pregnancy to late lactation, whereas cytochrome oxidase remained constant. No change in the distribution of these enzymes or of protein was found at the three stages of the lactation cycle studied.

3. In mammary gland succinate–neotetrazolium reductase increased rapidly over parturition and throughout lactation; cytochrome oxidase showed a smaller increase during lactation. These increases were accompanied by changes in the enzyme distribution among the mitochondrial and soluble fractions, more activity being found in the larger particles as lactation proceeds.

4. Mammary-gland mitochondria appear to change in size over the lactation cycle, being smallest in late pregnancy and largest in late lactation. In late lactation they sediment at approximately the same *g*-min. values as do liver mitochondria.

We acknowledge with gratitude the continual encouragement and advice offered by Dr A. L. Greenbaum. This work was done during the tenure of a Beit Memorial Fellowship by one of us (T.F.S.); the award of a grant from the Agricultural Research Council to D.N.P. is likewise gratefully acknowledged.

REFERENCES

Aldridge, W. N. (1957). *Biochem. J.* **67**, 423.
Bonner, W. D. (1955). In *Methods in Enzymology*, vol. 1, p. 722. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Brody, T. M., Wang, R. I. H. & Bain, J. A. (1952). *J. biol. Chem.* **198**, 821.
de Duve, C. & Berthet, J. (1953). *Nature, Lond.*, **172**, 1142.
de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
Dempsey, E. W., Bunting, H. & Wislocki, G. B. (1947). *Amer. J. Anat.* **81**, 309.
Dmochowski, L. & Strickland, L. H. (1953). *Brit. J. Cancer*, **7**, 250.
Eichel, H. J. (1957). *J. biophys. biochem. Cytol.* **3**, 397.
Greenbaum, A. L. & Slater, T. F. (1957*a*). *Biochem. J.* **66**, 148.
Greenbaum, A. L. & Slater, T. F. (1957*b*). *Biochem. J.* **66**, 155.
Greenbaum, A. L. & Slater, T. F. (1957*c*). *Biochem. J.* **66**, 161.
Holton, F. A., Hülsmann, W. C., Myers, D. K. & Slater, E. C. (1957). *Biochem. J.* **67**, 579.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Schneider, W. C. & Hogeboom, G. H. (1950). *J. nat. Cancer Inst.* **10**, 969.
Slater, T. F. & Planterose, D. N. (1958). *Biochem. J.* **69**, 417.
Slater, T. F. & Planterose, D. N. (1960). *Biochem. J.* **74**, 591.
Smith, T. C. & Richterich, B. (1958). *Arch. Biochem. Biophys.* **74**, 398.
Straus, W. (1956). *Biochem. biophys. Acta*, **19**, 58.
Tuba, J., Orr, P. F. & Wiberg, G. S. (1955). *Canad. J. Biochem. Physiol.* **33**, 904.
Weatherford, H. L. (1929). *Amer. J. Anat.* **44**, 199.

An Assay Procedure for a Succinate–Neotetrazolium-Reductase System

By T. F. SLATER* and D. N. PLANTEROSE†
Department of Biochemistry, University College London, Gower Street, London, W.C. 1

(*Received* 17 *July* 1959)

There is a rapid production of reduced neotetrazolium chloride (formazan) by rat-liver suspensions in the presence of succinate, vitamin C and vitamin K_3 (Slater, 1959*b*). The enzyme system coupling the reduction of neotetrazolium chloride with the oxidation of succinate is termed succinate–neotetrazolium reductase. The adoption of the word 'reductase' in connexion with neotetrazolium chloride as final electron acceptor must, of course, be interpreted with reserve. It is not intended to imply that there is only one route for the reduction of neotetrazolium by succinate as it seems probable that the pathway operating in the unsupplemented homogenate is different from that which operates in the presence of added vitamin C and vitamin K_3. This paper describes suitable conditions for the assay of such an enzyme system in the presence of added vitamin K_3 and vitamin C; under the conditions described formazan production increases proportionately with the amount of tissue added.

* Beit Memorial Fellow.
† Present address: Research Institute (Animal Virus Diseases), Pirbright, Surrey.

METHODS

The animals used were hooded Norwegian rats of the Medical Research Council strain. They were adult females, body weight 180–200 g., and were fed on a diet described by Parkes (1946). Liver homogenates were prepared either in water or in 0·25 M-sucrose at 0° in a conventional-type homogenizer with a plastic pestle (diam. difference 0·005 in.). Mammary-gland homogenates were prepared in

the type of homogenizer described by Chaikoff & Emanuel (1957). Vitamin K_3 (2-methyl-1:4-naphthaquinone) was added as a suspension (10 mM unless otherwise stated) as described for vitamin E suspensions by Deul, Slater & Veldstra (1958). The suspensions were freshly prepared on each occasion as follows: 25 mg. of vitamin K_3 was dissolved in 3 ml. of ethanol; this solution was then added to 12 ml. of 0·2% ox-plasma albumin in 0·1 M-sodium phosphate buffer, pH 7·4. The standard buffer mixture used in the incubations was prepared as follows: 20 ml. of sodium phosphate buffer (Na_2HPO_4–NaH_2PO_4), pH 7·4, 0·1 M; 2 ml. of disodium succinate (recrystallized from water), pH 7·4, 0·5 M; 1 ml. of sodium ethylenediaminetetraacetate (EDTA), pH 7·4, 0·1 M. All references to individual components of the standard mixture, i.e. phosphate buffer, imply that the molarity, pH, etc., are the same as given above.

Tissue (0–20 mg. wet wt.) was pre-incubated aerobically at 37° for 2 min. with standard buffer mixture and vitamin C unless it is specifically mentioned that vitamin C was omitted from this pre-incubation mixture. Except where otherwise stated, 1 μmole of vitamin C was added in a volume of 0·1 ml., and buffer mixture to give a pre-incubation volume of 1·1 ml. was added. Blank values to compensate for reduction of neotrazolium by enzymic and non-enzymic pathways other than via succinate oxidation were obtained by substituting disodium malonate (pH 7·4, 0·1 M) for disodium succinate in the standard buffer mixture. After the pre-incubation period, neotetrazolium chloride (0·15 ml. of aqueous 1% solution, unless otherwise stated) and vitamin K_3 (the quantity added is described at the appropriate position in the text) were added. The reaction was stopped 5–10 min. after the addition of neotetrazolium with 1 ml. of 10% trichloroacetic acid; the formazan was extracted by shaking with 4 ml. of ethyl acetate and the extinction was obtained at 510 mμ with a Hilger Uvispek spectrophotometer.

RESULTS

The production of formazan by succinate–neotetrazolium reductase in unsupplemented homogenates does not increase proportionately with the amount of tissue added (Slater, 1959*b*). Formazan production is greatly stimulated by the addition of vitamin K_3 although the response remains non-linear (Slater, 1959*b*). At three different amounts of liver tissue (5, 10 and 15 mg. wet wt.) maximum stimulation of formazan production by vitamin K_3 in the absence of vitamin C was obtained with a final vitamin K_3 concentration of approximately 1 mM. Table 1 gives the results of this investigation, for which the incubation time was 5 min. Higher levels of vitamin K_3 than 1–2 mM depress formazan production by low amounts of tissue possibly because of the inhibitory effect of ethanol on the enzyme system. The addition of 0·1, 0·2 and 0·3 ml. of ethanol to 20 mg. wet wt. of liver tissue in 1 ml. of standard buffer mixture inhibited formazan production by 14, 80 and 94% respectively during a 20 min. incubation. A portion (0·1 ml.) of vitamin K_3 suspension contained only 0·02 ml. of ethanol. Similar results to those reported for rat liver were obtained with rat-mammary-gland suspensions although on occasions a higher level (2 mM) of vitamin K_3 was required for optimum response; this is probably due to the high fat content of rat-mammary-gland homogenates.

Formazan production increases as the concentration of neotetrazolium in the incubation medium is increased. In a typical experiment 5 mg. wet wt. of liver tissue was incubated with 0·7 μmole of vitamin K_3 and varying amounts of neotetrazolium. Buffer mixture to give a final incubation volume of 1·1 ml. was added. Formazan production reached a plateau value when more than 1 mg. of neotetrazolium was added; no inhibition of the reaction was observed when up to 3 mg. of neotetrazolium was added to the incubation medium.

With 1 mM-vitamin K_3 in the incubation medium the effect of varying the final ascorbate concentration was investigated. Fig. 1 shows that there is an increase in formazan production as the final ascorbate concentration is increased to approx. 1–2 mM in rat-liver suspensions (5–15 mg. wet wt.). Varying the pre-incubation period from 0 to 10 min. in the presence of ascorbate was without effect on this ascorbate stimulation. It can be seen from Fig. 1 that the very marked non-linear response

Table 1. *Variation in the amount of formazan produced by the addition of vitamin K_3 to three different amounts of rat-liver tissue*

Incubation was at 37° (5 min.) in standard buffer mixture as described in the Methods section; vitamin K_3 was added as a suspension in ethanol + ox-plasma albumin + phosphate buffer as described in the Methods section. Formazan was extracted with ethyl acetate and measured at 510 mμ.

Amount of tissue added (mg. wet wt.)	Vitamin K_3 added in suspension (μmoles)					
	0·10	0·30	0·50	1·0	2·0	3·0
	Formazan produced with various amounts of vitamin K_3 (μg.)					
5	1	47	83	71	6	6
10	63	208	240	276	268	252
15	118	296	281	380	380	330

Table 2. *Effect of increasing concentration of ascorbic acid on the blank value in the presence of malonate as a percentage of the value obtained in the presence of succinate*

Formazan production was measured with three amounts of rat-liver tissue (5, 10 and 15 mg. wet wt.) at five different amounts of vitamin C (0·3 μmole–10 μmoles); pre-incubation was for 2 min. Incubation was in the presence of 1 mM-vitamin K_3 for 8 min. Other details were as described in the Methods section.

Final ascorbate concn. (mM)	Wt. of liver tissue (mg. wet. wt.) 5	10	15
	Formazan (μg.) produced in the presence of malonate / Formazan (μg.) produced in the presence of succinate × 100		
0·3	9	2	2
0·6	4	2	6
1·0	9	5	10
2·0	24	16	19
10·0	71	54	47

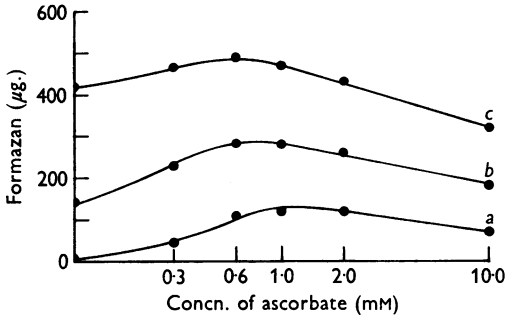

Fig. 1. Formazan production by succinate–neotetrazolium reductase in the presence of varying amounts of ascorbic acid (0–10 μmoles were added) and with three different amounts of rat-liver tissue (curve *a*, 5 mg. wet wt.; curve *b*, 10 mg. wet wt.; curve *c*, 15 mg. wet wt. of liver tissue). Each tube contained 1 mM-vitamin K_3, 0–0·2 ml. of vitamin C solution, 0·15 ml. of neotetrazolium chloride and buffer mixture to give a final incubation volume of 1·35 ml. Pre-incubation was for 2 min. in the presence of buffer mixture, tissue and vitamin C; incubation time after addition of vitamin K_3 and neotetrazolium chloride was 8 min. Other details are as given in the Methods section.

found when no vitamin C was added was largely eliminated when 1–2 mM-vitamin C was present in the incubation medium. Linearity of response in the presence of 2 mM-vitamin C is shown specifically in Fig. 2 for rat-liver and rat-mammary-gland suspensions. If high concentrations of ascorbate are used the succinate–neotetrazolium-reductase system becomes difficult to identify against a very high blank value resulting from the reduction of neotetrazolium by ascorbate and vitamin K_3. Table 2 shows that the blank value (i.e. formazan production in the absence of succinate as substrate) increases rapidly as the ascorbate concentration is increased. At a final ascorbate concentration of 10 mM the blank is 50–70 % of the value obtained in the presence of succinate. With 1 mM-ascorbate,

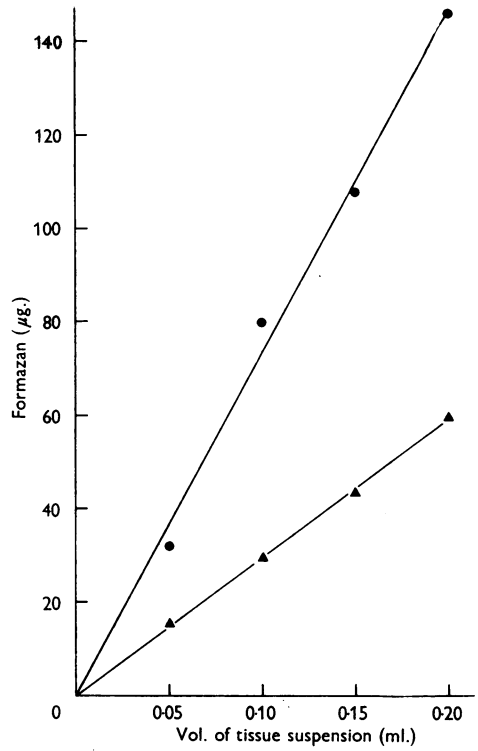

Fig. 2. Formazan production by rat-liver (●) and rat-mammary-gland (▲) suspensions with various amounts of tissue (0–20 mg. wet wt.). Tissue was pre-incubated for 2 min. with 1 ml. of standard buffer mixture, 2·2 μmoles of vitamin C and water to give a final volume of 1·5 ml. Incubation was started by the addition of 0·25 ml. of vitamin K_3–neotetrazolium chloride mixture (0·1 ml. of vitamin K_3 suspension in ethanol + ox-plasma albumin + phosphate buffer–0·15 ml. of 1 % neotetrazolium chloride solution). Incubation time was 8 min. Liver suspension, 1:20 in water; mammary-gland suspension, 1:10 in water, tissue at fourteenth day of lactation; formazan values were corrected for production in the absence of succinate.

however, the blank value is only some 10% of the value obtained in the presence of succinate with 0–15 mg. wet wt. of liver tissue. Formazan production in the presence of malonate appears to be largely the result of a non-enzymic coupling of vitamin C and vitamin K_3 (Slater, 1959*a*); this process is stimulated by tissue suspensions and tissue fractions. Boiling a liver homogenate, however, only decreases this stimulation by some 20%. The stimulatory power of tissue homogenates resides largely in the soluble fraction obtained after removing particulate fractions by differential centrifuging. Examination of the blank values obtained by Slater & Planterose (1960) indicated that the stimulatory power of both rat-liver and -mammary-gland suspensions was distributed as follows among the six fractions they described: M1, 13%; M2–M5, 18%; fraction S, 69%.

Formazan production via the succinate–neotetrazolium-reductase system varies with the time of incubation as shown in Fig. 3. It can be seen that for both liver and mammary-gland suspensions there is a preliminary time lag of approximately 4 min. before formazan production becomes appreciable.

In the presence of 1 mM-vitamin K_3 and 1 mM-vitamin C the assay system is suitable for determining the level of the succinate–neotetrazolium-reductase system in intracellular fractions of rat liver and mammary gland; Fig. 4 illustrates this. It can be seen that under the conditions used formazan production is proportional to the amount of tissue added for all fractions. The overall

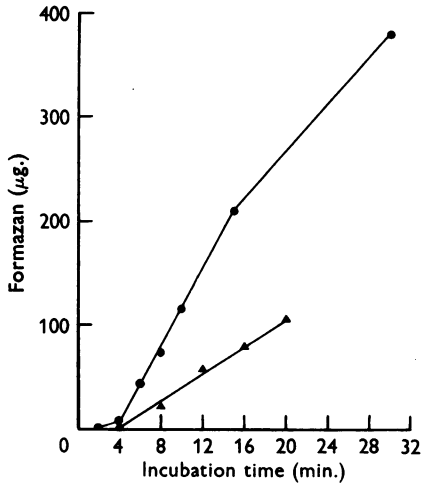

Fig. 3. Formazan production by rat-liver (●) and rat-mammary-gland (▲) suspensions after various times of incubation (0–30 min.). Standard buffer mixture (15 ml.), 3 ml. of 0·2% vitamin C and tissue suspension [2 ml. of liver suspension (1:20); 4 ml. of mammary gland (1:10)] were pre-incubated for 2 min. at 37°. Neotetrazolium chloride–vitamin K_3 mixture (5 ml.: 2 ml. of vitamin K_3 suspension in ethanol + ox-plasma albumin + phosphate buffer–3 ml. of 1% neotetrazolium chloride) was then added to start the incubation. Samples (2 ml.) were withdrawn at various times of incubation and pipetted into 1 ml. of cold 10% trichloroacetic acid. Formazan was extracted and estimated as described in the Methods section. Formazan values are corrected for production in the absence of succinate. The animal used was at the fourteenth day of lactation.

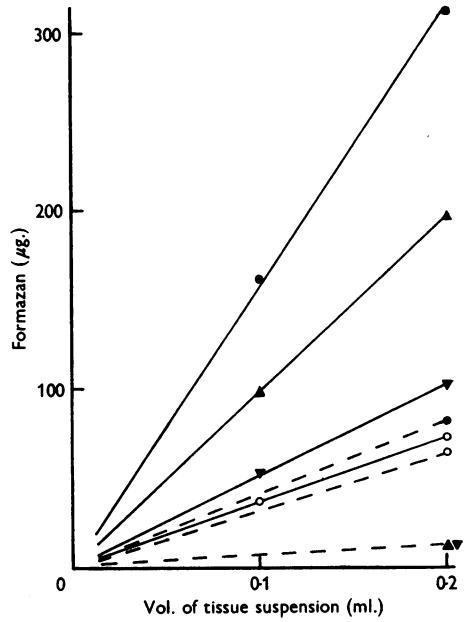

Fig. 4. Formazan production by various intracellular fractions of rat liver. For details of preparation of the fractions see Slater & Planterose (1960). Fraction H′ (●) is the whole-homogenate standard; fractions M1 (▲) and M2 (▼) are mitochondrial fractions; fraction S (○) is the supernatant remaining after the sedimentation of fraction M5 at approx. 340 000 *g*-min. Continuous lines show formazan production in the presence of sodium succinate as substrate; broken lines show the corresponding blank values, i.e. formazan production in the presence of sodium malonate. For clarity, the malonate points obtained with 0·1 ml. of suspension are omitted. The concentrations (fraction H′ in g./ml. of final suspension; other fractions relative to initial concentration in fraction H′) of the fractions used in the assay were: H′, 1:13·4; M1, 1:10·7; M2, 1:2·7; S, 1:1. Each tube contained 88 μmoles of phosphate buffer, 4 μmoles of EDTA, 40 μmoles of sodium succinate or 8 μmoles of sodium malonate, 1 μmole of vitamin C, 1 μmole of vitamin K_3 and 1·5 mg. of neotetrazolium chloride. The final incubation volume was 1·5 ml.; pre-incubation of tissue, buffer and vitamin C was for 2 min.; incubation time was 5 min.; other details were as described in the Methods section.

Table 3. *Formazan production after varying times of incubation at 37° in the absence of added vitamin K_3 and ascorbic acid*

Rat liver or mammary gland (17-days lactating), 20 mg. wet wt., was incubated with 2 ml. of phosphate buffer, 0·2 m of sodium succinate, 0·2 ml. of EDTA and 0·5 ml. of 1% neotetrazolium chloride.

	Incubation time (min.)					
	10	20	30	60	90	120
Tissue	Formazan produced (μg.)					
Liver	36	67	105	209	—	—
Mammary gland	—	—	25	46	65	105

difference between formazan production in the presence of succinate and that in the presence of malonate is small for fraction S; this difference can, however, be obtained with reasonable accuracy by assaying at several levels of tissue.

The formazan solution in ethyl acetate is stable over reasonable periods of time; the extinction at 510 mμ decreases approx. 6% per hr. The succinate–neotetrazolium-reductase system is destroyed by boiling and is inhibited by *p*-chloromercuribenzoate.

DISCUSSION

The short time lag which occurs before a linear production of formazan is obtained is similar to the time lags in tetrazolium reduction mentioned by Shelton & Rice (1957) for succinate oxidation and by Lagnado & Sourkes (1957) for monamine oxidation. This does not appear to be the result of permeability phenomena arising from the relatively large charged molecule of neotetrazolium chloride, since bursting intracellular structures by high-speed blending or by freezing and thawing has little influence on the reaction (Slater, 1959*b*). After this preliminary time lag, however, formazan production is linear until the concentration of formazan becomes limiting. For instance, in the presence of added vitamin K_3 and vitamin C, where the reaction is very fast, linearity is maintained under the conditions used only up to about 15 min. with liver tissue (Fig. 3). In tissue suspensions unsupplemented with vitamin K_3 and ascorbate the reaction is very slow and formazan production via succinate oxidation is linear for 1–2 hr. (Table 3).

The method has been applied to tissues other than rat liver: e.g. rat-kidney, rat-mammary-gland and mouse-liver suspensions. It seems that the concentration of vitamin C required for optimum response varies with the tissue. On a few occasions with rat mammary gland, for instance, a higher concentration of ascorbate was required for linearity of response with increasing amounts of tissue. Higher levels of ascorbate than approximately 2 mM in rat liver depress formazan production by succinate–neotetrazolium reductase (Fig. 1), at the same time greatly increasing formazan production by a process independent of succinate oxidation and probably utilizing a non-enzymic coupling of vitamin K_3 and vitamin C, which are known to interact directly (Slater, 1959*a*). Ascorbic acid has been previously reported to inhibit the succinic-oxidase complex to a larger extent than succinic dehydrogenase itself (Slater, 1949).

A routine assay of a succinate–neotetrazolium-reductase system can be performed under the conditions suggested. Under such conditions the distribution of the succinate–neotetrazolium–reductase system among intracellular fractions of rat liver and rat mammary gland has been examined (Slater & Planterose, 1960). The results showed that the succinate–neotetrazolium-reductase system is almost wholly associated with the mitochondrial fraction in rat liver; the distribution pattern found was identical with that found in many previous studies on the distribution of succinic oxidase in rat-liver suspensions.

SUMMARY

1. A method is described for estimating an enzyme system (succinate–neotetrazolium reductase) coupling the oxidation of succinate with the reduction of neotetrazolium chloride. In the presence of added vitamin K_3 (1 mM) and vitamin C (approximately 1 mM) the production of formazan increases proportionately with the amount of tissue added.

2. Assay conditions are described for liver homogenates applicable to 0–15 mg. wet wt. of liver tissue. The application of the method to intracellular fractions and to other tissues is discussed.

We are grateful to Dr A. L. Greenbaum for many helpful comments and advice. This work was done during the tenure of a Beit Memorial Fellowship by one of us (T. F. S.); the award of a grant from the Agricultural Research Council to D. N. P. is likewise gratefully acknowledged.

REFERENCES

Chaikoff, I. L. & Emanuel, C. F. (1957). *Biochim. biophys. Acta*, **24**, 254.
Deul, D., Slater, E. C. & Veldstra, L. (1958). *Biochim. biophys. Acta*, **27**, 133.
Lagnado, J. R. & Sourkes, T. L. (1957). *Canad. J. Biochem. Physiol.* **34**, 1095.
Parkes, A. S. (1946). *J. Hyg., Camb.*, **44**, 491.
Shelton, E. & Rice, M. E. (1957). *J. nat. Cancer Inst.* **18**, 117.
Slater, E. C. (1949). *Biochem. J.* **45**, 14.
Slater, T. F. (1959*a*). *Nature, Lond.*, **183**, 50.
Slater, T. F. (1959*b*). *Biochem. J.* **73**, 314.
Slater, T. F. & Planterose, D. N. (1960). *Biochem. J.* **74**, 584.

Amino Acid Sequence in Mycobacillin

By S. K. MAJUMDAR and S. K. BOSE
Department of Applied Chemistry, University College of Science and Technology, Calcutta, India

(*Received* 20 *July* 1959)

It has been observed from the study of physicochemical properties of mycobacillin that it is a polypeptide consisting of seven different amino acids (Majumdar & Bose, 1958). Studies on amino acid composition of mycobacillin show that the molecule is composed of five aspartic acid residues, two glutamic acid and two tyrosine residues and one residue each of serine, alanine, leucine and proline. The problem is how the amino acids are arranged in the mycobacillin molecule. The studies of Sanger and his collaborators (Sanger & Tuppy, 1951*a*, *b*; Sanger & Thompson, 1953) demonstrated the details of covalent structure of insulin by the application of fairly simple methods of separation and analysis, e.g. paper chromatography, paper electrophoresis and end-group analysis. This paper describes work done to obtain small peptides by a suitable method of hydrolysis followed by fractionation and identification of amino acid residues in each of the peptide fractions, and determination of *N*-terminal amino acids of the constituent peptides to discover the amino acid sequence of the mycobacillin molecule.

EXPERIMENTAL

Partial hydrolysis of mycobacillin. A sample of 30 mg. of mycobacillin was hydrolysed with 3 ml. of 11·4N-HCl for 7 days at 37° in sealed tubes. The hydrolysate was then freed from HCl by evaporating the sample to dryness *in vacuo* below 40° and the process was repeated thrice after addition of fresh water. Finally the hydrolysate was dissolved in 2 ml. of water.

Fractionation of peptide mixtures. After partial hydrolysis the next step was the separation of the complex mixture of peptides. By the application of descending two-dimensional paper chromatography with water-saturated phenol as the first solvent and butanol–acetic acid–water (4:1:1) as second, it was possible to have a satisfactory fractionation of the complex mixture of peptides. About 10 μl. of the sample was spotted at one corner (5 cm. away from both the edges) of the Whatman no. 1 filter paper (56 cm. × 46 cm.). The peptide mixture was allowed to run parallel in six pairs. One of each pair was developed with 0·2% of ninhydrin in acetone and used as a guide to locate peptide areas on the other undeveloped chromatogram. With the aid of the guide chromatogram, individual peptide areas were located, cut out and eluted with water (Consden, Gordon & Martin, 1947). The solution of each eluted peptide was evaporated *in vacuo* to give a residue.

Identification of constituent amino acids of a peptide. In the identification of constituent amino acids, a portion of the peptide residue eluted from the undeveloped chromatograms was completely hydrolysed in 0·5 ml. of 5·7N-HCl at 110° for 18 hr. The hydrolysate was then taken up in water and evaporated under reduced pressure to remove HCl, the operation being repeated twice. The final residue was dissolved in 0·05 ml. of 10% propan-2-ol. The solution so obtained was analysed chromatographically (Sanger & Tuppy, 1951*a*) and the constituent amino acids were identified.

Identification of N*-terminal amino acid of a peptide.* The *N*-terminal amino acid residue in each peptide from the partial acid hydrolysate of mycobacillin was determined in two ways: (*a*) by deamination with nitrosyl chloride followed by hydrolysis with hydrochloric acid; (*b*) by the dinitrophenyl (DNP) method of Sanger (1945, 1949).

(*a*) Deamination with nitrosyl chloride. In the deamination of each peptide, the method as followed by Consden *et al.* (1947) was used. A portion of the eluted peptide was dissolved in 0·5 ml. of 5·7N-HCl and deaminated with nitrosyl chloride at 30° for 30 min. The deaminated product was dried over solid KOH *in vacuo*, dissolved in water and evaporated *in vacuo* before complete hydrolysis. Finally, identification of amino acids remaining after hydrolysis of deaminated peptides with 5·7N-HCl at 110° for 18 hr. indicated the amino acid destroyed at the *N*-terminal end of the original peptide.

(*b*) Dinitrophenyl method. The DNP method of Sanger was next tried for amino end-group analysis of peptides. In practice, the peptides eluted from four paper chromatograms were dissolved in 1 ml. of water. To the solution was added 100 mg. of $NaHCO_3$ and then a solution of 0·1 ml. of dinitrofluorobenzene in 2 ml. of absolute ethanol (Schroeder & Legette, 1953). The DNP-peptide residue thus formed