# *Nucleotides. Part XXIV.* The Action of Some Nucleases on Simple Esters of Monoribonucleotides.*

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[Reprint Order No. 4576.]

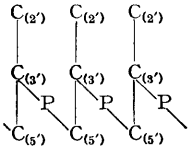
An enzymically active fraction from spleen converts adenosine-3′ benzyl phosphate and cytidine-3′ benzyl phosphate into adenosine-3′ phosphate (adenylic acid *b*) and cytidine-3′ phosphate (cytidylic acid *b*) respectively. The corresponding 2′-esters are not affected. The same fraction yields only the 3′(*b*)-mononucleotides from ribonucleic acids and polyribonucleotides. Taking these in conjunction with previous results on the specificity of ribonuclease (Brown and Todd, *J.*, 1953, 2040) it is concluded that the internucleotidic linkage in ribonucleic acids involves the 3′- and not the 2′-position in both purine and pyrimidine nucleotide residues, *i.e.*, that the ribonucleic acids are 3′ : 5′-linked polynucleotides.

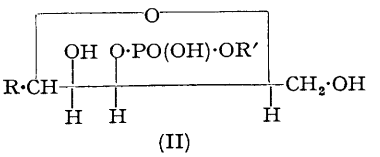
Comparable observations with diesterases from other sources are described.

Recent work on the structure of the ribonucleic acids has had as its objective the elucidation of the nature and position of the internucleotide linkage of the polynucleotide chain. From a consideration of the mechanism of chemical hydrolysis by acid and alkali it became clear that the nucleoside residues comprising the chain were linked through the phosphoryl group at $C_{(3')}$ or $C_{(2')}$ and $C_{(5')}$ (*e.g.*, I) (Brown and Todd, *J.*, 1952, 52). In practice, it has been necessary, previously, to refer to these positions as *a*, *b*, and $C_{(5')}$ since

* Part XXIII, preceding paper.

the orientation of the phosphoryl group in the isomeric monoribonucleotides derived from hydrolysates of ribonucleic acid had not been established. Recently, however, Khym, Doherty, Volkin, and Cohn (*J. Amer. Chem. Soc.*, 1953, **75**, 1262) have given chemical evidence that adenylic acid *b* is adenosine-3′ phosphate (II; R = adenine residue, R′ = H). Cytidylic acid *b* is considered to be cytidine-3′ phosphate (II; R = cytosine residue,


(I)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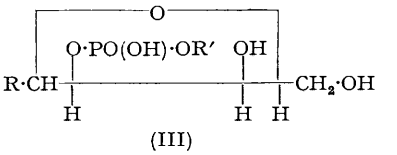

(III)

R′ = H) on physical grounds (Loring, Hammell, Levy, and Bortner, *J. Biol. Chem.*, 1952, **196**, 821; Cavalieri, *J. Amer. Chem. Soc.*, 1952, **74**, 5804), and hence uridylic acid *b* is the 3′-isomer (II; R = uracil residue, R′ = H) (Brown, Dekker, and Todd, *J.*, 1952, 2715). Recent evidence from enzymic studies (Shuster and Kaplan, *J. Biol. Chem.*, 1953, **201**, 535) confirms the view (Cohn, *J. Cell. Comp. Physiol.*, 1951, Suppl. 1, **38**, 21) that the *b* isomers of all four monoribonucleotides are alike in the position of the phosphoryl group in the ribofuranose residue. Any conclusions regarding the position of the internucleotidic linkage in the polynucleotides depend on the validity of the structures assigned to the mononucleotides and, in the present paper, we accept those indicated above.

The decision between $C_{(3')}$ and $C_{(2')}$ as a linkage point in the polynucleotides was reached in the case of the pyrimidine nucleoside residues by a study of the action of the pancreatic enzyme, ribonuclease, on some simple benzyl esters of the isomeric uridylic and cytidylic acids. Uridine-3′ and cytidine-3′ benzyl phosphate (II; R = uracil and cytosine residue respectively, R′ = $CH_2Ph$) were hydrolysed *via* the corresponding nucleoside-2′ : 3′ phosphate to uridine-3′ and cytidine-3′ phosphate respectively; the 2′-esters (III; R = uracil and cytosine residue respectively, R′ = $CH_2Ph$) were unaffected (Brown, Dekker, and Todd, *J.*, 1952, 2715; Brown and Todd, *J.*, 1953, 2040). Since the mechanism of hydrolysis of these simple esters by the enzyme was entirely analogous to that observed for the ribonucleic acids (Markham and Smith, *Biochem. J.*, 1952, **52**, 552, 558) it was concluded that the pyrimidine nucleotide residues were linked at the 3′-position in the ribonucleic acids. The specificity of ribonuclease clearly did not permit any conclusion concerning the linkage of the purine nucleotide residues. Evidence on this was sought in the action of other, related enzymes.

There are numerous indications in the literature that nucleases with specificities different from that of ribonuclease exist in plant and animal tissues. *E.g.*, Schmidt, Cubiles, and Thannhauser (*J. Cell. Comp. Physiol.*, 1951, Suppl. 1, **38**, 61) found that pancreas contains a fraction which hydrolyses further the limit polynucleotides produced by ribonuclease digestion of ribonucleic acids. Volkin and Cohn (*Fed. Proc.*, 1952, **11**, 303) showed that extracts of spleen prepared according to Maver and Greco (*J. Biol. Chem.*, 1949, **181**, 861) degrade ribonucleic acids to the 3′-mononucleotides. Fractionation of spleen has led to three distinct enzymically active fractions (Heppel and Hilmoe, *Fed. Proc.*, 1953, **12**, 217) of which that designated Fraction III has been used in the present work; degradation of ribonucleic acids and some ribonuclease-resistant polynucleotides by Fraction III has been studied (Heppel, Markham, and Hilmoe, *Nature*, 1953, **171**, 1152) concurrently. It was found that ribonucleic acids and certain polynucleotides are split by the enzyme preparation to mononucleotides of which the purine representatives were adenosine-3′ phosphate and guanosine-3′ phosphate. No evidence of intermediate nucleoside-2′ : 3′ phosphates was observed. It was concluded that the diesterase split the internucleotide linkages without the possibility of migration of the phosphoryl group, so that the purine nucleoside residues in the intact polynucleotide had been linked at the 3′-position. This, although plausible, would be rendered more certain by a study of the action of the enzyme on isomeric nucleotide esters in which the phosphoryl group is known to occupy the 2′- and the 3′-position of the nucleoside residue.

We have now studied the action of the spleen fraction on benzyl esters of adenosine-2′ and -3′ phosphate and cytidine-2′ and -3′ phosphate, following the reactions on paper chromatograms. The benzyl esters of the two adenylic acids, as their mixed barium salts, have previously been described (Brown and Todd, *J.*, 1952, 44); they have now been separated by ion-exchange chromatography, and the free acids obtained in crystalline form. The cytidine derivatives have already been described (Brown and Todd, *J.*, 1953, 2040).

The adenosine-3′ and cytidine-3′ benzyl phosphates, when treated with the spleen fraction, were completely converted into adenosine-3′ phosphate and cytidine-3′ phosphate; no evidence for a cyclic phosphate intermediate was found on paper chromatograms. The 2′-isomers were, however, unaffected, even though for adenosine-2′ benzyl phosphate the concentration was varied 80-fold. This was not due to enzyme inhibition, because incubation of a mixture of the 2′- and the 3′-ester resulted in approximately complete splitting of the latter. A stricter comparison of the two esters was made by using nearly comparable quantities of substrate and varying the concentration of enzyme widely (Table 1). This showed that hydrolysis of the 2′-isomer, if it occurred, must be at a rate less than 1/3000 that of the 3′-isomer.

TABLE 1. *Action of enzyme fractions from spleen, intestinal mucosa, potato, and rye grass on certain benzyl esters.*

| Incubation (hr.) | Enzyme solution (ml. per ml. incubation mixture) [1] | Substrate (benzyl phosphates) | Concn. of substrate (μmoles/ml.) initial | final | change | Hydrolysis (%) |
|---|---|---|---|---|---|---|
| | | (a) *Spleen fraction.* | | | | |
| 32 | 0·57 | Cytidine-2′ | 1·4 | 1·4 | 0 | 0 |
| 1 | 0·13 | Cytidine-3′ | 3·8 | 3·0 | 0·8 | 20 |
| 24 | 1·0 | Adenosine-2′ | 2·5 | 2·5 | 0 | 0 |
| 32 | 0·57 | ,, | 4·8 | 4·8 | 0 | 0 |
| 32 | 0·57 | ,, | 0·6 | 0·6 | 0 | 0 |
| 29 | 0·57 | ,, | 0·06 | 0·06 | 0 | 0 |
| 1 | 0·13 | Adenosine-3′ | 9·4 | 4·4 | 5·0 | 55 |
| 1 | 0·13 | ,, | 2·4 | 0·4 | 2·0 | 80 |
| 1 | 0·065 | ,, | 7·6 | 4·8 | 2·8 | 35 |
| 1 | 0·031 | ,, | 2·9 | 2·3 | 0·6 | 20 |
| 1 | 0·016 | ,, | 1·9 | 1·7 | 0·2 | 11 |
| 7 | 0·002 | ,, | 3·3 | 3·1 | 0·2 | 6 |
| 8 | 0·001 | ,, | 7·7 | 7·5 | 0·2 | 3 |
| | | (b) *Intestinal mucosa fraction.* | | | | |
| 32 | 0·65 | Cytidine-2′ | 1·9 | 1·9 | 0 | 0 |
| 7 | 0·65 | Cytidine-3′ | 4·8 | 3·9 | 0·9 | 20 |
| 32 | 0·65 | Adenosine-2′ | 1·7 | 1·7 | 0 | 0 |
| 7 | 0·65 | Adenosine-3′ | 3·9 | 2·5 | 1·4 | 35 |
| | | (c) *Rye grass fraction.* | | | | |
| 24 | 0·75 | Cytidine-2′ | 1·9 | 1·9 | 0 | 0 |
| 1 | 0·75 | Cytidine-3′ | 4·4 | 0·4 | 4·0 | 90 |
| 30 | 0·75 | Adenosine-2′ | 2·7 | 2·7 | 0 | 0 |
| 1 | 0·75 | Adenosine-3′ | 6·1 | 0·1 | 6·0 | 95 |
| 1 | 0·07 | ,, | 5·7 | 0·7 | 5·0 | 85 |
| | | (d) *Potato extract.* | | | | |
| 28 | 0·8 | Cytidine-2′ | 1·3 | 1·3 | 0 | 0 |
| 4 | 0·8 | Cytidine-3′ | 2·9 | 1·1 | 1·8 | 60 |
| 28 | 0·8 | Adenosine-2′ | 2·0 | 2·0 | 0 | 0 |
| 4 | 0·8 | Adenosine-3′ | 3·6 | 2·2 | 1·4 | 40 |

[1] The same enzyme solutions were used throughout (see Experimental section).

Although no evidence for the presence of nucleoside-2′ : 3′ phosphates in the solution during the enzymic hydrolysis was found, the enzyme preparation does split the cyclic phosphates under comparable conditions, yielding mononucleotides * (cf. Heppel,

* [Added, 24.9.53.] Observations made since submission of this paper by one of us (L. A. H.) with Mr. P. R. Whitfeld and Dr. R. Markham show that this enzyme preparation yields only nucleoside-2′ phosphates from the corresponding cyclic phosphates. This does not affect the conclusions drawn from the present work.

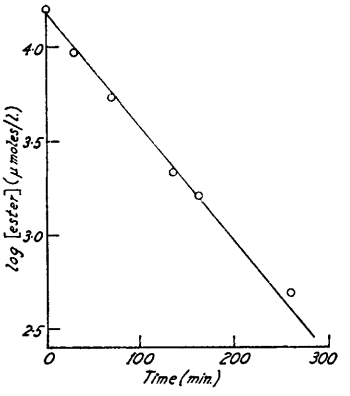
Markham, and Hilmoe, *loc. cit.*), and Table 2 shows that the rates are of a similar order of magnitude. The mechanism of hydrolysis of the esters is of some importance since ribonuclease, which has a closely related specificity, acts by a mechanism essentially

TABLE 2. *Action of enzyme fractions from spleen, intestinal mucosa, potato, and rye grass on guanosine-2′ : 3′ phosphate.*

| Incubation (hr.) | Enzyme solution (ml. per ml. incubation mixture) | Enzyme | Concn. of guanosine-2′ : 3′ phosphate (μmoles/ml.) initial | final | change | Hydrolysis (%) |
|---|---|---|---|---|---|---|
| 1 | 0·6 | Rye grass fraction | 2·2 | 1·1 | 1·1 | 50 |
| 1 | 0·6 | Potato extract | 2·9 | 2·2 | 0·7 | 25 |
| 1 | 0·09 | Spleen fraction | 2·7 | 2·3 | 0·4 | 15 |
| 1 | 0·65 | Intestinal mucosa fraction | 4·5 | 3·0 | 1·5 | 35 |

similar to that of chemical hydrolytic agents (Brown and Todd, *loc. cit.*) in which cyclic intermediates are involved. Present evidence is insufficient to allow more detailed discussion.

The same spleen fraction catalyses the nearly complete hydrolysis of ribonucleic acids, well over 90% being converted into mononucleotides (Heppel, Markham, and Hilmoe,




*Disappearance of adenosine-3′ benzyl phosphate when incubated with spleen fraction at* 37°. *Each ml. of incubation mixture contained* 0·2 *ml. of enzyme solution,* 16·3 *μmoles of the ester, and* 0·3 *ml. of* 0·5M-*potassium phosphate buffer* (pH 7·4).

*loc. cit.*). Since, then, only the nucleoside-3′ benzyl phosphates are hydrolysed by the enzyme it follows that the linkage point in the ribonucleic acids must also be at the 3′-position of the nucleoside residues, regardless of the mechanism of the enzymic hydrolysis.

Recently, it has been shown by Whitfeld and Markham (*Nature*, 1953, **171**, 1151) that only purine nucleoside-3′ phosphates are formed when dinucleotides derived from ribonuclease hydrolysates of yeast ribonucleic acid are subjected to a novel chemical degradation. The method (Brown, Fried, and Todd, *Chem. and Ind.*, 1953, 352) cannot involve phosphoryl migration.

Thus, if one accepts the structures assigned to the isomeric monoribonucleotides and the accumulated evidence that $C_{(5')}$ is involved as one of the linkage points of the nucleoside residues, the ribonucleic acids must be considered to be polynucleotides in which the individual nucleoside residues are linked by phospho-diester groupings at the $C_{(3')}$ and the $C_{(5')}$ positions, as in (I). Chain branching, if it occurs, either by incorporation of phosphotriester groupings or at $C_{(2')}$ of the sugar residues of the main chain (cf. Cohn, Doherty, and Volkin, " Phosphorus Metabolism," Johns Hopkins Univ. Press, Baltimore, 1952, Vol. II, p. 339) is not discussed here since the present experiments do not bear on the point.

Several other enzyme preparations which catalyse an extensive degradation of ribonucleic acids have been tested for diesterase activity. A fraction from intestinal mucosa (Heppel and Hilmoe, *loc. cit.*), a dialysed potato extract, and a relatively crude fraction from rye grass (Shuster and Kaplan, *loc. cit.*) rapidly hydrolysed the 3′-esters but had no

effect on the 2′-isomers (Table 1). This gives further support to the conclusions regarding the structural features of the ribonucleic acids reached in the spleen nuclease experiments. The intestinal mucosa fraction, like the spleen preparation, converts adenosine-3′ benzyl phosphate into adenosine-3′ phosphate; however, the plant extracts cause rapid conversion to the corresponding nucleoside. In the case of potato, A. Kornberg (personal communication) has shown that during extensive purification, the ratio of diesterase to non-specific phosphomonoesterase remained constant. Unless further work results in a dissociation of these activities, we have no simple way of determining the nature of the first reaction product with this diesterase preparation.

The rye grass fraction, kindly supplied by Drs. Shuster and Kaplan, converts the nucleoside-3′ benzyl esters into nucleosides under conditions where nucleoside-2′ phosphates would not be attacked. This fraction possesses monoesterase activity specific for 3′-nucleotides (Shuster and Kaplan, *loc. cit.*). It is probable that the conversion of the benzyl esters into nucleosides by this preparation involved the intermediate formation of nucleoside-3′ phosphate, *i.e.*, that the initial hydrolytic step was analogous to that catalysed by the spleen and the intestinal fraction.

Kinetic studies were not undertaken since the 2′-esters were not detectably split while the 3′-esters were completely hydrolysed. However, the Figure suggests that the hydrolysis of adenosine-3′ benzyl phosphate by the spleen fraction follows first-order kinetics. It was felt desirable to relate the activity of these four enzyme preparations towards the benzyl esters with their activity against a natural substrate. Table 2 indicates that the rates of hydrolysis of guanosine-2′ : 3′ phosphate are of a similar order of magnitude.

In the present investigation we have found that fractions from spleen, potato, and intestinal mucosa hydrolyse adenosine-3′ benzyl phosphate but not adenosine-5′ benzyl phosphate. Preliminary experiments with another diesterase derived from intestinal mucosa (Fraction V; Heppel and Hilmoe, *loc. cit.*) show the opposite specificity; the 3′-ester is not hydrolysed, but adenosine-5′ benzyl phosphate is attacked. Thus, it can be seen that with the synthetic alkyl esters one can obtain new information concerning the structural groups required for the specificity of diesterases. This can be used in following their separation from one another and for classification.

## Experimental

*Purification of Enzyme Fractions.*—(a) *From spleen and intestinal mucosa.* Further purification is being continued by one of us (R. J. H.) and a detailed description will be published later. For our experiments the procedure was as follows.

All operations were at 3° except as noted. Calf spleen was homogenized with three volumes of cold 0·25M-sucrose, and the mixture adjusted to pH 5·1 with acetic acid. A bulky precipitate formed which was collected by centrifugation and washed with cold (−10°) acetone. The dried powder was extracted with 20 parts (v/w) of 0·2M-acetate buffer (pH 6), and the solution fractionated with ammonium sulphate, first at pH 4·9, then at pH 8, each time selecting broad fractions containing most of the activity. The enzyme solution used in these experiments was dialysed against distilled water and contained 7 mg. of protein per ml.

Calf intestinal mucosa was homogenized with 3 volumes of cold 0·25M-sucrose, adjusted to pH 5·1 with acetic acid, and all insoluble material removed by centrifugation. The supernatant solution was twice fractionated with ammonium sulphate at pH 4·6. A fraction precipitated between 0·7 and 0·85 saturation was dialysed against distilled water and constituted the enzyme solution used for this work. It contained 9 mg. of protein per ml. and was free from phosphomonoesterase activity.

(b) *From potato extract.* Skinned potatoes were homogenized in a Waring blender with one-half of their weight of distilled water, then filtered through paper, and the filtrate was saturated with ammonium sulphate. The precipitated proteins were collected by centrifugation and dialysed against cold distilled water, to give a solution containing 3·0 mg. of protein per ml.

(c) *From rye grass fraction.* This was obtained from Drs. Shuster and Kaplan, and represented the stage of 2-fold purification described by them (cf. *J. Biol. Chem.*, 1953, **201**, 535). The solution used contained 1·5 mg. of protein per ml.

*Adenosine Benzyl Phosphates.*—Yeast adenylic acid (0·5 g.) was treated with phenyldiazomethane (from 2·3 g. of benzaldehyde hydrazone) in dimethylformamide (5 ml.) and kept for some time. Ether, water, and barium carbonate were then added. The solution was shaken for several hours with changes of ether. The aqueous phase was separated, filtered through Hyflo Supercel, and evaporated under reduced pressure. The product was twice reprecipitated from water (3—4 ml.) by acetone, and dried (0·44 g.). This material was dissolved in water (50 ml.), brought to pH 8, and run on to a column (9 cm.$^2$ × 11 cm.) of Dowex-2 resin in the formate form. After washing with water (500 ml.), elution was commenced with 0·1N-formic acid. Mononucleotides were removed with the first 5 l. of solvent, which were discarded. Adenosine-2′ benzyl phosphate was slowly removed from the column with the next 2 l., and the remainder rapidly removed by changing to 0·5N-formic acid. Continued elution with this solvent then removed adenosine-3′ benzyl phosphate. The course of fractionation was followed by observing the optical densities of the fractions (*ca.* 20 ml.) at 260 mμ.

The fractions containing *adenosine-2′ benzyl phosphate* were evaporated at 20 mm. and the residue crystallized from water. The product formed small irregular prisms from water which retained water tenaciously (Found, in material dried at 105°/0·1 mm.: C, 45·1; H, 4·5; N, 15·0; P, 7·8. $C_{17}H_{20}O_7N_5P,H_2O$ requires C, 44·8; H, 4·8; N, 15·3; P, 6·8%).

The fractions containing *adenosine-3′ benzyl phosphate* were evaporated at 14 mm. at room temperature, with additions of water at the later stages to reduce the formic acid concentration. The residual oil crystallized from water in rosettes of small needles (Found, in material dried at 105°/0·1 mm.: C, 43·2; H, 5·0; N, 14·5; P, 6·7. $C_{17}H_{20}O_7N_5P,2H_2O$ requires C, 43·1; H, 5·1; N, 14·7; P, 6·6%). The two substances gave single spots on paper chromatograms with butyl alcohol–acetic acid–water ($R_F$ 0·42 and 0·47) corresponding to adenosine benzyl phosphate *a* and *b* respectively (Brown and Todd, *J.*, 1952, 44).

*Guanosine-2 : 3′ Phosphate.*—This compound was obtained (Markham and Smith, *loc. cit.*) by heating dialysed ribonuclease digests of yeast ribonucleic acid with excess of barium carbonate at 100° for 1 hr., followed by chromatographic separation.

*Incubation of Enzyme Fractions with the Benzyl Esters and with Guanosine-2′ : 3′ Phosphate.*—All incubations were carried out at 37°. For experiments with spleen fractions the total volume was 0·07 ml.; this contained amounts of enzyme solution and concentrations of substrate as indicated in the Tables, and 0·015 ml. of 0·5M-potassium phosphate buffer of pH 7·4. Incubations with intestinal mucosa and rye grass fractions were similar except that the total volume was 0·06 ml. For experiments with potato extract the total volume was 0·05 ml. and the mixture contained 0·01 ml. of M-ammonium acetate buffer of pH 5. Control incubations were carried out, both with omission of enzyme and omission of substrate. It was also determined that the enzyme preparations remained active at 37° for at least 2 days.

In preliminary experiments it was found that the enzyme fractions completely hydrolysed the 3′-esters under conditions where no detectable splitting of the 2′-esters took place. The concentration of ester was 2—5 μmoles per ml., and the formation of 0·02 μmole of product per ml. could have been readily detected.

Quantitative experiments were then carried out as follows. At the end of incubation an aliquot portion was applied as a spot or thin line on Whatman No. 3 MM paper. Further reaction was halted by encircling the spot with acetone and allowing this to diffuse into the regions where applications had been made. After chromatography the sheets were dried and photographed in ultra-violet light (Markham and Smith, *Biochem. J.*, 1949, **45**, 294; 1951, **49**, 401). The ultra-violet-absorbing regions of the paper were cut out together with appropriate control regions. Elution was at room temperature for 20 hr., with 5 ml. of 0·1N-hydrochloric acid after which the absorption at 260 mμ was measured with the Beckman model DU quartz prism spectrophotometer. The results are shown in Tables 1 and 2.

For the paper chromatography of the compounds discussed in this paper several solvent systems were used (Whatman No. 3 MM paper, solvent descending). Good separation of the benzyl esters from nucleotides or nucleosides was obtained with the system *iso*propanol–water (7 : 3; v/v), with the further addition of 0·35 ml. of aqueous ammonia (*d* 0·880) per l. of gas space in the tank (poured into the bottom of the tank) (cf. Markham and Smith, *Biochem. J.*, 1952, **52**, 552).

The adenylic acid formed from its benzyl ester by fractions from intestine and spleen was identified as the 3′-isomer by migration with the solvent system saturated aqueous ammonium sulphate–M-sodium acetate–*iso*propanol (80 : 18 : 2; v/v) (Markham and Smith, *loc. cit.*) by comparison with an authentic specimen of adenosine-3′ phosphate.

The nucleotide formed from cytidine-3′ benzyl phosphate by the spleen fraction was shown

to be entirely cytidine-3′ phosphate by means of ion-exchange chromatography (Brown, Dekker, and Todd, *loc. cit.*).

One of us (L. A. H.) participated in this work as a member of the National Institutes of Health, Bethesda, temporarily assigned to the Molteno Institute. Thanks are offered to the John Simon Guggenheim Memorial Foundation for a Fellowship (to L. A. H.), to the American Cancer Society for a travel aid grant (to L. A. H.), to the Rockefeller Foundation for financial assistance (to D. M. B.), and to Professor A. R. Todd for his continued interest.

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National Institutes of Health, Bethesda. [*Received, August 11th,* 1953.]