an injection of paraldehyde. W.B.C. was 13,800 (P. 85%, L. 5%, M. 7%, E. 3%). C.S.F. cells, 1; total protein, 20 mg. per 100 ml.; globulin absent; sugar present; chloride 700 mg. per 100 ml. He was treated with sulphadiazine and alkaline potassium citrate mixture. The temperature subsided to normal on the third day of admission and he was discharged on May 11.

Case 2. Epistaxis as a Presenting Symptom.—A 5-year-old Chinese boy was admitted on May 15 with the complaint of fever for four days, bleeding from the nose the night before admission, and vomiting of dark-coloured material on the day of admission. On examination the temperature was 101° F. (38.3° C.). There was fresh bleeding from the nose. There were no petechiae or ecchymoses in the skin. Nothing abnormal was found in the heart, lungs, abdomen, or nervous system. In the ward he vomited coffee-ground material. The epistaxis was controlled by packing the nose with gauze soaked in adrenaline. W.B.C. was 3,600 (P. 67%, L. 27%, M. 6%, E. 0%). The platelet count was 90,000 per c.mm. and the next day it was 110,000 per c.mm. The temperature became normal and he was discharged on May 17.

Case 3.—A 3-year-old Chinese boy was admitted on May 20 at 1 a.m. He had fever and cough for one day, and on the day of admission he passed loose motions on four occasions. He had a fit during the night. On examination he was conscious. The temperature was 104° F. (40° C.). The skin was hot and dry; the throat was red and granular, and the tonsils were enlarged. The pulse was 140 a minute. There was nothing abnormal in the heart, lungs, or abdomen. There was no neck rigidity, and Kernig's sign was negative. W.B.C. was 8,900 (P. 83%, L. 12%, M. 4%, E. 1%). He died the same day at 9.15 a.m. *Necropsy Report* (Dr. C. S. Muir): The heart appeared normal, as were the pharynx and larynx. The trachea was oedematous and showed slight congestion. The right lung showed a violaceous mottling with a large collapsed area 3 by 9 by 3 cm. at the base of the lower lobe. The cut surface showed bronchopneumonic change. The left lung showed bronchopneumonic change. The large intestine showed a few small ulcerated areas about 0.5 cm. across. The liver showed fatty change. The renal tract appeared normal. The pancreas, adrenals, thyroid, and spleen appeared normal. The brain was congested. The cause of death was influenzal bronchopneumonia.

Case 4. Case with Peripheral Circulatory Collapse.—An 8-months-old male Chinese child was admitted on May 20. For the last three days he had diarrhoea, passing watery yellow stools 10 times a day, and for two days had slight fever and vomited his feeds. On examination he was sitting up in bed. His temperature was 101° F. (38.3° C.) and he was not dehydrated. The throat was red and the tonsils were enlarged. There was nothing abnormal in the heart, lungs, abdomen, and central nervous system. On the morning of May 21 the temperature was normal. He was taking his feeds well. The stools were still watery. At 2.10 p.m. he suddenly had peripheral circulatory collapse. He was comatose. There was peripheral cyanosis. The pulse was not palpable. Respiration was rapid and shallow. The axillary temperature was 99° F. (37.2° C.), but the rectal temperature was 107° F. (41.7° C.). In spite of active sponging he died at 2.30 p.m. *Necropsy Report* (Dr. T. Balasingham): The child was well nourished. The heart showed slightly dilated right chamber. The muscle and valves were normal. The mucosa of the larynx and trachea was injected, and the lumen contained excess mucus. The lungs were slightly voluminous and reddish in colour. Both lungs showed patches of bronchopneumonia, and these areas were friable. The mucosa of the bronchi was injected. The spleen was twice the normal size, its capsule being tense. The pulp was swollen, red, and firm. Lymph follicles were indistinct. The liver and kidneys showed cloudy swelling. The stomach and intestines were normal. The mesenteric glands were prominent. The brain was injected. The cause of death was bronchopneumonia, probably influenzal.

Summary

An account is given of the clinical impressions of the influenzal epidemic as seen in 250 cases admitted into the paediatric unit of the General Hospital, Singapore.

The common presenting symptoms were sudden onset of fever, cough, and fits. The 1–5-year age group appeared to be most affected.

The main complications were pulmonary and neurological.

The number of admissions and deaths increased considerably during the epidemic, and the causes of death as revealed by necropsy are given.

There was no specific treatment.

Four typical cases with different features are described.

We wish to acknowledge the guidance and encouragement of Dr. C. Elaine Field, paediatric specialist, General Hospital, Singapore, who provided the stimulus for this paper. We are also indebted to Dr. G. Smith and Dr. Quah Quee Guan for invaluable advice and criticism; to Dr. A. Heah for compiling the figures and drawing the graph for the admissions and mortality; to our colleagues in the paediatric unit for their co-operation and assistance; to Dr. Lim Kok Ann, of the bacteriology department, University of Malaya, for the virus studies; and to the Director of Medical Services, Singapore, for permission to publish.

RAPID COLORIMETRIC MICRO-METHOD FOR ESTIMATING GLUCOSE IN BLOOD AND C.S.F. USING GLUCOSE OXIDASE

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The availability of glucose oxidase (notatin), first described by Müller (1928) and studied in detail by Keilin and Hartree (1948a), an enzyme which specifically oxidizes β -glucose to gluconic acid with the formation of hydrogen peroxide, has resulted in the development of rapid qualitative tests for glucose in biological fluids and especially in urine (Adams, Burkhart, and Free, 1957; Luntz, 1957).

The use of this enzyme for the quantitative estimation of glucose has also been described: Keilin and Hartree (1948b) measured the oxygen uptake during the reaction, and Froesch and Renold (1956) the decrease in copper-reducing ability after complete destruction of the glucose by the enzyme. In both these methods the hydrogen peroxide formed was decomposed by catalase. However, while they are accurate these procedures are too time-consuming for routine clinical work.

Keston (1956) and Teller (1956) showed that when peroxidase is employed instead of catalase and a suitable chromogenic oxygen acceptor is added to the system, the colour produced is a measure of the amount of glucose originally present. The commercial urine-testing strips "clinistix" and "tes-tape" utilize this reaction, and Kohn (1957) has described the use of clinistix for the rapid but approximate determination of blood glucose.

The work here described was initiated with the object of devising a rapid and simple enzyme method which could be employed when a large number of blood

samples have to be analysed in a short time, as is necessary, for instance, in a diabetic clinic. We describe how, with a suitably buffered solution of glucose oxidase, peroxidase, and *o*-tolidine, glucose can be estimated in blood and C.S.F. with the accuracy of the best copper-reducing methods, more simply and quickly and without the use of heat.

Method

Reagents Used.—(1) Phosphate buffer, 0.5 M, pH 5: to 100 volumes of 0.5 M KH_2PO_4 (analytical reagent) are added 3.4 volumes of 0.5 M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, "Sørensen's salt." The pH should be checked. (2) Glucose oxidase: glucose oxidase powder (Sigma Chemical Co., potency 1,500 units per g.) and the liquid preparation "fermcozyme" (Fermco Chemicals Inc., potency 750 units per ml.) are equally suitable. (3) Peroxidase: "POD" (Boehringer and Soehne G.M.B.H.), a horse-radish peroxidase. This is expensive but economical in use. A stock solution containing 20 mg. per 100 ml. of the phosphate buffer may be kept in the refrigerator for at least two weeks. (4) *o*-Tolidine: a 1% solution of *o*-tolidine (A.R.) in ethanol. This should be kept in a brown bottle away from light. (5) NaCl, 0.9%. (6) $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 5%. (7) NaOH, 0.3 N.

(8) Buffered Enzyme-dye Solution.—(a) Using glucose oxidase powder: 250 mg. of the powder is dissolved in about 80 ml. of the phosphate buffer, 5 ml. of the stock peroxidase solution is added and the volume made up to 100 ml. with the buffer solution. This will keep in the refrigerator for at least two weeks. On the day of use 1 ml. of the *o*-tolidine solution is added to 100 ml. of this buffered enzyme solution with shaking and the mixture filtered; the filtrate should be protected from strong light, and if subsequently a fine precipitate forms this must be filtered off before use. The final concentration of peroxidase is 1 mg. per 100 ml. (b) Using fermcozyme: 0.5 ml. of this solution can replace the 250 mg. of glucose oxidase powder in the preparation described under (a), the amounts of peroxidase and *o*-tolidine remaining the same. Fermcozyme can be kept in the refrigerator for many months without loss of potency.

All commercial preparations of glucose oxidase contain catalase, which, if present in sufficient concentration, will prevent colour formation by competing with peroxidase for hydrogen peroxide. In the above solution, however, the peroxidase is present in excess and the colour production is adequate.

(9) Glucose Standards.—A permanent stock solution containing 100 mg. of pure glucose per 100 ml. is prepared in water saturated with benzoic acid. From this, standards containing 5, 10, 20, 30, and 40 mg. of glucose per 100 ml. are prepared by dilution with water saturated with benzoic acid; these are kept cold. The stock glucose solution should be prepared about 24 hours before use to allow the equilibrium mixture of α - and β -glucose to be attained. The solid sugar is usually predominantly the α form in commercial preparations, and, since glucose oxidase reacts only with β -glucose, a freshly prepared solution will not produce maximum colour if there should be insufficient glucomutarotase in the sample of glucose oxidase used.

Standardization of the Buffered Enzyme-dye Solution

The blue colour produced on the addition of glucose to the buffered enzyme-dye solution rapidly reaches a maximum and then fades at a slower rate. At the concentration of glucose oxidase recommended (375 units per 100 ml.) the addition of 0.2 ml. of glucose standards containing 5 to 40 mg. per 100 ml. to 4 ml. of the buffered enzyme-dye solution results in colour development which reaches a maximum at 10 to 12 minutes (Fig. 1). To counter possible variations in the potency of commercial enzyme preparations the following test should be made: to 4 ml. of the buffered enzyme-dye solution in a 6 by $\frac{3}{4}$ in. (15 by 2.2 cm.) test-tube is added 0.2 ml. of a 20 mg./100 ml. standard glucose solution. The colour is read at intervals of one minute on a photoelectric absorptiometer at 680 $m\mu$ and the

time taken to reach maximum absorption noted. If this is not 10 to 12 minutes the amount of glucose oxidase is either increased or decreased until the right amount is found. Under the foregoing conditions the logarithm of the time taken to reach maximum colour is approximately proportional to the logarithm of the glucose oxidase concentration. The amount of peroxidase recommended should be ample in all cases.

Procedure for Blood and C.S.F.

To 1 ml. of 0.9% NaCl is added 0.2 ml. of blood (capillary, oxalated, or heparinized venous blood; sodium fluoride is suitable in concentrations of not more than 10 mg. per 1 ml. of blood), 0.4 ml. of 5% ZnSO_4 is then added, followed by 0.4 ml. of 0.3 N NaOH, with shaking after each addition.

Deproteinization should be carried out promptly, but, if for any reason this cannot be done, when using freshly shed blood the 0.9% NaCl should contain 50 mg. of potassium oxalate per 100 ml. to prevent clotting. The mixture is either centrifuged or filtered through a 4.25-cm. Whatman No. 41 filter paper.

Then 4 ml. of the buffered enzyme-dye solution is pipetted into a 6 by $\frac{3}{4}$ in. test-tube and 0.2 ml. of clear blood filtrate is added and mixed by a gentle swirl. Suitable standards, 0.2 ml. of each, are likewise added to 4 ml. of the buffered enzyme-dye solution at intervals of one minute. Up to 10 analyses, including the standards, may be carried through in one batch. After standing at room temperature for 10 minutes the blue colour is read at 680 $m\mu$ against a blank of buffered enzyme-dye solution. For greatest accuracy both standards and filtrates should be read at 10 minutes precisely, as was done in these studies; little error is incurred, however, by reading over the period of 10 to 16 minutes. The unknowns are read from a curve constructed from the standard values, which should be determined daily and for each new batch of buffered enzyme-dye solution so as to eliminate minor errors due to temperature fluctuation and slightly varying enzyme concentration. The blue colour is linear for blood-glucose values up to at least 400 mg. per 100 ml. If the blood glucose is found to be higher than this, 0.1 ml. of blood filtrate should be added to 4.1 ml. of enzyme-dye solution for greatest accuracy.

In the C.S.F. procedure 0.4 ml. of C.S.F. is added to 0.8 ml. of 0.9% NaCl, the solution is deproteinized, and the colour produced by 0.2 ml. of the filtrate is measured as in the procedure for blood; the glucose concentration found, divided by two, gives the C.S.F. value.

Results

It is well known that many methods in common use for the determination of blood sugar are really a measure of the total copper-reducing substances, which, in addition to glucose, include fructose, amino-acids, glutathione, phos-

FIG. 1.—Graphs of colour development, using 4 ml. of buffered enzyme-dye solution and 0.2 ml. of standard glucose solutions: A, 5 mg.; B, 10 mg.; C, 20 mg.; D, 40 mg.; and E, 50 mg. per 100 ml. Spekker absorptiometer, 1 cm. cell, Kodak No. 570 (680 $m\mu$) filters.

phorylated hexoses, glyceraldehyde, etc. Harding and Downs (1933) devised a method which does not include these non-glucose reducing substances; this was modified by Asatoor and King (1954), and it is their method which was used in comparison with the enzyme method described in this paper. In many instances the sugar was also determined simultaneously by the method of MacLean. The results are shown in Fig. 2. There is statistically a high degree of

FIG. 2.—Blood glucose by enzyme method versus those of Asatoor and King (full line=observed regression ($y=6.86+0.980x$)) and of MacLean.

correlation ($r=+0.995$) between the enzyme method and that of Asatoor and King (based on 64 parallel estimations). MacLean's method, on the other hand, gave generally higher values. This proves not only the accuracy of the enzyme method but, since this is specific for glucose, the virtue of this particular copper method for the estimation of true glucose.

Discussion

A modified Somogyi method for deproteinization, whereby blood is delivered first into an isotonic solution, was adopted in this work so as to be comparable with the procedure of Asatoor and King (1954), in which the red cells are also not lysed. However, the specificity of the enzyme method is such that the values obtained are the same whether or not the blood is haemolysed before deproteinization. We favour the method described in this paper because it produces a less bulky precipitate and more filtrate is obtained. No difference was found when deproteinization was performed immediately, compared with a delay of five minutes to allow theoretically for complete diffusion of glucose from the intact red cells. If, however, the usual order of addition of $ZnSO_4$ and $NaOH$ to laked blood was reversed, values consistently 3 to 4% higher were obtained. This may have been due to the liberation of glucose from some complex combination under the action of alkali.

Huggett and Nixon (1957a, 1957b) have published a method also based on that of Keston (1956) and Teller (1956), using glucose oxidase powder and peroxidase. They used *o*-dianisidine as the chromogenic oxygen acceptor and carried out the reaction at pH 7 at 35° C. Their method has the advantage that the orange-brown colour developed during the reaction is stable for some hours; on the other hand, a thermostatically controlled water-bath is necessary and full colour is not attained until after the lapse of 60 minutes. It is not therefore so well adapted to the purpose we had in mind as the method described above.

It would appear that the enzyme method gives true values for the glucose content of the fluids analysed. In the case of blood and C.S.F. these values are lower than are given by such methods as those of MacLean, Shaffer-Hartman, and

Folin and Wu, and the normal fasting blood glucose will be found to be from 50 to 90 mg. per 100 ml. The maximum value after an oral dose of 50 g. of glucose in the normal person is approximately 160 mg. per 100 ml.

The accuracy of the enzyme method is of such a high order that it will be found invaluable not only in clinics but in laboratories, where it can replace the usual copper-reducing methods with great economy in time and materials.

Summary

A rapid and accurate colorimetric micro-method for the estimation of glucose in blood and C.S.F. is described in which no heat is required. The procedure utilizes a suitably buffered solution of glucose oxidase, peroxidase, and *o*-toluidine of pH 5.

In the case of blood it has been shown that there is a close correlation between the values given by this method and that of Asatoor and King. Since the enzyme is specific for glucose the values obtained are lower than those given by copper-reducing methods such as that of MacLean, which include reducing substances other than glucose; this also applies to C.S.F.

The normal fasting blood glucose is found to be from 50 to 90 mg. per 100 ml., which after oral glucose rises to a maximum of approximately 160 mg. per 100 ml. in normal subjects.

We wish to thank Mr. L. J. Taylor, of Hughes and Hughes Ltd., for his helpful co-operation and for samples of fermcozyme.

The British agents for the enzyme preparations are: fermcozyme solution, Hughes and Hughes Ltd., 35, Crutched Friars, London, E.C.3; glucose oxidase powder, G. T. Gurr Ltd., 136-8, New King's Road, London, S.W.6; peroxidase powder, Courtin and Warner Ltd., Ballards Old Brewery, Lewes, Sussex.

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Dr. C. A. BOUCHER, speaking at the annual meeting of the Distressed Gentlefolk's Aid Association on October 24, pointed out that old age did not begin suddenly at a certain time. Gradual deterioration in hearing and vision might commence in middle age. He said that the rather prevalent attitude of regarding old people as different beings needing special care and attention was not necessarily the right one. Old people, with a lifetime's experience behind them, were used to helping others, and therefore needed help and companionship rather than "sugary sympathy." The Distressed Gentlefolk's Aid Association clearly recognized the needs of the infirm in its homes, where it provided well-qualified staff and gave security to its residents. The building of a new home in Vicarage Gate, London, for 42 nursing cases and seven ambulant cases was to be welcomed. Contributing also to a solution of this problem is the Friends of the Poor and Gentlefolk's Help Society, which has ten homes for old people. An anonymous gift of £40,000 was announced at a recent meeting of this society, at which PRINCESS MARGARET presided. With this gift another residential home had been bought in Hampshire, providing accommodation for 25 to 30 people. Princess Margaret said that the generosity of the trustees of the London Parochial Charities had enabled them to add a new wing to the home in Hampstead. The society was now spending more than £100,000 a year, of which sum it had to find more than half: over the past ten years there had been an average deficit of £10,000 a year.