Some Properties of the Insulin Core

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It has been shown (Butler, Dodds, Phillips & Stephen, 1948) that insulin is split by the action of chymotrypsin into a number of peptide fragments of molecular weight about 800 (hereinafter referred to as the filtrate), and a large residue (hereinafter referred to as the core), which has a molecular weight between 4000 and 5000. A more accurate determination of the diffusion coefficient of the core material has been kindly made by Dr K. O. Pedersen of the Physico-Chemical Institute, Upsala, who has obtained at 20° the figures

$$D_A = 18.6 \times 10^{-7}, \quad D_M = 18.3 \times 10^{-7},$$

corresponding to a molecular weight of approximately 5000, assuming a spherical and unhydrated molecule. Details of this and further determinations will be published later by Dr Pedersen. In this paper we record further experiments on the nature of the residual core.

In the work cited, it was found that the extent of the hydrolytic splitting, before it became very slow, varied markedly with the concentration of enzyme used (Butler *et al.* 1948; Fig. 1). With the smaller concentrations of enzyme the action becomes a slow one when only about 30% of non-protein nitrogen has been formed, and even after long-continued action of the enzyme the extent of splitting is not so great as with the larger enzyme concentrations. This kind of behaviour is probably due to inhibition of the enzyme by the products of reaction, the action being slowed up at an earlier stage with the smaller enzyme concentrations than with the larger. Since the size of the residual molecule produced would appear to depend on the enzyme concentration, we have examined this point in greater detail.

The core has also been oxidized by the method of Toennies & Homiller (1942), as employed by Sanger (1947, 1949) on insulin, and it has been shown that three or four separate cysteic acid peptides are formed, which are believed to be derived from the chains united by S-S linkages in the core itself. The dinitrophenyl derivative of the core has been prepared and its terminal amino-acids identified.

In view of the suggestion by Bergmann & Fruton (1941), recently confirmed by Kaufman, Neurath & Schwert (1949), that chymotrypsin acts on peptide bonds in which the carboxyl group is that of an aromatic amino-acid, a further study has been made

of the distribution of the aromatic amino-acids between the core and the filtrate. The data are in reasonable accordance with this hypothesis.

EXPERIMENTAL

Preparation of the core. All the cores used were made in very similar ways, but breakdown of the insulin varied to some extent in the various preparations. The following is a typical preparation. Crystalline zinc insulin (5 g.), from ox pancreas (Boots Pure Drug Co.), was dissolved in a mixture of 75 ml. of phosphate buffer (1.36 g. KH_2PO_4 and 32.2 g. $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 200 ml. water), 200 ml. water and 150 ml. 0.02 N-NaOH. The whole was made up to 750 ml. with water and filtered. The insoluble residue weighed 36.5 mg. The filtrate was diluted to 984 ml. with water and the pH brought to about 8 with 1 ml. of N-NaOH; 16.3 ml. of chymotrypsin solution was added ($= 3.7 \times 10^{-4}$ units/ml. of digest) and the whole put in a bath at 25° for 26.25 hr. The digest was then brought to pH 5.5 with 2.4 ml. of glacial acetic acid. This produced a slight ppt. (unchanged insulin) which was filtered off (138 mg.). To the filtrate, one-third of its volume of N-trichloroacetic acid was added and the ppt. filtered after 30 min. The non-protein nitrogen (N.P.N.) in the filtrate was 59% of the original total nitrogen (T.N.). The ppt. (2.96 g.) was then extracted with ether in a Soxhlet apparatus for about 48 hr. The product gave only a faint colour when 5 mg. were boiled with 5 ml. of 20% (w/v) NaOH and 2 ml. of pyridine, representing a trichloroacetic acid content of less than 0.5% of the weight of solid. This test for trichloroacetic acid is a modification, developed in this laboratory by G. L. Mills (1948), of one given by Clarke (1928). The solid after extraction represented 60% of the original ppt., i.e. 35.5% of the original insulin.

Dinitrophenyl (DNP) derivative of the core. Core (1 g.), containing a negligible amount of trichloroacetic acid, was treated with 1 g. of NaHCO_3 in 10 ml. of water. The mixture was ground up in a mortar with 1.1 ml. of 1-fluoro-2:4-dinitrobenzene (FDNB) dissolved in 20 ml. of ethanol, and allowed to stand for several days with occasional shaking. A yellow solid was filtered off, washed twice with water and ethanol, then with ether until the ether washings were colourless, and dried in air. This solid was suspended in 10 ml. of 0.5 N-HCl, stirred thoroughly, centrifuged down and dried in a desiccator. Its weight was 1.09 g.

Direct oxidation of the core. To 500 mg. of core material, which still contained approximately 5% trichloroacetic acid, 18 ml. of formic acid and 2 ml. of H_2O_2 (100 vol.) were added. After 15 min., with occasional stirring, the solid had dissolved. Water (20 ml.) was added, and the solution was concentrated to about 5 ml. *in vacuo* on a water bath at about 60°, and acetone (40 ml.) added, which produced a white

ppt. This was allowed to settle and then filtered off through a sintered glass funnel, washed well with acetone and then ether, and dried (wt. 408 mg.). (A small amount of N, detected in the filtrate, was not examined further.)

End group analysis. Four different preparations of insulin core have been examined for their terminal amino groups by the method of Sanger (1945). The DNP derivatives of C 11B, C 15B, C 16B and C 19B were hydrolysed in 6N-HCl for various times up to 17 hr. at 120–130° (oil-bath temperature), and the breakdown corrections given by Sanger applied. The amounts of DNP amino-acids, after separation on silica columns, were determined colorimetrically. Solutions in a 90% acetone, 10% 0.1N-HCl solution (v/v) were examined in a Spekker absorptiometer using a dark-blue filter, and the quantities read from a graph calibrated for the particular derivative.

Determination of phenylalanine and tyrosine. Phenylalanine was determined by the Hess & Sullivan (1944) modification of the Kapeller-Adler nitration method, in hydrolysates prepared with both 5N-NaOH and 6N-HCl. Insulin does not contain tryptophan, so that tyrosine could be determined directly with the Folin-Ciocalteu (1927) reagent on hydrolysates of insulin products.

Determination of cystine. The fractions from insulin digests were hydrolysed with a mixture of equal volumes of 6N-HCl and 90% (v/v) formic acid in an oil-bath at 130–140° for 8 hr. This method of hydrolysis was adopted after it had been shown to give quantitative results for insulin itself. Preliminary hydrolyses with 6N-HCl in the presence of urea and in an atmosphere of CO₂ (Kassell & Brand, 1941) gave low values for cystine in insulin. The hydrolysate was evaporated to dryness *in vacuo*, the residue dissolved in a known volume of water, and cystine was determined on a sample by the method of Kassell & Brand (1938).

Formol titration. The progress of digestion of the insulin core by trypsin was followed by measuring the increase in the formol titration. To 1 ml. of the digest, 1 ml. of 36% (w/v) formaldehyde solution was added and the mixture titrated with 0.01N-NaOH to the first pink colour of phenolphthalein.

RESULTS

The action of chymotrypsin on the core

Fig. 1B shows the course of the digestion (C13) of insulin at pH 8.3 with chymotrypsin (3.7×10^{-4} units/ml. prepared from crystalline chymotrypsinogen). After 46 hr., when the N.P.N. was 53%, the digestion had become very slow and a quantity of enzyme sufficient to double the original concentration was added. In the next 48 hr. the N.P.N. rose to nearly 70%. This shows that the stationary state reached in the first 46 hr. is not due to the residue being indigestible, but to the inhibition or inactivation of the enzyme. The extent of chymotrypsin digestion thus depends on the concentration of enzyme, so the size of the 'core' obtained might be expected to depend on the conditions of the digestion.

The action of chymotrypsin on the isolated core was also examined. The two experiments shown (Fig. 1A, C) were made with a core (C 11B) which was the result of a 64% digestion (i.e. the core was 36% of the original insulin). The two experiments

differ only in the way the core was dissolved. In A the core was added to a pH 7.7 phosphate buffer, and 0.02N-NaOH added to give pH 8.3. It was difficult to dissolve the core in this way. In the experiment shown in Fig. 1C the core was dissolved in 0.02N-NaOH and the pH then adjusted to 8.3. In either case, it is seen that an appreciable breakdown of the core to peptides soluble in trichloroacetic acid occurs,

Fig. 1. Action of chymotrypsin on insulin and the core of insulin. A, core only partly in solution; B, insulin control digestion; C, core completely in solution.

the initial rate of digestion depending on the pre-treatment. The reaction is slow and there is no sign of inactivation of the enzyme after 48 hr. At this time fresh enzyme was added and the final result after 96 hr. was much the same in the two cases. The samples of core were then 35% digested, which is about the same proportion (calculated on the amount of core produced) as the rise of N.P.N. in Exp. 1B. There is no doubt, therefore, that the cores as usually prepared are still digestible by chymotrypsin, but at a very much reduced rate. The trichloroacetic acid-soluble peptides produced during the digestion of insulin have been shown (unpublished experiments) to exert a mild inhibitory action on chymotrypsin in the presence of insulin, which will account in part for the rapid fall in rate of digestion of insulin by this enzyme.

The action of trypsin on the core

Fig. 2 shows the increase in N.P.N. when a core (C 16B) from insulin was digested at pH 8 with 6×10^{-4} trypsin units/ml. of crystalline trypsin (a specially pure sample kindly given by Dr Northrop). The core could only be brought into solution by dissolving in a more alkaline buffer and immediately bringing back to pH 8. Also plotted in Fig. 2 is the number of bonds broken per core molecule of weight

5000, as determined by the increase of formol titration. The breaking of very few bonds results in a great increase in trichloroacetic acid-soluble material (N.P.N.) and the subsequent action apparently achieves further breakdown of the soluble peptides produced.

Fig. 2. Action of trypsin on the core of insulin. ●, percentage N soluble in 0.25N-trichloroacetic acid (N.P.N.); ×, peptide bonds broken/mol. of core of weight 5000 (determined by formol titration).

Previous study of the action of trypsin on insulin (Butler, Dodds, Phillips & Stephen, 1949) showed that the action was very slight. The considerable action reported here on a split product of insulin is in accord with the general experience that trypsin attacks denatured or degraded proteins far more readily than native proteins.

The action of pepsin on the core

The core material can only be partially dissolved at pH 2. Crystalline pepsin (6×10^{-4} pepsin units/ml.) will act on the suspension, slowly producing

Fig. 3. Action of pepsin on the core of insulin. Percentage N soluble in 0.25N-trichloroacetic acid. The break in the curve indicates the effect of solution of the core and continued digestion.

about 20 % trichloroacetic acid-soluble nitrogen, the action then having stopped. Further action cannot be initiated by adding extra enzyme. If, however, at

this stage the suspension is completely dissolved by adding alkali and immediately bringing back to pH 2, the N.P.N. value is found to be very much greater. Such treatment of core without enzyme present does not produce N.P.N., so that the effect here seems to be the liberation of small peptides which are bound to the solid material of the suspension. The action of pepsin is shown in Fig. 3, where the broken line represents the conversion of the digest from a suspension to a clear solution. The maximum figure of trichloroacetic acid solubility was 72 %, so that chymotryptic digestion of insulin followed by peptic digestion of the core of the insulin resulted in a total of 90 % of the insulin becoming soluble in 0.25N-trichloroacetic acid.

Electrophoretic examination of the core and its oxidation products

The core material was dissolved in a sodium phosphate buffer of pH 8.0 and ionic strength 0.13, at a concentration of 0.9 g./100 ml.; a small proportion was insoluble under these conditions and was discarded. The solution, when examined in the Tiselius electrophoresis apparatus, showed only one migrating component (Fig. 4a). This evidence of homogeneity, when considered with the diffusion evidence

Fig. 4. *a*, Electrophoresis of the core material C11B. Ascending boundary pattern (anode limb), obtained after 404 min. at 2.19 V./cm. Migration is from left to right. δ , the δ anomaly. *b*, Electrophoresis of the oxidized core. Descending boundary pattern (cathode limb), obtained after 130 min. at 3.25 V./cm. Migration is from right to left.

(p. 74) obtained on this substance, indicates [that the core material is probably a single molecular species.

Oxidized core. This material, when dissolved in a sodium phosphate buffer of pH 7.0 and ionic strength 0.20 at a concentration of 0.7 g./100 ml., gave an electrophoretic pattern displaying four peaks (Fig. 4b). As was the case with the core material, the solution could not be equilibrated with the supernatant buffer by dialysis, owing to the low molecular weight of the peptides, and it is accordingly possible that the slowest boundary, although it is rather large, may be due solely to an ϵ -type of boundary anomaly. Allowing for this uncertainty, the electrophoretic behaviour thus supports the view that the core consists of four peptide chains, united by S.S links.

Terminal amino-acids of core

Table 1 gives the results of determinations with four different cores, calculated as g.mol. of amino-acid in 5000 g. of the core, which is taken provisionally as its g.mol.wt. It is seen that the terminal acid is mainly glycine. The correction for the decomposition of DNP-glycine is very uncertain and may account for the low values obtained in some cases. There is a small quantity of valine, but less than would be expected if one peptide chain in each core molecule terminated in valine. This must be regarded as evidence of more than one species of core.

Table 1. *Estimation of the free amino groups of the insulin cores*

(Groups/5000 g. insulin core.)

Core no.	Glycine	Valine	Lysine as ϵ -DNP derivative	Dinitroaniline
C 11 B	Present	Present	Absent	Present
C 15 B	2.5	0.4	0.5	Present
C 16 B	2.4	0.3	0.2	1.2
C 19 B	3.7	0.8	0.2	1.5

It has been reported (Butler, Phillips & Stephen, 1948) that histidine was present as a terminal amino-acid. This was based on the finding of a brownish band, running fast on a methyl ethyl ketone-hydrochloric acid column. However, it has since been noted that DNP-glycine, when heated with 6N-hydrochloric acid gives considerable amounts of a coloured substance, insoluble in ether, which runs ahead of ϵ -DNP-lysine like the brownish substance obtained from the cores. Since all the DNP cores examined contain DNP-glycine, it is likely that this is the origin of the material in question, although

this is not definitely proved. A substance running at the same rate as 2:4-dinitrophenol has been found in the very fast fraction on chloroform columns, admixed with another substance which may be 2:4-dinitroaniline. Dinitroaniline values are reported in Table 1, without correction for hydrolytic breakdown, since they probably originate from DNP-histidine (substituted in the imidazole ring) and may thus be an index of the histidine content. DNP-proline has not been looked for since we have been unable to prevent the complete decomposition of DNP-proline when hydrolysed under the special conditions recommended by Porter & Sanger (1948).

The distribution of tyrosine and phenylalanine in a chymotryptic digest of insulin

Table 2 shows the distribution of phenylalanine between a core and the corresponding filtrate. This core was 38 % of the original weight of the insulin taken and is assumed to have a molecular weight of 4600, while the combined weights of the filtrate molecules are taken as 7400. The distribution is approximately one molecule in the core to four or five in the filtrate.

Table 3 shows the distribution of tyrosine between the same core and filtrate. The distribution is approximately three to four molecules in the core and five or six in the filtrate. It is to be noted that the value of tyrosine in insulin determined by the same method is rather low, so probably the true values for the fractions tend towards the higher limits.

For both of these aromatic amino-acids, we have totals of four or five in the core and nine to eleven in the filtrate.

Table 2. *Distribution of phenylalanine between core and filtrate*

Material	Phenylalanine-N (% of total N)	Hydrolysis conditions	Groups/mol. of wt. stated
Core C 8 D	2.3	18 hr., 6N-HCl	1.2/4,600
Core C 8 D	1.5	10 hr., 5N-NaOH	0.8/4,600
Filtrate C 8 F1	5.5	18 hr., 6N-HCl	4.5/7,400
Filtrate C 8 F1	4.4	16 hr., 5N-NaOH	3.6/7,400
Insulin	4.7*	8 hr., 6N-HCl	6.4/12,000
Insulin	5.0	8 hr., 5N-NaOH	6.7/12,000

* Chibnall's (1946) value for insulin is 4.41 %, equivalent to six groups/mol. of wt. 12,000.

Table 3. *Distribution of tyrosine between core and filtrate*

Material	Tyrosine N (% of total N)	Hydrolysis conditions	Groups/mol. of wt. stated
Core C 8 D	7.5	1 part formic acid (90 % v/v) + 1 part 6N-HCl, 10 hr.	3.8/4,600
Core C 8 D	5.9	6N-HCl, 18 hr.	3/4,600
Filtrate C 8 F1	7.7	1 part formic acid (90 % v/v) + 1 part 2N-HCl, 10 hr.	6.3/7,400
Filtrate C 8 F1	6.5	6N-HCl, 18 hr.	5.3/7,400
Insulin	5.5*	6N-HCl, 8 hr.	7.6/12,000

* Chibnall's (1946) value for insulin is 6.49 %, equivalent to nine groups/mol. of wt. 12,000.

Distribution of cystine

Two cores and the corresponding filtrates containing the smaller peptide fragments were examined, and a third filtrate which came from a similar digestion. The results are shown in Table 4, and compared with those for insulin under identical hydrolysis conditions. The figures for the two cores C 16B, and C 19B are higher than those obtained in

values which were also determined. The decrease in the percentage of sulphur in the DNP derivative corresponds to an increase in molecular weight of 1490 taking the original core as 5000, i.e. to the addition of nine DNP groups. On our other evidence there should be about nine groups in the core which react with FDNB, i.e. the four terminal amino groups, four tyrosine hydroxyl groups and some lysine and histidine, which are generally present.

Table 4. *Distribution of cystine between core and filtrate*

No.	Material	Percentage of original insulin	Cystine-N $\times 100$ Total N	Cystine as percentage of original insulin	Percentage of total cystine	Residues of cystine/2 per mol. insulin (12,000)
C 19B	Core	41.5	13.1	5.43	61	7
C 19F	Filtrate	58.5	5.9	3.47	39	5
C 16B	Core	41	14.5	5.95	67	8
C 16F	Filtrate	59	4.93	2.91	33	4
C 11F	Filtrate	64	5.4	3.46	39	5
—	Insulin*	100	9.1	9.1	—	(12)
—	Insulin	100	8.5	8.5	—	(12)
—	Insulin	100	9.2	9.2	—	(12)

* The value of twelve residues of cystine/2 per mol. insulin (12,000) is taken from Chibnall (1946). His value for cystine-N in insulin is 9.36%.

experiments reported previously by Butler, Dodds, Phillips & Stephen (1948), but in these early determinations some discrepancy in the total amount of cystine present was also noticed. In no case was any cysteine found.

The DNP derivative of the core

The DNP derivative of the core (C 15B), examined by paper chromatography with phenol as solvent, gave a main yellow spot at $R_f = 0.92$ and a faint trace at $R_f = 0.61$. The DNP derivative of C 16B behaved similarly, the positions being 0.95 and 0.60. The fainter yellow spots could easily be washed off the paper with water, and were probably impurities such as dinitrophenol, but the quantity was so small that this was not proved. This chromatographic behaviour also indicates the homogeneity of the preparations.

Quantitative analysis of a core and its DNP derivative was used as a guide to the number of groups in the core which had reacted with FDNB.

Table 5. *Analysis of core and dinitrophenyl derivative*

	C 16B	C 16B/DNP
Moisture (%)	6.45	4.82
Ash (%)	5.00	0.27
Sulphur (%)	3.76	2.90
Nitrogen (%)	14.1	14.79

The results, obtained by Drs Weiler and Strauss, are given in Table 5. The figures given for nitrogen and sulphur have been corrected for the ash and moisture

The qualitative composition of the cores

Most of the cores prepared from insulin have been examined qualitatively by paper partition chromatography after hydrolysis with 6N-hydrochloric acid. The results are given in Table 6.

Table 6. *Qualitative amino-acid composition of the insulin cores*

(+, signifies present, giving a good spot with ninhydrin; -, indicates a weak spot; =, a very weak spot and 0, indicates absent.)

Acid	C 11B	C 15B	C 16B	C 19B
Cystine	+	+	+	+
Aspartic	+	+	+	+
Glutamic	+	+	+	+
Serine	+	+	+	+
Glycine	+	+	+	+
Alanine	+	+	+	+
Valine	+	+	+	+
Leucines	+	+	+	+
Tyrosine	+	+	+	+
Histidine	-	+	+	+
Lysine	=	0	+	0
Arginine	0	-	0	0
Threonine	0	=	0	0
Phenylalanine	0	+	0	0
Proline	0	0	0	0

It must be noted that since lysine, arginine, threonine and proline are the rarest amino-acids in insulin, their ninhydrin spots on chromatograms will of necessity be weak relative to the others, and duplicate chromatograms have not always agreed in

the case of these acids, or phenylalanine. With phenol and butanol-acetic acid as chromatographic solvents phenylalanine runs with leucine, and, even when somewhat separated from leucine, phenylalanine always gave a poor ninhydrin reaction. Cystine was usually demonstrated by oxidation to cysteic acid just before the run.

DISCUSSION

The fact that the number of bonds split by chymotrypsin is approximately the same as the total number of aromatic residues in insulin suggests that the specificity found by Bergmann & Fruton (1941) holds. On this basis, the chymotrypsin will remove successive peptides, each of which terminates at the carboxyl end with an aromatic residue. It is known, however, that two of the four peptide chains of insulin terminate in phenylalanine at the amino end, and two terminate in glycine. The terminal amino groups of the core no longer contain phenylalanine, but there is still much terminal glycine. It has also been found (Phillips, unpublished work), by the use of Sanger's (1945) method, that the amino-acid residues with free amino groups in the trichloroacetic acid-soluble peptides from the digest belong almost entirely to acids other than phenylalanine and tyrosine.

The simplest picture of chymotrypsin action which will accommodate all these facts is shown in Fig. 5, where ϕ indicates an aromatic residue, i.e. tyrosine or phenylalanine, and the core is enclosed by the broken lines. The core might either contain the two original terminal glycines, as shown in Fig. 5*a*, or not, as in Fig. 5*b*. The latter structure is possible only if the glycine chains also contain other glycine residues adjacent to the aromatic residues. Sanger (1948*a*), however, has suggested that the only glycine residues in his glycine-terminated chains are the terminal ones. This has been confirmed on a sample of his fraction *A* which he kindly provided for us. After coupling with FDNB, no free glycine could be detected in the hydrolysate by paper chromatography. The scheme (Fig. 5*b*) is in conflict with these facts, and the evidence so far obtained favours the view that the core is derived from residues near the amino ends of the original peptide chains. According to Sanger (1948*b*) at least one of the chains terminating in phenylalanine has next to it a valine residue, which fits in with the finding of valine as a terminal amino-acid of the core. The enzyme may be regarded as working along the chains until no more bonds of this type remain. The residual portion, the core, which as we have seen contains six or eight half-cystine residues, is made up of those sections of the original chains which remain united by these S.S. links.

The slow final stage of the action of the enzyme is apparently either unspecific or of a different speci-

city, since it must concern the breakage of some bonds not involving aromatic residues. Nevertheless, chymotrypsin appears to provide a valuable method of structure analysis of proteins since these non-specific actions are relatively slow.

Fig. 5. Possible positions of the core within the insulin submolecule. ϕ , indicates a tyrosine or phenylalanine residue; Gly, indicates a glycine residue; Val, indicates a valine residue. The free amino groups of the peptide chains are on the left of the diagrams. Cystine disulphide linkages are omitted.

SUMMARY

1. The core of the insulin molecule, produced by the action of chymotrypsin, has been further studied.
2. On oxidation of the core three or four peptides can be distinguished by electrophoresis.
3. Additional analyses of the distribution of cystine and the aromatic amino-acids have been made.
4. The number of peptide bonds broken and the distribution of aromatic amino-acids between the core and the filtrate suggests that chymotrypsin attacks preferentially peptide bonds involving the carboxyl side of an aromatic residue. This is in accordance with the specificity observed by Bergmann & Fruton (1941).
5. The end groups of the core are mainly glycine with a smaller proportion of valine. The implications of this are discussed.

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Studies on the Composition of Horse Oil

1. COMPOSITION OF HORSE OIL IN RELATION TO THE DEPOT FATS OF OTHER PASTURE-FED ANIMALS

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Many investigators (cf. Schuette, Garvin & Schwoegler, 1934) have determined the characteristics of fat from various parts of the horse, indicating in particular a relatively high iodine value (74.8–110.7) (cf. Lewkowitsch, 1922). There is, however, little exact information concerning the fatty-acid composition. Heiduschka & Steinruck (1921) deduced the presence of oleic, linoleic and linolenic acids by the preparation of hydroxy and bromo derivatives, and by fractional crystallization showed the saturated acids to be palmitic and stearic. They also gave the relative proportions of the fatty acids present, but it is doubtful whether the methods used were quantitative. More recently, Schuette *et al.* (1934) confirmed the results of these investigators and determined the relative proportions of the acids by the Kaufmann (1926) thiocyanometric procedure, together with the ester fractionation analysis of the 'solid' acids from the lead salt-ethanol separation. Of particular interest is the fact that these workers recorded the presence of 4.48% linolenic acid; a result which is consistent with the yield and melting point of the hexabromides of horse oil recently recorded by Crowell (1944). According to Bloor (1943), however, linolenic acid is not normally found in animal fats.

It has been stated in the literature (Armstrong & Allan, 1924; Hilditch, 1947) that horses fed continuously on grass lay down fat very similar to that

of mutton or beef tallow. This, no doubt, has led to the general impression that horse oil contains little or no linolenic acid, as has been shown to be the case with beef and mutton tallow.

Recently, Beadle, Wilder & Kraybill (1948) observed that rats fed on flax seed, equivalent to 6.6% linolenic acid in the diet, deposit up to 27.57% of linolenic acid in their depot fats. In addition, they noted that yellow fat from hogs may contain up to 11.4% linolenic acid. Vickery (1928), quoted by Banks, Hilditch & Jones (1933), found 9.0% of linolenic acid, based on the weight of hexabromide isolated, in the perinephric fat of the rabbit. However, taking into consideration the fact that not more than one-quarter to one-third of the linolenic acid is precipitated as ether-insoluble polybromide (cf. White & Brown, 1949), it may be estimated that the true linolenic acid content was 27–36%. From these results it is apparent that certain species, far from rapidly metabolizing the dietary linolenic acid, readily store considerable proportions of this acid in their depots.

Further to a preliminary note published elsewhere by one of us (Shorland, 1949a), it will now be shown that oils from pasture-fed horses contain considerably greater proportions of linolenic acid than has hitherto been generally suspected, and that the fatty-acid composition of such oils differ markedly from that of beef and mutton tallow.