted material	Diet code	%				
			Meat meal	$Ca_3(PO_4)_2$	Gluten*	Lysine	Protein
Experiment 5:							
Mm D	starch	5	5.00	2.68	29.50	-	22.89
		6	10.00	2.30	29.50	-	25.78
		7	20.00	1.50	29.50	-	31.56
	gluten	8	5.00	2.68	25.24	0.040	20.00
		9	10.00	2.30	20.98	0.080	20.00
		10	20.00	1.50	12.46	0.160	20.00
Mm E	starch	11	4.00	2.30	29.50	-	22.23
		12	8.00	1.55	29.50	-	24.46
		13	16.00	0.00	29.50	-	28.93
Mm F	starch	14	3.45	3.03	29.50	-	22.07
		15	6.90	2.95	29.50	-	24.15
		16	13.80	2.85	29.50	-	28.29
	gluten	17	3.45	3.03	26.45	0.029	20.00
		18	6.90	2.95	23.38	0.058	20.00
		19	13.80	2.85	17.27	0.115	20.00
Mm G	starch	20	4.17	2.50	29.50	-	22.48
		21	8.33	2.00	29.50	-	24.96
		22	16.66	0.85	29.50	-	29.91
Experiment 6:							
Mm F	starch	5,7**	2.79	3.03	29.50	-	21.68
		6,8**	5.58	2.98	29.50	-	23.35
	gluten	9,11**	2.79	3.03	27.03	0.023	20.00
		10,12**	5.58	2.98	24.55	0.047	20.00

* Batch 3.

** Diets 7 and 8, 11 and 12 had 2% corn oil added.

Appendix 1: TABLE I

Supplements (%) added to basal diet D for the estimation of metabolic amino acid excretion and % apparent retention of dietary amino acids from wheat gluten (experiment 7)

		Supplements (%)				
Diet code	% protein	Casein	Gluten*	Lysine	Arginine	Methionine
Metabolic excretion diets:						
2% LPD	2.0	2.44	-	-	-	-
4% LPD	4.0	4.88	-	-	-	-
Test diets:						
T-X	10.0	-	13.50	-	-	-
T-Y	10.0	-	13.50	0.21	0.21	0.18
T-Z	12.5	-	16.88	-	-	-

* Batch 1.

Appendix 1: TABLE J

Supplements (%) added to basal diet D for the estimation of metabolic amino acid excretion and % apparent retention of dietary amino acids from meat meals, Mm A - Mm F, (experiment 8)

Diet code	% protein	Meat meal code	Supplements (%)		
			Casein	Meat meal	$Ca_3(PO_4)_2$
Metabolic excretion diets:					
PFD	0.0		-	-	3.06
2% LPD	2.0		2.44	-	3.06
Test diets:					
T - A	10.0	Mm A	-	19.10	0.13
T - B	10.0	Mm B	-	14.20	3.45
T - C	10.0	Mm C	-	17.90	0.50
T - D	10.0	Mm D	-	17.30	2.45
T - E	10.0	Mm E	-	17.90	0.40
T - F	10.0	Mm F	-	16.70	3.50

APPENDIX 2

TABLE A

Analysis of variance, experiment 3

Weight gain

Sources of variation	Degrees of freedom	Sum of squares	Mean squares
Correction of mean		4679925.0	
Regression	5	704585.0	
Blanks	1	1720.5	1720.5
Intersection	4	1321.8	330.5
Between treatments	10	707628.0	
Between replicates	1	173.0	173.0
Error	272	127917.0	470.3
Total	283	835718.0	

Food conversion efficiency*

Sources of variation	Degrees of freedom	Sum of squares	Mean squares
Correction of mean		18167.703	
Regression	5	1210.400	
Blanks	1	6.525	6.525
Intersections	4	45.800	11.450
Between treatments	10	1262.664	
Between replicates	1	9.413	9.413
Error	10	54.249	5.425
Total	21	1326.326	

* Values multiplied by 100 for analysis

Appendix 2: TABLE B

Analysis of variance, experiment 4

Weight gain

Sources of variation	Degrees of freedom	Sum of squares	Mean squares
Correction of mean		1605832.0	
Regression	3	363016.1	
Blanks	1	23.25	23.25
Intersections	2	1209.70	604.80
Between treatments	6	364249.00	
Between replicates	1	1260.00	1260.00
Error	180	87601.00	486.70
Total	187	453110.00	

Food conversion efficiency *

Sources of variation	Degrees of freedom	Sum of squares	Mean squares
Correction for mean		7828.623	
Regression	3	953.813	
Blanks	1	14.652	14.652
Intersections	2	8.995	4.497
Between treatments	6	977.460	
Between replicates	1	0.345	0.345
Error	6	16.285	2.714
Total	13	994.090	

* Values multiplied by 100 for analysis

Appendix 2: TABLE C

Analysis of variance, experiment 5

Weight gain

Sources of variation	Degrees of freedom	Sum of squares	Mean squares
Correction for mean		1848710.0	
Regression	7	248193.4	
Blanks	1	453.7	453.7
Intersections	6	1353.8	225.6
Between treatments	14	250001.0	
Between replicates	1	776.0	776.0
Error	373	82840.0	222.1
Total	388	333617.0	

Food conversion efficiency *

Sources of variation	Degrees of freedom	Sum of squares	Mean squares
Correction for mean		23251.968	
Regression	7	1197.621	
Blanks	1	12.070	12.070
Intersections	6	25.750	4.292
Between treatments	14	1235.439	
Between replicates	1	3.974	3.974
Error	14	61.374	4.384
Total	29	1300.787	

* Values multiplied by 100 for analysis

Appendix 2: TABLE D

Analysis of variance, experiment 6

Weight gains

Sources of variation	Degrees of freedom	Sum of squares	Mean squares	
Correction for mean		922724.26		
Regression	5	114224.75		
Blanks	1	1795.89	1795.89	4.68**
Intersections	4	1901.64	475.41	
Between treatments	10	117922.33		
Between replicates	2	4736.68	2368.34	6.17***
Error	449	172439.73	384.05	
Total	461	295098.74		

** Significant at 5%

*** Significant at 1%

Food conversion efficiency *

Sources of variation	Degrees of freedom	Sum of squares	Mean squares
Correction for mean			
Regression	5	1194.34350	
Blanks	1	0.12041	0.1204
Intersections	4	8.26169	2.0654
Between treatments	10	1202.75000	
Between replicates	2	14.14000	7.0700
Error	20	62.86000	3.1430
Total	32	1279.75000	

* Values multiplied by 100 for analysis

APPENDIX 3

TABLE A

Amino acid* sequence and tryptic peptides of alpha and beta chains of bovine haemoglobin (Dayhoff, 1972)

Tryptic peptide	Alpha chain
1	VAL LEU SER ALA ALA ASP LYS
2	GLY ASN VAL LYS
3	ALA ALA TRP GLY LYS
4	VAL GLY GLY HIS ALA ALA GLU TYR GLY ALA GLU ALA LEU GLU ARG
5	MET PHE LEU SER PHE PRO THR THR LYS
6	THR TYR PHE PRO (HIS PHE) ASP LEU SER HIS GLY SER ALA GLN VAL LYS
7	GLY HIS GLY ALA LYS
8	VAL ALA ALA ALA LEU THR LYS
9	ALA VAL GLU (HIS LEU ASP ASP) LEU PRO GLY ALA LEU SER GLU LEU SER ASP LEU HIS (ALA HIS) LYS
10	LEU ARG
11	VAL ASP PRO VAL ASN PHE LYS
12	LEU LEU SER HIS SER LEU LEU VAL THR LEU ALA SER HIS LEU PRO SER ASP PHE THR PRO ALA VAL HIS ALA SER LEU ASP LYS
13	PHE LEU ALA ASN VAL (SER THR VAL) LEU THR SER LYS
14	TYR ARG

Tryptic peptide	Beta chain
1	MET LEU THR ALA GLU GLU LYS
2	ALA ALA VAL THR ALA PHE TRP SER LYS
3	VAL HIS VAL ASP GLU VAL GLY GLY (GLU ALA LEU GLY) ARG
4	LEU LEU VAL VAL TYR PRO TRP THR GLN ARG
5	PHE PHE GLU SER PHE GLY ASP (LEU SER THR ALA ASP ALA VAL MET ASP ASN PRO) LYS
6	VAL LYS
7	ALA HIS GLY LYS
8	LYS
9	VAL LEU ASP SER PHE SER (ASP GLY MET) LYS
10	HIS LEU ASP ASP LEU LYS
11	GLY (THR PHE ALA ALA LEU SER GLU LEU HIS CYS ASP) LYS
12	LEU HIS VAL ASP PRO GLU ASN PHE LYS
13	(LEU LEU GLY ASN VAL LEU VAL VAL VAL LEU ALA) ARG
14	ASN PHE GLY ASN (GLU PHE THR PRO VAL LEU GLN ALA ASP PHE GLN) LYS
15	VAL VAL ALA GLY VAL ALA ASN ALA LEU ALA HIS ARG
16	TYR HIS

( ) Sequence not determined experimentally

* List of abbreviations for amino acids given in Table B, Appendix 3.

Appendix 3:

TABLE B

Abbreviations of amino acids

ALA	alanine
ARG	arginine
ASN	asparagine
ASP	aspartic acid
CYS	cysteine
GLN	glutamine
GLU	glutamic acid
GLY	glycine
HIS	histidine
LEU	leucine
LYS	lysine
MET	methionine
PHE	phenylalanine
PRO	proline
SER	serine
THR	threonine
TRP	tryptophan
TYR	tyrosine
VAL	valine

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