10^{-6}. In one subject the investigation was repeated one hour after the subcutaneous administration of 1/75 grains of atropine sulphate, and no alteration in the response was noted, suggesting that the sweating is due to the direct stimulation of the sweat glands by the injected solutions, and that cholinergic fibres do not play a part in its production.

A. J. BARNETT

Clinical Research Unit,
Alfred Hospital,
Melbourne.
Nov. 9.

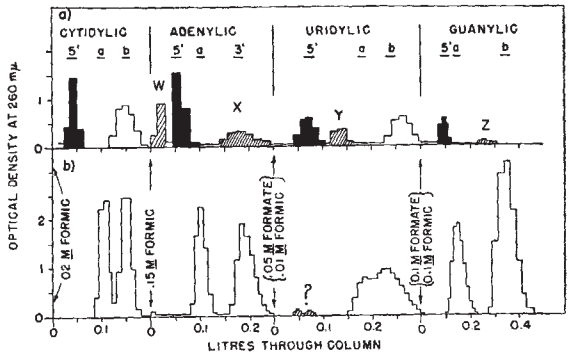
[1] Best, C. H., and Taylor, N. B., "The Physiological Basis of Medical Practice", 724 (5th edit., 1950).
[2] Barnett, A. J., Blackett, R. B., Depoorter, A. E., Sanderson, P. H., and Wilson, G. M., *Clin. Sci.*, **9**, 151 (1950).
[3] Haimovici, H., *Proc. Soc. Exp. Biol. and Med.*, **68**, 40 (1948).
[4] Wada, M., *Science*, **111**, 376 (1950).
[5] Barger, G., and Dale, H. H., *J. Physiol.*, **41**, 19 (1910–11).
[6] Bacq, Z. M., *Ann. physiol. et de physico-chim.*, **10**, 467 (1934).
[7] Blashko, H., *J. Physiol.*, **101**, 337 (1942).
[8] Bacq, Z. M., and Fischer, P., *Arch. Internat. Physiol.*, **55**, 73 (1947).
[9] Von Euler, U. S., *Acta Physiol. Scand.*, **16**, 63 (1948).
[10] Holton, Pamela, *J. Physiol.*, **108**, 525 (1949).

Nucleoside-5′-Phosphates from Ribonucleic Acid

THOUGH the manner in which the four nucleotides of ribonucleic acid are combined in the intact molecule is not completely settled, the data obtained from analyses of the chemical degradation products are most compatible with phosphoryl-group attachments through the C_2 and C_3 positions of the ribose group[1]. The exclusion of C_5 phosphoryl linkages rests principally upon the failure to isolate 5′ nucleotides or the acid-stable ribose-5-phosphate from acid hydrolysates of ribonucleic acid. However, Gulland and Jackson[2], hydrolysing yeast ribonucleic acid with a snake venom containing a phosphodiesterase and a specific 5′ nucleotidase, found 35 per cent of the total phosphate liberated as inorganic phosphate, suggesting that a large number of the phosphoryl groups were attached to the ribose C_5 position. Recently, Schmidt[3], utilizing periodate titrations of ribonuclease-hydrolysed yeast ribonucleic acid, concluded that some nucleotide groups had been linked to positions other than ribose C_2 or C_3 in the original ribonucleic acid. The experiments described below indicate that intact 5′ nucleotides can be isolated from enzymatically hydrolysed calf liver ribonucleic acid.

Calf liver ribonucleic acid, prepared by a technique avoiding the use of alkaline solutions[4], was hydrolysed successively by crystalline ribonuclease and by alkaline intestinal phosphatase in the presence of sodium arsenate to inhibit excessive dephosphorylation of the derived nucleotides, as originally described by Klein[5] for the enzymatic production of deoxyribonucleotides. The reaction was allowed to proceed until about 75–80 per cent of the total titratable phosphoryl groups were released, after which the digestion was stopped by the addition of formic acid to a concentration of 0·1 *N*. The nucleotide products of the digest were then isolated by the anion-exchange procedure described by Cohn[6].

The accompanying diagram shows the ion-exchange elution curve of the nucleotides isolated from the digest. The identification of the 5′ nucleotides indicated on the figure was made chiefly by comparing their properties with those of the corresponding synthetic 5′ nucleotides (we are indebted to Dr. D. M. Brown and Prof. A. R. Todd for generous samples of synthetic guanosine-5′-phosphate, uridine-5′-phosphate and cytidine-5′-phosphate) as follows: (*a*) the ion-exchange elution positions are identical with those of the analogous 5′ nucleotides (and deoxynucleotides) and are readily differentiated from the *a* and *b* isomeric forms[6,7]; (*b*) the spectrophotometric absorption characteristics of the isolated pyrimidine-5′-phosphates show definite differences from those of the *a* and *b* forms, but are exactly those of the corresponding synthetic substances; (*c*) the isolated adenosine-5′-phosphate and muscle adenylic acid were both deaminated at exactly the same rate by a preparation of Schmidt's muscle adenylic acid deaminase[8], whereas adenylic acids *a* and *b* were unaffected by the enzyme; (*d*) a highly specific preparation of bull semen 5′ nucleotidase (kindly supplied by Dr. L. A. Heppel) readily and rapidly dephosphorylated all of the isolated 5′ nucleotides, as well as the synthetic 5′ nucleotides, but was completely inactive towards all *a* and *b* mononucleotides; (*e*) the isolated 5′ adenylic acid contained adenine, pentose (orcinol reaction) and phosphate in a 1 : 1 : 1 proportion. Deoxypentose (diphenylamine reaction) was not present in any of the 5′ compounds nor was acid-labile phosphate (1 *N* hydrochloric acid, 1 hr., 100° C.).



Analyses of (*a*) enzymatic digest, (*b*) alkaline digest of 20 mgm. of calf-liver ribonucleic acid by ion-exchange. Exchanger: 'Dowex-1' (formate form), 200–400 mesh, 7 cm. × 0·88 sq. cm. Influent: formic acid and sodium formate, as shown; 0·6 ml./min.

Enzymatic resynthesis of smaller fragments, rearrangements of mononucleotides to yield the 5′ compounds during the enzymatic hydrolysis and preexistence of the free 5′ compounds were excluded by the following experiments: (1) a similar enzymatic hydrolysis of ribonucleic acid carried out in the presence of isotopic inorganic phosphate yielded 5′ and 3′ nucleotides containing none of the isotope (nor was arsenate incorporated); (2) incubation of a mixture of the nucleosides or nucleotides with the phosphatase in a phosphate medium resulted in no measurable synthesis of any of the 5′ nucleotides; (3) acid and alkaline hydrolysates yielded neither 5′ nucleotides nor ribose-5-phosphate; (4) ribonuclease alone or ribonuclease followed by alkaline hydrolysis yielded no 5′ nucleotides; these appeared only after phosphatase treatment. Thus, if isomerization during digestion does indeed take place, it must occur at the polynucleotide level.

It is our intention to use ion-exchange chromatography, already responsible for the isolation of many new nucleotides[6] from nucleic acid hydrolysates, to determine the generality of this finding, as well as in the search for hitherto unknown constituents (for example, *W*, *X*, *Y*, *Z* and ? in the diagram).

This work was performed under Contract Number W-7405-Eng.-26 for the U.S. Atomic Energy Commission.

Waldo E. Cohn
Elliot Volkin

Biology Division,
Oak Ridge National Laboratory,
Oak Ridge, Tennessee. Dec. 22.

[1] Levene, P. A., and Bass, L. W., "Nucleic Acids" (Chemical Catalog Co., 1931). Gulland, J. M., *J. Chem. Soc.*, 1722 (1938). Tipson, R. S., "Advances in Carbohydrate Chemistry", **1**, 193 (Academic Press, Inc., 1945).
[2] Gulland, J. M., and Jackson, E. M., *J. Chem. Soc.*, 1492 (1938).
[3] Schmidt, G., Cubiles, R., and Thannhauser, S. J. (in the press).
[4] Volkin, E., and Carter, C. E., *J. Amer. Chem. Soc.* (in the press).
[5] Klein, Willibald, *Z. physiol. Chem.*, **218**, 164 (1933).
[6] Cohn, W. E., *J. Amer. Chem. Soc.*, **72**, 1471 and 2811 (1950).
[7] Carter, C. E., *J. Amer. Chem. Soc.*, **72**, 1466 (1950).
[8] Kalckar, H. M., *J. Biol. Chem.*, **167**, 461 (1947).

Nature of the Activation Process in Enzymatic Reactions

The study of enzyme-catalysed reactions has thrown considerable light on the geometrical factors involved; but little is as yet known concernining the mechanism of the activation process. The experiments reported here were designed to yield information on the transformations which occur during enzyme action.

In the first investigation, carbobenzoxy-L-phenylalanine was incubated with and without chymotrypsin in oxygen-18 water ($H_2{}^{18}O$). In one experiment, crystalline bovine serum albumin was included. Following incubation, the reaction mixture was acidified (*p*H 3); the carbobenzoxy derivative was centrifuged, washed with water, and converted to phenylalanine by hydrogenation in aqueous methanol in the presence of palladium black. After crystallization from water, the amino-acid was decarboxylated at 270–290° in a mixture of diphenylamine and diphenylmethane[1], and the oxygen-18 concentration of the resulting carbon dioxide determined.

Phenylalanine was similarly incubated in oxygen-18 water with and without chymotrypsin; the acidified reaction mixture was taken to dryness, the phenylalanine hydrochloride extracted from the salts with absolute ethanol and the free amino-acid precipitated with pyridine. The results are given in the accompanying table.

Compound	Oxygen-18 (atom per cent excess)
L-Phenylalanine	0·000
Cbz-L-Phenylalanine	0·009
Cbz-L-Phenylalanine + chymotrypsin	0·470
Cbz-L-Phenylalanine + chymotrypsin	0·524
L-Phenylalanine + chymotrypsin	0·028
*Cbz-L-Phenylalanine + chymotrypsin	0·412
*Cbz-L-Phenylalanine + chymotrypsin + 25 mgm. bovine serum albumin	0·392

* 4-hr. incubation.

100 mgm. of Na_2HPO_4 and 41 mgm. of KH_2PO_4 were dissolved in 5 c.c. of $^{18}H_2O$ (1·13 atom per cent excess ^{18}O) and 100 mgm. of carbobenzoxy-L-phenylalanine or 65 mgm. of L-phenylalanine were dissolved in this buffer (*p*H 7·0). Following the addition (where indicated) of 20 mgm. of crystalline chymotrypsin (Armour), the solution was incubated at 37° for 6 hr.

It can be seen that, under the influence of chymotrypsin, the carboxyl group of an N-acyl aromatic amino-acid is activated and the oxygen atoms exchange with those of the medium. The presence of serum albumin has no marked effect. In the absence of enzyme the exchange does not occur. The free amino-acid is apparently not activated by chymotrypsin.

It is of interest that N-benzoyl D- and L-phenylalanine are inhibitors of chymotrypsin[2] (cf. also ref. 3).

In another investigation, the nature of the activation process in transamination reactions was studied. Konikova, Dobbert and Braunstein[4] had previously indicated, on the basis of densimetric estimation of the amount of deuterium passing into the medium, that the deuterium atom of α-deuterio α-amino-acids undergoes exchange with the hydrogen of the solvent water under the influence of glutamic–alanine transaminase. This has now been investigated by direct isolation and analysis. When L-leucine, containing 4·5 atomic per cent excess nitrogen-15 and 82 atomic per cent excess deuterium in the α-position, was incubated for 90 min. with minced pig heart, it lost more than 98 per cent of the deuterium but none of the nitrogen-15[5]. With D-leucine, neither deuterium nor nitrogen-15 was lost. When the L-leucine was incubated with 1 equivalent of α-ketoglutaric acid, more than 98 per cent of its deuterium was lost and the concentration of nitrogen-15 dropped to 3·8 atomic per cent excess[6].

In both cases the interaction of the enzyme and substrate results in an activated structure which can in the first example exchange with the hydroxyl ions, and in the second with the hydrogen ions of the water. In neither case is the usual reaction associated with these enzyme systems observed, namely, the hydrolysis of a peptide bond or the conversion of the amino-acid to the corresponding keto-acid. Were it not for the changes in the isotopic composition of some of the components of the system, no chemical reaction would have been observed. These changes result from the nature of the activated structure, the formation of which precedes and is essential for the complete chemical reaction. The ensuing steps, for example, the hydrolysis of the peptide bond, would then be spontaneous. For this reason it may be appropriate to call the reactions observed *virtual reactions*.

Further work is necessary to elucidate the detailed mechanisms responsible for the observations reported here.

This investigation was supported by a grant from the American Cancer Society on the recommendation of the Committee on Growth (National Research Council).

David B. Sprinson
D. Rittenberg

Department of Biochemistry,
College of Physicians and Surgeons,
Columbia University, New York. Nov. 13.

[1] Johnson, T. B., and Daschavsky, P. G., *J. Biol. Chem.*, **62**, 725 (1925).
[2] Kaufman, S., and Neurath, H., *J. Biol. Chem.*, **181**, 623 (1949).
[3] Johnston, R. B., Mycek, M. J., and Fruton, J. S., *J. Biol. Chem.*, **185**, 629 (1950).
[4] Konikova, A. S., Dobbert, N. N., and Braunstein, A. E., *Nature*, **159**, 67 (1947).
[5] Sprinson, D. B., and Rittenberg, D., *J. Biol. Chem.*, **184**, 405 (1950).
[6] Tanenbaum, S. W., and Shemin, D., *Fed. Proc.*, **9**, 236 (1950).

Final Stages of the $A \rightarrow BCD$ Route to Cholesterol

The ketone (I), termed Inhoffen ketone[1], has been obtained from cholestenone by improved methods. When pure, it has a melting point of 52°; it was originally described as an oil (semicarbazone, m.p. 225°, which we confirm) and was crystallized in this laboratory by R. P. A. Sneeden (m.p. 42°).

The Inhoffen ketone might well have been convertible to cholestenone by direct condensation with diethylaminobutanone methiodide under suitable conditions. However, this reaction did not succeed,