

Chemistry of Insulin

Determination of the structure of insulin opens the way to greater understanding of life processes.

F. Sanger

In 1943 the basic principles of protein chemistry were firmly established. It was known that all proteins were built up from amino acid residues bound together by peptide bonds to form long polypeptide chains. Twenty different amino acids are found in most mammalian proteins, and by analytical procedures it was possible to say with reasonable accuracy how many residues of each one was present in a given protein. Practically nothing, however, was known about the relative order in which these residues were arranged in the molecules. This order seemed to be of particular importance since proteins, although they all contained approximately the same amino acids, differed markedly in both physical and biological properties. It was thus concluded that these differences were dependent on the different arrangement of the amino acid residues in the molecules. Although very little was known about amino acid sequence, there was much speculation in this field. The most widely discussed theory was that of Bergmann and Niemann, who suggested that the amino acids were arranged in a periodic fashion, the residues of one type of amino acid occurring at regular intervals along the chain. At the other extreme there were those who suggested that a pure protein was not a chemical individual in the classical sense but consisted of a random mixture of similar individuals.

Due largely to the work of Chibnall and his colleagues (1), insulin had been studied in considerable detail. It had a somewhat simpler composition than most proteins in that two of the commonly occurring amino acids, tryptophan and methionine, were absent and an accurate

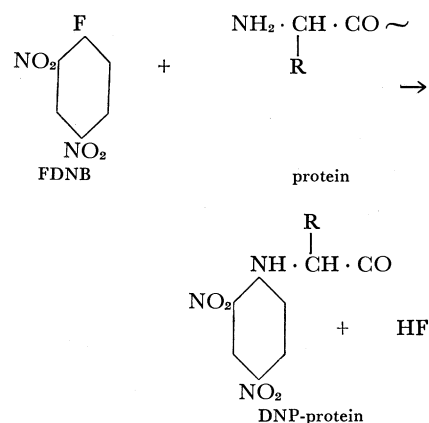
analysis was available. Moreover, using the van Slyke procedure, Chibnall had shown that insulin was peculiar in having a high content of free α -amino groups. This indicated that it was composed of relatively short polypeptide chains, since free α -amino groups would be found only on those residues (the N-terminal residues) which were present at one end of a chain. Thus, the number of chains could be determined from the number of these N-terminal residues. The nature of one of these N-terminal residues was in fact known. Jensen and Evans (2) had shown that the phenylhydantoin of phenylalanine could be isolated from an acid hydrolyzate of insulin that had been treated with phenyl isocyanate, thus indicating that phenylalanine was at the end of one of the chains. At that time this was the only case where the position of an amino acid in a protein was known.

There was considerable doubt about the actual molecular weight of insulin and hence about the number of amino acid residues present. Values varying from 36,000 to 48,000 derived by physical methods had been reported, but it was shown by Gutfreund (3) that these high values were due to aggregation, and it was suggested that the real molecular weight or subunit was 12,000. This indicated that there were about 100 residues in the molecule. More recently, Harfenist and Craig (4) have shown that the actual value is about 6000; however, during most of our work it was believed to be 12,000.

Dinitrophenyl Method

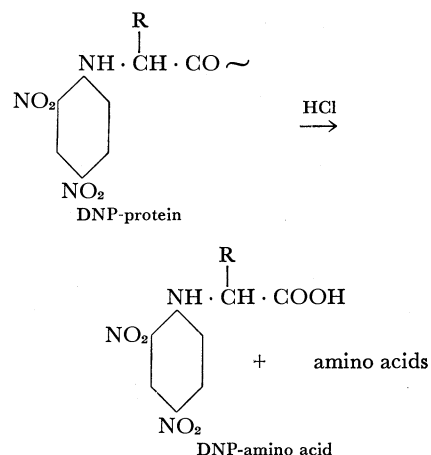
In order to study in more detail the free amino groups of insulin and other proteins, a general method for labeling them was worked out (5). This was the dinitrophenyl (or DNP) method. The reagent used was 1,2,4-fluorodinitroben-

zene (FDNB), which reacts with the free amino groups of a protein or peptide to form a DNP derivative.



The reaction takes place under mildly alkaline conditions which normally do not cause any breakage of the peptide bonds.

The DNP-protein is then subjected to hydrolysis with acid which splits the peptide bonds in the chain, leaving the N-terminal residue in the form of its DNP-derivative.



The DNP-amino acids are bright yellow substances and can be separated from the unsubstituted amino acids by extraction with ether. They could be fractionated by partition chromatography, a method which had just been introduced by Gordon, Martin, and Synge (6) at that time. The DNP-amino acids could then be identified by comparison of their chromatographic rates with those of synthetic DNP-derivatives. In the original work on insulin, silica gel chromatography was used, though more recently other systems, particularly paper chromatography, have been found more satisfactory. When the DNP derivatives had been separated and identified they could be estimated colorimetrically.

When the method was applied to in-

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Table 1. Results of partial acid hydrolysis of the DNP derivative of fraction B. The yield from DNP insulin is expressed in moles of peptide as a percentage of the total N-terminal phenylalanine residues of insulin.

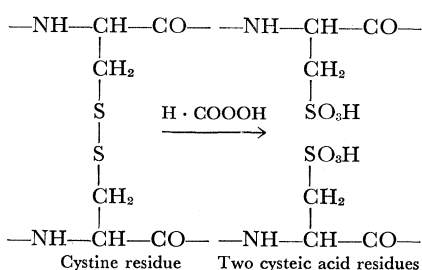
Peptide	Products of complete hydrolysis of peptide	Products of partial hydrolysis	Structure	Yield from DNP insulin
B1	DNP-phenylalanine		DNP-Phe	13
B2	DNP-phenylalanine, valine	B1	DNP-Phe-Val	16
B3	DNP-phenylalanine, valine, aspartic acid	B1, B2	DNP-Phe-Val-Asp	13
B4	DNP-phenylalanine, valine, aspartic acid, glutamic acid	B1, B2, B3	DNP-Phe-Val-Asp-Glu	30
Other bands giving B4 on partial hydrolysis				20
Total				92

ulin, three yellow DNP-derivatives were found in the hydrolyzate of the DNP-insulin. One of these was not extracted into ether; this was ϵ -DNP-lysine, which was formed by reaction of the FDNB with the free ϵ -amino group of lysine residues which are bound normally within the polypeptide chain. The others were identified as DNP-phenylalanine and DNP-glycine, and estimation on the basis of an assumed molecular weight of 12,000 showed that there were two residues of each. This suggested to us that insulin was composed of four polypeptide chains, two with phenylalanine and two with glycine end groups. This method has now been applied widely to many proteins and peptides and, together with the Edman phenylisothiocyanate method, is the standard method for studying N-terminal residues. In general it has been found that the chains of other proteins are much longer than those of insulin. All pure proteins appear to have only one or two N-terminal residues.

Separating the Peptide Chains

It seemed probable that the chains of insulin were joined together by the disulfide bridges of cystine residues. Insulin is relatively rich in cystine, and this was the only type of cross linkage that was definitely known to occur in proteins. It was thus next attempted to separate the peptide chains by splitting the disulfide bridges. Earlier attempts to do this by reduction to $-SH$ derivatives had not proved successful and had given rise to insoluble products which were probably the result of some type of polymerization. More satisfactory results were

obtained by oxidation with performic acid (7). The cystine residues were converted to cysteic acid residues, and thus the cross links were broken.



Performic acid also reacts with residues of methionine and tryptophan, the two amino acids which fortunately were absent from insulin.

From the oxidized insulin two fractions could be separated by precipitation methods. One (fraction A) contained glycine; the other (fraction B), phenylalanine N-terminal residues. Fraction A was acidic and had a simpler composition than insulin in that the six amino acids lysine, arginine, histidine, phenylalanine, threonine, and proline were absent from it. It thus had no basic amino acids; these were found only in fraction B. From a quantitative determination of the end groups it was concluded that fraction A contained about 20 residues per chain, four of these being cysteic acid, and that fraction B had 30 residues, two of which were cysteic acid. Since the yield of each fraction was greater than 50 percent in terms of the N-terminal residues present and since the fractions appeared to be homogeneous, it seemed likely that there was only one type of glycol chain and one type of phenylalanyl chain. This was confirmed by a study of the N-terminal sequences (8).

Results of Acid Hydrolysis

When the DNP derivative of fraction B was subjected to complete acid hydrolysis, DNP-phenylalanine was produced. If, however, it was subjected to a milder acid treatment so that only a fraction of the peptide bonds were split, DNP-phenylalanine peptides were produced which contained the amino acid residues near to the N-terminal end, and by an analysis of these peptides it was possible to determine the N-terminal sequence to four or five residues along the chain. The results for fraction B are shown in Table 1. It was concluded from these results that all the N-terminal phenylalanine residues of insulin were present in the sequence

Phe-Val-Asp-Glu

This suggested that if there were in fact two phenylalanyl chains, then these two were identical. Similar results were obtained with fraction A, and it was shown that the N-terminal glycine residues were present in the sequence

Gly-Ileu-Val-Glu-Glu

These results, besides giving information about the position of certain residues in the polypeptide chains, showed for the first time that the molecule was composed of only two types of chains and that if the molecular weight were 12,000, as was then believed, then the molecule was built up of two identical halves. The alternative conclusion was that the actual molecular weight was 6000, and this was later shown to be the case. At any rate the structural problem was somewhat simplified, since we were now concerned with determining the sequence in two chains containing 20 and 30 residues, respectively.

The main technical problem was the fractionation of the extremely complex mixtures that resulted from partial hydrolysis of a protein. However, Consden, Gordon, Martin and Synge (9) had shown that small peptides could be well fractionated by paper chromatography and had determined the sequence in the pentapeptide gramicidin S from the composition of peptides produced on acid hydrolysis.

At this point (1949) I was joined by Hans Tuppy, who came to work in Cambridge for a year. Although we did not seriously envisage the possibility of being able to determine the whole sequence of one of the chains within a year, we considered it worth while to investigate the small peptides from an acid hydrolyzate, using essentially the methods that had

been applied to gramicidin S. Studies were initiated on both the chains at the same time, but it soon became clear that there would be more difficulties with fraction A, although it was the shorter chain, and the work on fraction B progressed so favorably and Tuppy worked so hard that by the end of the year we were virtually able to deduce the whole of the sequence of its 30 residues (10).

Sequences of Fraction B

Fraction B was subjected to partial hydrolysis with acid. Since the mixture was too complex for direct analysis by paper chromatography it was necessary to carry out certain preliminary group separations in order to obtain fractions containing five to 20 peptides that could then be separated on paper. This was accomplished by ionophoresis, ion exchange chromatography, and adsorption on charcoal. These simplified mixtures were then fractionated by two-dimensional paper chromatography. The peptide spots were cut out, and the material was eluted from the paper, subjected to complete hydrolysis, and analyzed for its constituent amino acids. Another sample of the peptide was then investigated by means of the DNP technique to determine the N-terminal residue. Table 2 illustrates the results obtained with a very acidic fraction obtained by ion-exchange chromatography. This contained only peptides of cysteic acid. Since there are only two such residues in fraction B, all these peptides must fit into two sequences. The way in which the two sequences

Leu-CySO₃H-Gly

and

Leu-Val-CySO₃H-Gly

were deduced from the results obtained with the peptides is illustrated in the table.

In this way about 45 peptides were identified in various fractions of the partial acid hydrolyzate, and the following five sequences were deduced as being present in the phenylalanine chain:

Phe-Val-Asp-Glu-His-Leu-
CySO₃H-Gly (N-terminal sequence) (1)
Gly-Glu-Arg-Gly (2)
Thr-Pro-Lys-Ala (3)
Tyr-Leu-Val-CySO₃H-Gly (4)
Ser-His-Leu-Val-Glu-Ala (5)

These five sequences contain all but four of the amino acid residues of frac-

tion B. It was not possible to determine from the small peptides derived from acid hydrolyzates the position of the remaining four residues or the manner in which the five sequences given above were joined together. There were two reasons for this. First, there was considerable technical difficulty involved in fractionating the peptides containing two or more of the nonpolar residues such as tyrosine or leucine. It happened that these residues were grouped together in the chain (see below) and gave rise to a mixture of peptides that moved fast on paper chromatograms and were not well resolved. The second difficulty was due to the great lability in the presence of acid of the bonds involving the amino groups of the serine and threonine residue. It was never possible to find a peptide containing this bond and hence to know what residues preceded the serine and threonine.

It was thus necessary to use another hydrolytic agent that would differ in specificity from concentrated acid. Hydrolyzates prepared by the action of dilute acid at high temperatures or of alkali were studied but yielded little further information. Much more successful, however, was the use of proteolytic enzymes (11). Initially we had refrained from using them, since it was considered that they might bring about rearrangement of the peptide bonds by transpeptidation or by actual reversal of hydrolysis. Subsequent work has, however, showed that this is not a very serious danger and that in fact proteolytic enzymes are the most useful hydrolytic agent for studies of amino acid sequences.

Proteolytic enzymes are much more specific than is acid, since only a few of the peptide bonds are susceptible. They give rise to larger peptides, which in general are more difficult to fractionate by paper chromatography. However, there are relatively few of them, so the mixtures are less complex. In this initial work we used essentially the same methods for studying the enzymic peptides that we had used for the acid ones, depending largely on paper chromatography for fractionation, although more recently it has been shown that better separations can be obtained by ion-exchange chromatography and by ionophoresis.

As an example we may consider a peptide Bp3 obtained by the action of pepsin. It had the following composition:

Phe (CySO₃H, Asp, Glu, Ser,
Gly, Val, Leu, His)

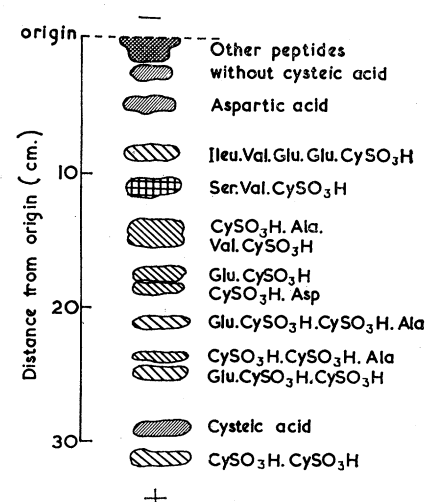


Fig. 1. Ionophoresis of partial acid hydrolyzate of fraction A at pH 3.5, showing separation of cysteic acid peptides.

Of these components the most important are aspartic acid and serine, since they occur only once in the chain. Aspartic acid is present only in the N-terminal sequence (1), and serine is in sequence 5. This shows that all of sequence 1 and at least the N-terminal part of sequence 5 are present in peptide Bp3. That none of the other sequences are present follows from the fact that Bp3 contains no arginine (sequence 2); threonine, proline, or lysine (sequence 3); or tyrosine (sequence 4). One may thus conclude that the two sequences are joined together. By studying other peptides obtained by the action of pepsin, trypsin, and chymotrypsin, it was possible to find out how the various sequences were arranged and to deduce the complete sequence of the phenylalanine chain, which is shown below:

Phe-Val-Asp-Glu-His-Leu-CySO₃H-
Gly-Ser-His-Leu-Val-Glu-Ala-Leu-
Tyr-Leu-Val-CySO₃H-Gly-Glu-Arg-
Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala

In this work many more peptides were

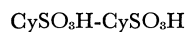
Table 2. Cysteic acid peptides identified in a partial acid hydrolyzate of fraction B. The inclusion of residues in parentheses indicates that their relative order is not known.

Peptide	Peptide
CySO ₃ H-Gly	CySO ₃ H-Gly
Val-CySO ₃ H	Leu-CySO ₃ H
Val-(CySO ₃ H, Gly)	Leu-(CySO ₃ H, Gly)
Leu-(Val, CySO ₃ H)	
Leu-(Val, CySO ₃ H, Gly)	
Sequences deduced	
Leu-Val-CySO ₃ H-Gly	Leu-CySO ₃ H-Gly

studied from both acid and enzymic hydrolyzates than were actually necessary to deduce the sequence. This was considered essential since the methods used were new and were qualitative rather than quantitative. The fact that all the peptides fitted into the unique sequence given above added further proof of its validity.

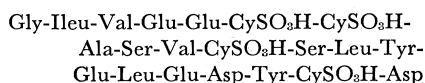
Sequence of Fraction A

Essentially similar methods were used to determine the sequence of fraction A (12). Although it was the shorter of the two chains, the determination of its structure was more difficult. Fraction B contains several residues that occur only once in the molecule, and this helps considerably in interpreting the results, whereas fraction A has only a few such residues, and these are all near one end. Also, fraction A is much less susceptible to enzymic hydrolysis. It is not attacked by trypsin, and there is a sequence of 13 residues which is not split by chymotrypsin or by pepsin, either. Considerable difficulty was at first experienced with the cysteic acid peptides. Fraction A contains the sequence



and this gave rise to a number of peptides which were very soluble in water and which would not fractionate easily by paper chromatography. However, it was found that by paper ionophoresis, at pH 2.75, they could be well separated, since they were the only acidic peptides present. At this pH , $-COOH$ groups are uncharged, while $-SO_3H$ groups carry a negative, and $-NH_2$ groups a positive, charge. Peptides without cysteic acid were all positively charged, those with one cysteic acid were neutral and could be separated as a group, and those with two cysteic acids were negatively charged. If a slightly higher pH (3.5) is used for the ionophoresis, the $-COOH$ groups become slightly charged and all the peptides containing one cysteic acid residue move slowly towards the anode and can be fractionated in this way. This method was found very useful for the separation and identification of cysteic acid peptides. Figure 1 is a tracing of an ionogram of an acid hydrolyzate of fraction A achieved in this way.

The sequence of fraction A was found to be as follows:



When a protein is hydrolyzed with strong acid, it gives rise not only to amino acids but also to a certain amount of ammonia. This is present in the form of amide groups on some of the aspartic and glutamic acid residues. It was thus necessary to determine the position of these groups (13). This was done by studying the ionophoretic rates and amide contents of peptides derived from enzymic hydrolyzates, since the amide groups are not split off by enzymes, whereas they are by acid. The position of the amide groups is indicated in Fig. 2 by the symbol NH_2 .

Arrangement of Disulfide Bridges

When the structures of the two chains of insulin had been determined, the only remaining problem was to find how the disulfide bridges were arranged. About this time it was shown by Harfenist and Craig that the molecular weight of insulin was of the order of 6000 and thus that the molecule consisted of two chains containing three disulfide bridges, and not of four chains as we had originally thought. The fact that fraction A contained four cysteic acid residues whereas fraction B had only two indicated that two bridges must connect the two chains together and that one must form an intrachain bridge connecting one part of the A chain with another part of the same chain.

In order to determine the distribution of the disulfide bridges, it was necessary to isolate, from unoxidized insulin, peptides containing intact cystine residues. These could then be oxidized to give cysteic acid peptides, which could be recognized since they had been found in the hydrolyzates of the oxidized chains. However, an unexpected difficulty arose in that, during hydrolysis, a reaction occurred which caused a random rearrangement of the disulfide bonds, so that cystine peptides were isolated which were not actual fragments of the original insulin, and it would have appeared from the results that every half-cystine was combined to every other half-cystine residue.

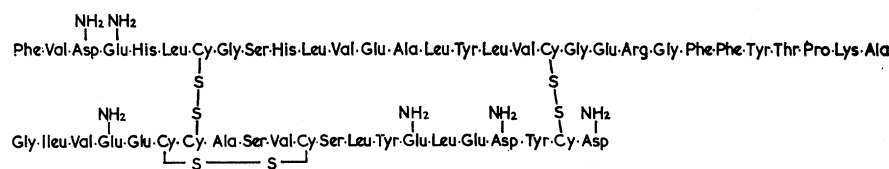
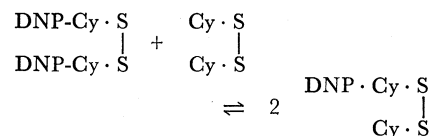


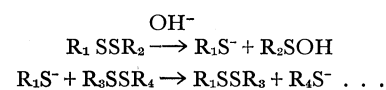
Fig. 2. Structure of insulin.

This disulfide interchange reaction could be demonstrated and studied when we used as a model system a mixture of cystine and bis-DNP-cystine, which reacted together to give mono-DNP-cystine



An ether-soluble colored substance was thus converted to a water-soluble colored substance, and the course of the reaction could be studied by measuring the distribution of color between ether and water.

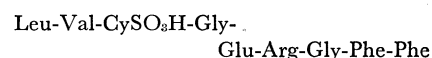
It was found that there were two types of disulfide interchange reactions (14). One took place in neutral and alkaline solution and was catalyzed by —SH compounds. It is probably due to initial hydrolysis of the disulfide, which then catalyzes a chain reaction:



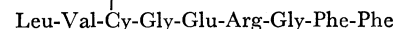
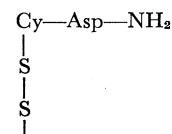
In neutral conditions the reaction could be inhibited by —SH inhibitors; hence, it was possible to use enzymic hydrolysis to obtain cystine peptides (15). Thus, for instance, with chymotrypsin, a peptide was obtained which on oxidation gave the two cysteic acid peptides



and



The structure of the cystine peptide was thus:



establishing the presence of a disulfide bridge between the two half-cystine residues nearest the C-terminal ends of the two chains.

It was not, however, possible to deter-

mine the positions of the other two disulfide bonds by means of enzymic hydrolysis, since no enzyme would split between the two consecutive half-cystine residues of the A chain. It was therefore necessary to reinvestigate the possibility of using acid hydrolysis.

The disulfide interchange reaction that occurred in acid solution was found to be different from that occurring in neutral and alkaline solution and instead of being catalyzed by —SH compounds was actually inhibited by them. This not only showed that a different reaction was involved but it also made it possible to prevent its occurring during acid hydrolysis. Thus, when insulin was treated with concentrated acid to which a small amount of thioglycolic acid was added, cystine peptides could be isolated which were in fact true breakdown products and from which the distribution of the remaining two disulfide bonds could be deduced. These are shown in Fig. 2, which shows the complete structure of insulin.

Of the various theories concerned with protein chemistry, our results supported only the classical peptide hypothesis of Hofmeister and Fischer. The fact that all our results could be explained on this theory added further proof, if any were necessary, of its validity. The results also showed that proteins are definite chemical substances possessing a unique structure in which each position in the chain is occupied by one, and only one, amino acid residue.

Examination of the sequences of the two chains reveals no evidence of peri-

odicity of any kind, nor does there seem to be any basic principle which determines the arrangement of the residues. They seem to be put together in an order that is random, but nevertheless unique and most significant, since on it must depend the important physiological action of the hormone.

Biological Activity and Chemical Structure

As yet little is known about the relationship of the physiological action of insulin to its chemical structure. One approach to this problem was to study the insulins from different animal species (16). Since all insulins show the same activity, it could be concluded that differences would be found only in parts of the molecule that were not important for activity.

All the results given above were obtained on insulin from cattle. When insulins from four other species were studied by essentially the same methods, it was found that the whole of the B chain was identical in all species, and the only differences that were found in the three amino acids contained within the disulfide ring of the A chain, which in the cattle are

Ala-Ser-Val

and in the other species are as follows:

Pig, Thr-Ser-Ileu
Sheep, Ala-Gly-Val
Horse, Thr-Gly-Ileu
Whale, Thr-Ser-Ileu

These results suggest that the exact structure of the residues in this position is not important for biological activity, but it does not follow that the whole of the rest of the molecule is important.

The determination of the structure of insulin clearly opens up the way to similar studies on other proteins, and already such studies are going on in a number of laboratories. These studies are aimed at determining the exact chemical structure of the many proteins that go to make up living matter and hence at understanding how these proteins perform their specific functions on which the processes of life depend. One may also hope that studies on proteins may reveal changes that take place in disease, and that our efforts may be of more practical use to humanity.

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Elmer Martin Nelson, Government Scientist

Elmer Martin Nelson will be remembered for his important contributions to science, particularly in the field of nutrition. Evidence that eminence in the field of science can be attained in the service of government is clearly demonstrated in the career of this man, who was born in Clark, South Dakota, more than 66 years ago.

A man of stature, both physically and mentally, he possessed a delightful sense of humor, as well as the dignity and modesty befitting a "servant of the people." His firm but friendly attitude earned for him the respect and admiration of his associates. In the key position he held for many years in the Food and Drug Administration, his philosophy of law

enforcement was to promote to the highest degree possible a voluntary compliance with pure food and drug law, through mutual understanding and respect. His straightforward discussion of difficult problems with scientists in industry led to increased confidence and developed a spirit of willingness to cooperate with the Food and Drug Administration. He continuously sponsored this theme of education and practiced frank discussion of enforcement problems with representatives of the food and pharmaceutical industries. In this association he helped to guide with integrity and knowledge the development and utilization of research in nutrition. The efforts of those who preyed upon consumers by exploiting new developments in nutrition science to their own commercial benefit were repugnant to his