- McKittrick, D. S. & Schmidt, C. L. A. (1945). *Arch. Biochem.* **6**, 411.
- Masters, M. (1939). *Biochem. J.* **33**, 1313.
- Masters, M. & McCance, R. A. (1939). *Biochem. J.* **33**, 1304.
- Metcalf, G. F. & Thompson, B. J. (1930). *Physiol. Rev.* **36**, 1489.
- Meulen, H. ter (1922). *Rec. Trav. chim. Pays-Bas*, **41**, 112.
- Niederl, J. B., Baum, H., McCoy, J. S. & Kuck, J. A. (1940). *Industr. Engng Chem. (Anal. ed.)*, **12**, 428.
- Owen, E. C. (1936). *Biochem. J.* **30**, 352.
- Patrick, S. J. & Young, L. (1943). Report to Directorate of Chemical Warfare, Canada.
- Pirie, N. W. (1932). *Biochem. J.* **26**, 2041.
- Seligman, A. M. & Fine, J. (1943). *J. clin. Invest.* **22**, 265.
- Sherman, H. C. (1941). *Chemistry of Food and Nutrition*, 6th ed. New York: Macmillan.
- Silberstein, M. L. (1934). *Bull. Soc. Chim. biol., Paris*, **16**, 285.
- Tarver, H. & Schmidt, C. L. A. (1939). *J. biol. Chem.* **130**, 67.
- Young, L. (1941). Report to Directorate of Chemical Warfare, Canada.

A Method for the Colorimetric Micro-estimation of Thymine

By D. L. WOODHOUSE, *Cancer Research Laboratory, Medical School, University of Birmingham*

(Received 13 January 1948)

Thymine is the distinctive pyrimidine of the nuclear deoxyribosenucleic acid. A sensitive method for its estimation would be of service in the analysis of nucleic acid, in conjunction with other analytical techniques. Thymine can be distinguished from uracil or cytosine by the stable red colour which is produced when it is coupled with the diazo reagent of Koessler & Hanke (1919) in a solution made alkaline with sodium carbonate and afterwards reduced with hydroxylamine in presence of sodium hydroxide (Hunter, 1936). This reaction has been examined as regards sensitivity, specificity and suitability for quantitative work.

EXPERIMENTAL

Micro-estimation of pure thymine

Reagents and procedure. Slight modifications were made of Hunter's (1936) procedure. The *diazo reagent* used was that of Koessler & Hanke, viz. (i) 1.5 ml. sulphanilic acid solution (4.5 g. in 45 ml. conc. HCl, made up to 500 ml. with water), and (ii) 1.5 ml. NaNO_2 solution (25 g. in 500 ml. water). Solutions (i) and (ii) are quickly mixed and cooled; after 5 min., 6 ml. NaNO_2 solution are added and the solution made up to 50 ml. It is kept cold in ice water, and the reagent used not less than 20 min. after making the final dilution. If kept cold it is stable for 24 hr. and can conveniently be prepared daily from the stock solutions.

Coupling was performed at 20° and allowed to proceed for exactly 20 min., after which the NaOH was added, followed by hydroxylamine. During the coupling, the tubes should be immersed in a water bath at the prescribed temperature. The extension of time as compared with Hunter's qualitative test (5 min.) gives a more intense colour, but no extra tint in similarly conducted blank tests in which distilled water replaces the thymine solution. It is advisable to use stoppered vessels to exclude NH_3 or acid fumes, and to mix the solutions quickly and thoroughly at all stages.

Intensity and stability of the colour was measured by means of a Spekker absorptiometer, using the following amounts of reactants, mixed in the order given: Na_2CO_3 (2.0 ml.; 2.4 (w/v) % solution of anhydrous salt); distilled water (2.0 ml.); diazo reagent (2.0 ml.); thymine solution (2.0 ml. containing 0.0125–0.100 mg./ml.); NaOH (2.0 ml. of 3N); hydroxylamine hydrochloride (0.2 ml. of 20% w/v).

Colour values were read using a blue filter (Chance OB2). Beer's law was obeyed up to thymine concentrations of 0.1 mg./ml. Curves have been made with specimens of thymine kindly given by Genatosan Ltd. (Loughborough) and Schwarz Laboratories Inc. (New York). The samples gave identical readings.

The time of coupling must be measured exactly and the temperature should not vary by more than $\pm 0.5^\circ$. If the reaction is carried out at a higher temperature, e.g. 45° , more intense colours are produced even with very dilute solutions; 10 μg . in a final volume of 10 ml. can be detected with ease, but these conditions are not recommended for quantitative estimations.

Substances reacting under similar conditions. (i) When tested by the above technique none of the purines or pyrimidines known to occur in nucleic acids gives the pink colour except thymine. The other bases yield yellow tints so that if relatively large amounts of these substances are present together with thymine, they affect the colour produced. The presence of uracil in the proportion of 0.1 mg. to 0.1 mg. thymine occasions little error, however, when the prescribed conditions are observed.

(ii) The reaction yields yellow colours of various intensities with amino-acids or proteins at concentrations of 0.2 mg./ml. With cystine a tint very similar to the thymine colour is produced.

(iii) Certain aldehydes, including furfural, and decomposition products produced by mild hydrolysis of nucleic acids, produce pink tints so that precautions must be taken to exclude such substances when estimating thymine. In these circumstances it was not found possible to apply the method directly to hydrolysates of nucleic acid.

Comparison with the method of Harkins & Johnson (1929). These writers described a test for thymine in which the

acetol obtained by refluxing brominated thymine with $\text{Ba}(\text{OH})_2$ is distilled and combined with a reagent produced by heating *o*-nitrobenzaldehyde with FeSO_4 in presence of concentrated NH_3 followed by steam distillation. After acidifying the mixture with HCl and then making alkaline with NaHCO_3 , a blue fluorescence results which is more intense when viewed in the mercury arc light.

I have confirmed the statement that, by this technique, 1 mg. thymine can be detected if the volume of the completed test is reduced to 1 ml., but experiments showed that the Hunter (1936) test was much more delicate. Also the 'diazo coupling' method requires only simple apparatus and is much more convenient for routine analyses.

Application of the method to nucleic acid

(a) Technique

The Hunter (1936) reaction cannot be applied directly to a solution of hydrolyzed nucleic acid, since certain reaction products, particularly from ribonucleic acid, also produce red-coloured compounds. Efforts to eliminate these, or to extract the thymine quantitatively with various solvents, were not successful. It was, however, found possible to apply the reaction after separating the pyrimidine as a silver compound. The method was based partly on the technique of Levene (1922) for separation of purines, and partly on that of Barnes & Schoenheimer (1943) for the isolation of thymine from thymus nucleic acid. Levene showed that, when HCl gas is passed freely for some time into a suspension of nucleic acid in methanol, heat is liberated, the nucleic acid dissolves and the purines are precipitated. After standing in the cold overnight, they can be removed by centrifugation. Barnes & Schoenheimer evaporated such a supernatant from the purine precipitate to a small volume, hydrolyzed the products by 20% HCl in a sealed tube, and isolated some crystalline thymine.

In the present work the hydrolysate from 50 mg. of deoxyribonucleic acid was evaporated to dryness under reduced pressure on the water bath and free HCl removed as thoroughly as possible. The residue was dissolved in 3 ml. of 25% (v/v) H_2SO_4 , 1.5 ml. transferred to a combustion tube (5-6 in. long) and heated in a furnace at 175° for 2.5 hr. Sulphates and phosphates were removed by adding hot, saturated $\text{Ba}(\text{OH})_2$ to pH 9 and centrifuging. The precipitate also carried down all coloured carbonized materials. The supernatant liquid was concentrated to about 10 ml., acidified with HNO_3 to pH 6 and 1 ml. of 5% (w/v) AgNO_3 added—shown to be a slight excess by testing externally with $\text{Ba}(\text{OH})_2$ solution. The silver-purine compounds, together with any remaining AgCl , were removed on the centrifuge after standing in the cold. The supernatant was then made alkaline to pH 9 with cold $\text{Ba}(\text{OH})_2$, added drop by drop. A flocculent precipitate resulted, consisting of pyrimidine-silver salts together with some silver hydroxide, which, on standing, gave a brownish appearance to the precipitate. After chilling overnight, the mixture was centrifuged, the precipitate washed with a few drops of distilled water, heated to 80° with 5 ml. of N-HCl to decompose the Ag compounds, and, after cooling, the insoluble AgCl was removed by centrifuging. The solution containing the thymine was made up to 10 ml., solid Na_2CO_3 added until effervescence ceased, the volume adjusted to 25 ml., and 2 ml. portions were submitted to the modified Hunter test.

(b) Thymine in commercial nucleic acids

(i) *Thymus nucleic acid.* Samples of 20 and 50 mg. of the material were subjected to the procedure outlined above, and the results compared with the P content as determined by Holman's (1943) method. The latter gave a figure of 7.3% P as compared with the theoretical of 9.2% for the tetrasodium salt of deoxyribonucleic acid, indicating 80% purity.

Colorimetric estimation of thymine showed a content of 0.128 mg. in 20 mg. of nucleic acid corresponding to 70% of the theory, or 90% of the expected amount based on the P content.

(ii) *Yeast nucleic acid.* Repeated analyses of such material demonstrated that only a trace of thymine was present, the final colour being tinged only very slightly pink. The negative diphenylamine reaction (Dische, 1930) obtained with the sample supported the assumption that the deoxyribonucleic acid content was very small.

(c) Thymine in nucleic acid samples from fresh calf thymus

These were prepared by a modification of Hammarsten's (1924) method. In order to remove the cytoplasmic constituents the minced tissue was extracted three times with physiological saline which removes a high proportion of the cytoplasmic nucleoprotein without affecting the nuclear nucleoprotein (Mirsky & Pollister, 1946). The isolation of the nucleic acid was then completed as described by Hammarsten (1924). This method, which yields fibrous nucleic acid, was used since it does not require a high-speed centrifuge such as must be employed to clarify the highly viscous solutions of nucleoprotein obtained in Mirsky's method of extraction with M-NaCl solution.

The P content of the product was 7.2%, which agreed closely with the value of 7.09% obtained by Hammarsten (1924), but the colour obtained in the quantitative diphenylamine reaction (Dische, 1930; Siebert, 1940) was definitely greater than that given by commercial thymus nucleic acid. When solutions of the same P content were compared, a ratio of 1.13:1.00 was found. The thymine content was 7.05%. These figures suggest that the material was probably free from ribonucleic acid but still retained some impurity. In order to remove this a solution in 2N-NaOH was warmed on a water bath for 1 hr. at 70° and rendered faintly acid with acetic acid. The precipitate was dissolved in 0.1N-NaOH, reprecipitated from ethanol, washed several times with ethanol-ether and dried in a desiccator to constant weight.

Portions on analysis gave the following values: (i) P content 8.8% (cf. theoretical 9.18% and for commercial specimen 7.57%); (ii) Dische diphenylamine reaction colour relative to an equivalent solution of commercial nucleic acid was 1.25:1.00; (iii) N content 15.2% (theory 15.6%); (iv) thymine content 9.0% (theory 9.3%); (v) as a further check on the deoxyribose content compared with the commercial specimen, the cysteine reaction (Stumpf, 1947) was carried out, yielding a relative colour value of 1.20:1.00.

The material thus appears to have been 96% deoxyribonucleic acid, and the method for thymine estimation, which has been described, is satisfactory for applying to nucleic acid in quantities as small as 20 mg.

Further work is being done to compare the thymine content of deoxyribonucleic acid isolated from animal tumours with that derived from normal tissues.

SUMMARY

1. A colorimetric method is described for the quantitative estimation of thymine which is suitable for quantities between 50 and 200 $\mu\text{g.}$ in 2 ml. of solution.

2. The application of this method to the estimation of thymine in 50 mg. samples of deoxyribose-nucleic acid is reported.

This work was carried out under the auspices of the Birmingham Branch of the British Empire Cancer Campaign.

REFERENCES

- Barnes, F. W. & Schoenheimer, R. (1943). *J. biol. Chem.* **151**, 123.
Dische, Z. (1930). *Mikrochemie*; **8**, 4.
Hammarsten, E. (1924). *Biochem. Z.* **144**, 383.
Harkins, H. M. & Johnson, T. B. (1929). *J. Amer. chem. Soc.* **51**, 1237.
Holman, W. I. M. (1943). *Biochem. J.* **37**, 256.
Hunter, G. (1936). *Biochem. J.* **30**, 745.
Koessler, K. K. & Hanke, M. T. (1919). *J. biol. Chem.* **39**, 407.
Levene, P. A. (1922). *J. biol. Chem.* **53**, 441.
Mirsky, A. E. & Pollister, A. W. (1946). *J. gen. Physiol.* **30**, 117.
Siebert, F. B. (1940). *J. biol. Chem.* **133**, 593.
Stumpf, P. K. (1947). *J. biol. Chem.* **169**, 367.

Anaerobic Leakage of Potassium from Brain

By K. C. DIXON, *Department of Pathology, University of Cambridge*

(Received 21 May 1948)

Interruption of blood supply to tissues and organs is one of the main causes of organic disease. Characteristic histological changes follow such vascular occlusion, and it seems likely that these have their origin in chemical and metabolic disturbances produced by deprivation of oxygen and food substances from the tissue cells. The present communication describes a study of some of the chemical events taking place when the supply of oxygen and glucose to the cells of the brain is interrupted. These two deficiencies are probably the immediate determinants in the dislocation of cerebral function following vascular accidents, and thus may be regarded as the primary factors in ischaemic damage.

The intracellular concentration of potassium is many times greater than that outside the cells. Danowski (1941) showed that this intracellular potassium diffuses out of red blood cells when the blood sugar is used up, or when glycolysis is inhibited by fluoride. Harris (1941) reported similar results. Dickens & Greville (1933) found that brief anaerobic incubation of slices of cerebral cortex, without substrate, causes inhibition of glycolysis, and Macfarlane & Weil-Malherbe (1941) ascertained that pyrophosphate is destroyed at the same time. Colfer & Essex (1947) observed a loss of potassium from brain during convulsions. Potassium ions are known to cause intense aerobic stimulation and anaerobic inhibition of the glycolytic metabolism of cerebral cortex (Ashford & Dixon, 1935; Dickens & Greville, 1935). For these reasons the present work was designed to find out if leakage of potassium also

occurs from brain when deprived of oxygen and glucose. In this tissue a rise in the external concentration of potassium might produce profound metabolic changes in neighbouring areas outside the initial region of ischaemia as well as affecting radically the internal milieu of the cells primarily affected.

METHODS

Slices of rabbit cerebral cortex were suspended in bicarbonate-Ringer solution without glucose made up according to Dickens & Greville (1935) and previously saturated with N_2 containing 5% CO_2 . After washing in this solution, the slices were transferred to Warburg manometer cups containing 1.5 ml. of fluid. In general, half the slices were incubated in bicarbonate-Ringer without substrate, and the remaining slices in the same volume of the same salt solution containing in addition 0.2% glucose. In some experiments half the slices were incubated in this glucose-Ringer, and the remainder in the same glucose-Ringer containing 0.05% NaF. In a few experiments three samples of the slices were used, one without substrate, one with glucose and one with glucose and fluoride. The manometers were filled at room temperature with N_2 containing 5% CO_2 the gas being passed through for 3–3.5 min.

Potassium estimations were made as follows. Practically the whole of the 1.5 ml. of fluid remaining in the manometer cups after incubation at 38° was pipetted away from the slices into centrifuge tubes using Pasteur pipettes. This fluid was then centrifuged at high speed for 15 min. and 1 ml. of the clear supernatant fluid was pipetted off into Pyrex crucibles and evaporated to dryness in an oven at 100–120°. Conc. HNO_3 (0.5 ml.) was then added to each, and again the crucibles were heated to dryness. The crucibles were then incinerated for 3 hr. in an electric muffle furnace at 400–450°, to destroy ammonium salts.