day of the week, presumably due to the influence of a weekly routine common to all subjects. It is probable that this weekly periodicity is a factor to be generally considered in all experiments on creatinine excretion in humans, and some such treatment of observations as that described must be used to eliminate this periodicity and also to reduce the effect of short-term fluctuations if particular deviations from the mean excretion level are being sought.

From excretion curves based on estimations made at intervals of 2–3 days, Smith (1942) has reported a regular premenstrual rise in excretion followed by a fall during the menstrual flow. Such curves smooth out the day to day fluctuations without taking into account their origin or their possible composition as a summation of several separately operating fluctuations, and thus give a false impression of the nature of creatinine excretion. To illustrate this, the data for the six subjects in this study have been interpreted in a similar manner, the representative results for subject C being given. In Fig. 2*b* are shown the three corresponding curves of values for every third day only, constructed from the complete excretion curve shown in Fig. 2*a*, and which resemble closely the curves obtained by Smith. It is obvious that not only are these 'periodic' curves unsatisfactory approximations of the complete excretion curve but that they bear no consistent relation to one another, and deductions drawn from them would have little significance. A connexion between menstruation and creatinine elimination has not been conclusively proved, and the data available indicate that if it is existent, it is by no means a clear-cut relationship as was proposed. The further long-term investigation of many subjects is clearly necessary, including examination of the excretion characteristics of males, before this relationship can be established with certainty. It is thought that treatment of results along the lines presented in this paper may be of value in clarifying this problem.

SUMMARY

1. Studies of the day to day creatine and creatinine excretion of a number of men and women, aged 19–26 years, revealed the absence of creatinuria in the males and its presence, in amounts up to 150 mg./day, in a proportion of the females.

2. No evidence has been obtained to relate creatinuria, whenever it appeared, to any phase of the menstrual cycle.

3. A weekly periodicity in creatinine excretion was observed, but there was no conclusive evidence to link the level of urinary creatinine with menstruation.

4. The nature of the daily fluctuations in creatinine output is discussed.

The author is indebted to Dr R. B. Fisher for his invaluable help in the analysis of the data obtained and to the University of London for a grant from the Dixon fund.

REFERENCES

Cameron, J. D. (1933). *Quart. J. exp. Physiol.* **23**, 351.
Dill, D. B. & Horvath, S. M. (1941). *Amer. J. Physiol.* **133**, 520.
Espersen, T. & Thomsen, A. (1937). *Acta med. Scand.* **92**, 39.
Folin, O. (1914). *J. biol. Chem.* **17**, 469.
Hodgson, P. & Lewis, H. B. (1928). *Amer. J. Physiol.* **87**, 288.
Hyde, E. C. (1942). *J. biol. Chem.* **143**, 301.
Krause, R. A. & Cramer, W. J. (1910). *J. Physiol.* **40**, 61*P*.
Krause, R. A. & Cramer, W. J. (1911). *J. Physiol.* **42**, 35*P*.
Rose, W. C. (1917). *J. biol. Chem.* **32**, 1.
Smith, O. W. (1942). *J. clin. Endocrinol.* **2**, 1.
Stearns, G. & Lewis, H. B. (1921). *Amer. J. Physiol.* **56**, 60.
Wilkins, L., Fleischmann, W. & Block, W. (1941). *J. clin. Endocrinol.* **1**, 3.

Oxidation of Glucose, Glycerol and Acetate by *Staphylococcus aureus**

By DOROTHY M. POWELSON, P. W. WILSON and R. H. BURRIS
College of Agriculture, University of Wisconsin, Madison, Wisconsin, U.S.A.

(*Received* 2 *January*, 1947)

Because of the importance of *Staphylococcus aureus* as a test organism in assay of antibiotics, detailed information about its metabolism is desirable. While comparing the oxidation of glycerol and glucose in microrespiration experiments, we observed that resting suspensions rarely consumed enough oxygen for complete oxidation of these two substrates. Krebs (1937) also reported incomplete oxidation of glucose, glycerol, lactate and pyruvate by 18–24 hr. old cells of *Staph. aureus*. Usually about 2·5 mol. of oxygen/mol. of glucose is rapidly consumed followed by a slow uptake. The observed uptake of oxygen and respiratory quotients in our experiments could be accounted for if acetic acid is assumed to be an

* Supported in part by grants from the Wisconsin Alumni Research Foundation.

intermediate in the oxidation. This report summarizes results from quantitative respiration experiments that were undertaken to test this explanation and to determine if complete oxidation-reduction and carbon balances of the aerobic metabolism of *Staph. aureus* could be made.

METHODS

Preparation of cells. 100 ml. of the standard medium (10 g. Bacto-tryptone, 10 g. Bacto yeast-extract/l. distilled water, pH 7·2–7·4) in a 500 ml. Erlenmeyer flask was inoculated with 0·1 ml. of a 24 hr. broth culture of *Staph. aureus* NRRL-B 313. The cultures were shaken mechanically at 30° until the desired density of cells was reached. After 6 hr. the cells were in the early phase of logarithmic growth and at 10 hr. in the middle of the logarithmic phase (see Fig. 3 for standard growth curve). When harvested, the cultures were cooled in an ice bath, then spun down in a Sharples or an angle-head centrifuge. The cells were washed twice with cold distilled water and resuspended in water to a density ($2 - \log G$) of 0·96 (G is galvanometer reading) using a 720 mμ. filter and an 18 mm. tube in an Evelyn colorimeter. This optical density represents 3 mg. dry weight/ml. for cells in the period of logarithmic increase. The cell suspensions were used immediately. All distilled water was CO_2-free.

Oxygen and carbon dioxide. Warburg's method was used to estimate oxygen consumed and carbon dioxide liberated. To a 125 ml. Warburg flask were added 12·5 ml. of 0·1 M-phosphate buffer pH 7·0, 4 ml. distilled water, and 8 ml. cell suspension: 0·5 ml. of substrate solution was contained in a Keilin tube hooked by a platinum wire to the centre well. For determining O_2 uptake the well held 0·7 ml. of 10% KOH; for CO_2 liberated, KOH was omitted and 1·0 ml. of approximately 6 N-H_2SO_4 was placed in a side-arm.

Carbon. Total carbon at the start and end of the oxidation period was determined on the contents of the flasks used for estimation of CO_2. Immediately following the final manometric readings the flasks were removed to an ice bath. Analyses were made by a modification of the wet carbon method of Friedemann & Kendall (1929) on (*a*) samples of the flask contents, (*b*) cells centrifuged from the suspensions, and (*c*) the substrate added. The contents of the flasks that measured O_2 uptake were used to determine the remaining substrate, the acetate formed, and the carbon in the cells after respiration. Each flask was cooled at the end of a run, and the contents were transferred quantitatively to cold test tubes and centrifuged. The cells were removed and washed once with cold distilled water; the washing was added to the first supernatant fluid. Either a few drops of dilute acid were added to the cells to prevent slow respiration during storage in the refrigerator or the cells were kept frozen in the freezing compartment until analyses for carbon were made. The supernatant fluid was analyzed within 6–12 hr. Initial figures were obtained from analyses of controls kept in 125 ml. Erlenmeyer flasks that were treated in the same manner as the experimental material until the time of substrate addition. The controls were then cooled, the cells were removed, substrate was added to the supernatant fluid, and analyses were made.

Acetate. After removal of the cells, the supernatant fluid was adjusted with dilute NaOH and phenol red to pH 7·2 and concentrated *in vacuo* to about 10 ml. The concentrate was acidified to pH 2·5 with dilute H_2SO_4 and distilled under a residual pressure of 30–40 mm. Hg; the distilling flask was immersed in a water bath at 75–80°. Distilled water (50 ml.) was added from a delivery tube during distillation so that the volume of liquid in the distilling flask remained constant. The distillate was caught in 35 ml. dilute NaOH (190 μmol.); excess NaOH was titrated with standard 0·01 N-HCl. A blank with 10 ml. distilled water was run in the same manner. When standardized with known quantities of acetic acid, recovery with 50 μmol. of acetic acid ranged from 85 to 92%, but with 10 μmol. the variation was greater; 85% recovery was arbitrarily taken as the standard for the apparatus. The volatile acid in the titrated distillate was recovered by vacuum distillation into cold distilled water, made to 100 ml., and its identity checked by the Duclaux method. The distillate and the control acetic acid fractions were titrated with standard 0·01 N-NaOH.

Glycerol and glucose. Glycerol or glucose was determined on the residue in the distilling flask. Samples were analyzed for glycerol by the periodate method of Voris, Ellis & Maynard (1940). Using 0·002 M-KIO_4, 3% KI, and 0·005 N-$Na_2S_2O_3$, we could accurately determine 0·1–2·0 mg. of glycerol. In most trials glucose in the residue after distillation was determined by the copper-iodometric method of Somogyi (1937). In later experiments glucose was estimated by Folin & Malmros's (1929) micromethod, in a sample of the supernatant fluid before distillation.

RESULTS

In the first three experiments the oxidation of glycerol and glucose by *Staph. aureus* NRRL-B 313 was studied. The results summarized in Figs. 1 and 2 and Tables 1 and 2 show that cells in the logarithmic period of growth (about 10 hr.) oxidized both glycerol and glucose to CO_2 and H_2O, but younger cells accumulated acetate. This finding suggested a test of the ability of cells of various ages to oxidize glycerol, glucose and acetate. As is evident in Fig. 3 and Exp. IV (Table 1), young cells of this strain hardly attack acetate, but later oxidize it (Fig. 4) at a rate comparable to those of glycerol and glucose. This physiological difference is accompanied by few other variations. The younger cells are about 1·5 times larger (in diameter) than the 10 hr. organisms, but both are encapsulated, Gram-positive and produce similar colonies.

It was thought that the physiological differences in the cells might be connected with a change in the medium used for their culture. Its initial pH of about 7·4 always decreased for 8–9 hr., then rose steadily to a final pH of 8·6–8·8, first reached at 24 hr. A change in colour of the cells from white to orange-yellow during this period did not appear to be an effect of the pH since cells grown for 12 hr. in a medium containing glycerol or glucose whose final pH was below 6·0 also showed this change in colour. The initial decrease in pH resulted from formation of a volatile acid by the growing cells; a Duclaux distillation identified the acid as acetic. Acetic acid

Table 1. *Respiratory balances for oxidations by* Staph. aureus *NRRL-B* 313

Experiment ...	I		II	III		IV		
	a	*b*		*a*	*b*	*a*	*b*	*c*
Substrate ...	Glycerol	Glucose	Glycerol	Glucose	Glucose	Acetate	Acetate	Acetate
μmol. substrate at start	93	50	88	51	49	102	48	98
μmol. substrate at end	17	1	7	2	1	97	5	64
μmol. O_2 respired	158	117	261	236	217	14	106	61
μmol. CO_2 formed	129	136	222	228	218	14	120	60
μmol. acetate formed	38	51	0	0	2	—	—	—
R.Q.	0·82	1·16	0·82	0·97	1·00	1·00	1·13	0·98
O/R*	1·06	1·16	1·00	0·97	1·00	1·00	1·13	0·98
Age of cells (hr.)	6	6	10	10	10	6	10	24
Growth density	0·126	0·126	0·611	0·620	0·620	0·111	0·638	0·959

* *Ratio* (*O*/*R*). The balance of oxidation and reduction during metabolism calculated as described by Johnson, Peterson & Fred (1931).

Table 2. *Carbon balances for oxidation of glycerol and glucose by* Staph. aureus *NRRL-B* 313

				Distribution of carbon					
		Total C in medium						Total	
Substrate	Time (min.)	(mg.)	Recovery (%)	Cells (mg.)	Substrate (mg.)	Acetate (mg.)	CO_2 (mg.)	(mg.)	Recovery (%)
				Experiment I. 6 hr. cells					
Endogenous	0	9·68	102*	9·01	—	0·07	0·01	9·09 94†	99‡
	180	9·85		8·73	—	0·12	0·17	9·02 92	
Glycerol	0	16·29	98	10·05	3·81	0·16	0·01	14·06 86	94
	290	14·37		9·90	0·60	1·08	1·55	13·13 83	
Glucose	0	15·54	102	10·44	3·66	0·18	0·01	14·29 92	93
	215	14·23		10·20	0·08	1·39	1·64	13·31 84	
				Experiment II. 10 hr. cells					
Endogenous	0	10·48	102	9·53	—	0·09	0·02	9·64 92	99
	380	10·10		8·91	—	0·08	0·57	9·56 89	
Glycerol	0	15·18	102	10·21	3·44	0·09	0·02	13·76 91	92
	380	13·43		10·00	0·34	0·09	2·10	12·53 81	
				Experiment III. 10 hr. cells					
Endogenous	0	11·40	99	9·78	—	0·00	0·02	9·80 86	99
	440	10·60		9·01	—	0·00	0·71	9·72 86	
Glucose	0	14·15	103	9·78	3·56	0·00	0·03	13·37 95	93
	440	11·82		9·52	0·16	0·00	2·73	12·41 85	
Glucose	0	15·15	99	9·54	3·66	0·01	0·02	13·23 88	93
	305	12·59		9·42	0·14	0·08	2·62	12·26 81	

* Recoveries in this column based on total carbon in medium + CO_2 at beginning and end. Total carbon in medium estimated by wet carbon method and would include cells, substrate, fermentation products other than CO_2, soluble products as lysed cells or polysaccharide.

† Figures in this column are (total C in analyzed products/total carbon in medium + CO_2) × 100.

‡ Recoveries in this column based on carbon found in products analyzed for at beginning and end.

equal to the quantity of volatile acid formed during the initial period decreased the pH of the sterile medium from 7·45 to 7·0.

Experiments were made to determine the effect of a higher initial hydrogen-ion concentration and the presence of acetate in the medium used for growth. The results (Exp. V, Table 3) show that production of the enzyme systems for oxidation of acetate was stimulated neither by a decrease in pH nor presence of acetate. Attempts to adapt 'resting' cells by allowing 6 hr. organisms to respire in the presence of acetate for 6–7 hr. were unsuccessful. As a possible source of a necessary coenzyme, boiled cells from a suspension which rapidly oxidized acetate were added but had no effect. Neither did growing the cells in a richer medium such as 10 % liver extract-2 % Bacto-tryptose broth.

The data of Exp. VI (Table 3) suggest that some change occurring in the medium during the growth of the organism is responsible for initiation of oxidation of acetate. Cells were grown in the standard medium until the growth density reached 0·620; such

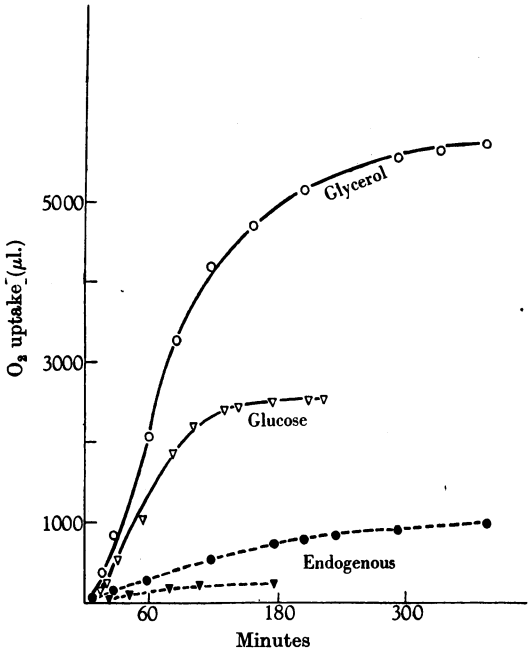

Fig. 1. Oxidation of glycerol and glucose by 6 hr. resting suspensions of *Staph. aureus* (Exp. I).

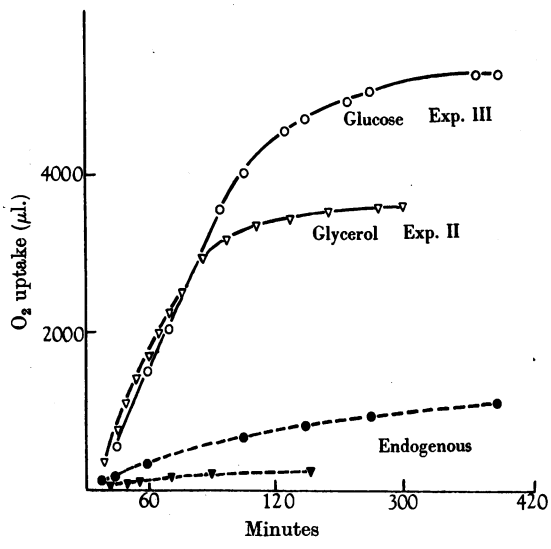

Fig. 2. Oxidation of glycerol (Exp. II) and glucose (Exp. III) by 10 hr. resting cells of *Staph. aureus*.

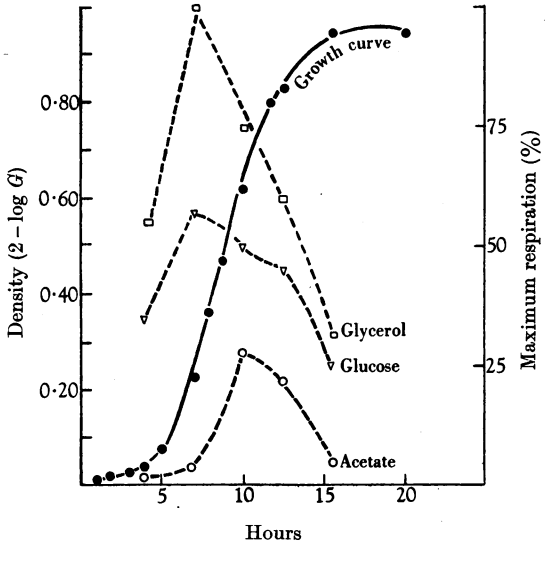

Fig. 3. Oxidation of glycerol, glucose and acetate by resting suspensions of *Staph. aureus* harvested at different phases of growth. ●——● growth curve.

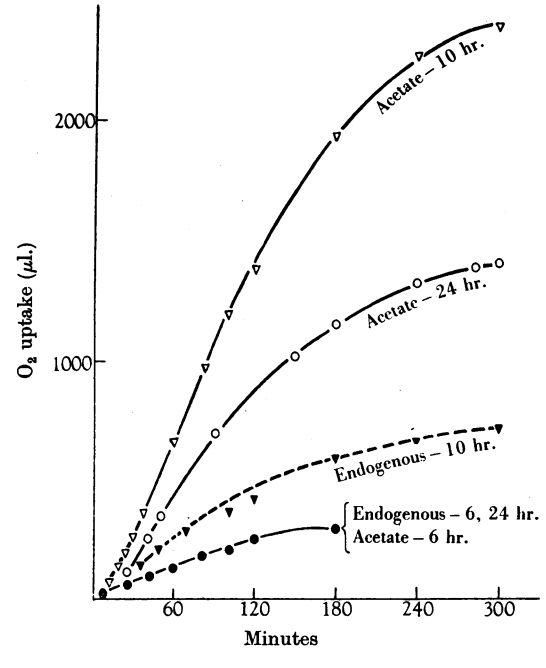

Fig. 4. Oxidation of 0·5 ml. 0·2 M-sodium acetate in 25 ml. total volume by 6, 10 and 24 hr. cells of *Staph. aureus*. Growth densities: 6 hr., 0·1024–0·1107; 10 hr., 0·629–0·643; 24 hr., 0·959.

Table 3. *Effect of changing growth medium on oxidation of acetate by young cells of* Staph. aureus *NRRL-B* 313

Medium	Incubation period (hr.)	Growth density	Q_{O_2} Endogenous	Q_{O_2} Acetate
		Experiment V		
1	6·5	0·1635	9·6	9·9
2	6·5	0·1177	6·4	5·7
3	6	0·1265	5·7	5·9
4	7	0·1357	5·6	6·5
		Experiment VI		
5	5·5	0·0605	6·7	6·8
6	9	0·0969	9·7	15·0
7	8·5	0·1369	6·5	16·8

Media used: (1) Standard, initial pH, 7·44, final, 6·90.
(2) Standard, initial pH, 7·08, final, 6·73.
(3) Standard plus 0·5% glycerol.
(4) Standard plus 0·5% acetate.
(5) Standard.
(6) Supernatant of 10 hr. culture, autoclaved, adjusted to pH 7·2.
(7) Supernatnat of 10 hr. culture, aseptically centrifuged. The cells from the 10 hr. culture (growth density 0·602) had an endogenous Q_{O_2} of 11·3; with acetate, 39·6.

cells are capable of oxidizing acetate. These were removed by centrifugation, and the supernatant fluid resterilized and inoculated as usual. The growth was slower than in the fresh medium; it required 9 hr. to reach a growth density (*c.* 0·10) that corresponds to a physiological age of about 6 hr. for cells seeded into fresh medium. If, instead of sterilizing the supernatant, the centrifugation was performed aseptically and the residual cells allowed to serve as inoculum, a growth density of about 0·10 was reached in 8·5 hr. Such physiologically young cells from either treatment, in contrast to those grown in the standard medium, did oxidize acetate. This peculiar behaviour toward acetate is not possessed by all strains of *Staph. aureus*; tests of three others, one recently isolated from a case of osteomyelitis, revealed that acetate was oxidized by neither very young nor older cells.

DISCUSSION

Consideration of the quantitative aspects of the data in Table 2 leads to the conclusion that the major products of oxidation of glycerol and glucose by *Staph. aureus* NRRL-B 313 are simply CO_2 and H_2O. It also appears probable that at least part of the oxidation goes by way of acetate. If other products are formed, they do not accumulate enough to be regarded as a significant part of the oxidative metabolism. If based on total carbon in the medium (plus CO_2 evolved) before and after incubation, recoveries of 98–103% are obtained, usually slightly more than 100%. If, however, the recovery is based on carbon in identified constituents of the medium—cells, glucose, glycerol, acetate and CO_2—it averages about 93% in the presence of substrate and 99% in its absence. As is evident by a comparison of the figures in the third and last columns of Table 2, this disagreement apparently originates from an incomplete analysis for the constituents in the medium. Even before respiration begins (0 min.), the sum of the carbon in the individual constituents for which analyses were made accounts for only 90–95% of the total carbon found in the medium, that is, in the suspension of cells plus substrate. After the oxidation, the discrepancy is about the same in the absence of substrate but increases in its presence.

Since incomplete recovery occurs even in the absence of substrate or oxidation, and since most of the carbon present is in the cells, it appears likely that the part unaccounted for arises from autolyzed cells and those not removed by centrifugation or from soluble cellular polysaccharide. Support for this view was secured in two experiments in which about 1·5 mg. of carbon was found in the supernatant fluid from 8 ml. of the cell suspension. This strain of *Staph. aureus*, as did many others tested, produced considerable quantities of gum in early growth, and capsules were easily demonstrated by the usual methods; chilled, concentrated suspensions formed a slimy mass.

Attempts to demonstrate polysaccharide formation sufficient to account for the missing carbon met, however, with only limited success. The supernatant fluid from cells after endogenous respiration and after oxidation of glucose was hydrolyzed with N-H_2SO_4 for 30 min. at 121°; only 10% of the missing carbon appeared as glucose after the hydrolysis. This result might not be conclusive, however, since Fellows & Routh (1944) found that young cultures of two pathogenic strains of *Staph. aureus* produce antigenic polysaccharides that are difficult

to hydrolyze. Our attempts to block synthesis of polysaccharide with dinitrophenol were unsuccessful.

Kendall, Friedemann & Ishikawa (1930) have also reported incomplete recovery of carbon from alanine, glucose and pyruvate when resting cells of *Staph. aureus* supplied with these substrates were aerated with CO_2-free air in tall cylinders. They report as both aerobic and anaerobic end products: lactate, volatile acid, CO_2 and small quantities of pyruvate. Test for lactate by the method of Barker & Summerson (1941) revealed less than 0·4 μmol. after oxidation of 50 μmol. of glucose by 10 hr. cells of *Staph. aureus* NRRL-B313.

SUMMARY

1. In microrespiration experiments resting cells of *Staphylococcus aureus* from the logarithmic period of growth (10 hr. cells) oxidized glycerol and glucose almost completely to CO_2 and H_2O. Younger organisms (6 hr. cells) oxidized these substrates to acetate, CO_2 and H_2O.

2. In these products 10% of the glycerol and 15% of the glucose did not appear. Lactate was not formed in appreciable quantity, but synthesis of polysaccharide might account for the missing source of carbon. Encapsulation of young cells favours this explanation, but direct quantitative evidence in its support was not obtained.

3. Study of this strain of *Staph. aureus* at different stages of growth established that only after the organism was well in the logarithmic phase did resting suspensions oxidize acetate. The effect was not one of simple adaptation but was associated with some as yet unknown change in the growth medium.

REFERENCES

Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
Fellows, O. N. & Routh, J. I. (1944). *J. Lab. clin. Med.* **29**, 1054.
Folin, O. & Malmros, H. (1929). *J. biol. Chem.* **83**, 115.
Friedemann, T. E. & Kendall, A. I. (1929). *J. biol. Chem.* **82**, 45.
Johnson, M. J., Peterson, W. H. & Fred, E. B. (1931). *J. biol. Chem.* **91**, 569.
Kendall, A. I., Friedemann, T. E. & Ishikawa, M. (1930). *J. infect. Dis.* **47**, 223.
Krebs, H. A. (1937). *Biochem. J.* **31**, 661.
Somogyi, M. J. (1937). *J. biol. Chem.* **117**, 771.
Voris, L., Ellis, G. & Maynard, L. A. (1940). *J. biol. Chem.* **133**, 491.

Stability of Haemoglobin and of Certain Endoerythrocytic Enzymes *in vitro*

By D. KEILIN and Y. L. WANG, *Molteno Institute, University of Cambridge*

(*Received* 17 *January* 1947)

In the present paper we propose to deal with those aspects of the stability of haemoglobin and of certain enzymes which can be expressed in terms of time during which these proteins may retain their specific biological activities and therefore their structural integrity. The study of this aspect of protein stability, which can be described as the life span of a protein, can hardly be based upon experiments solely designed for this purpose since it must depend to a very great extent upon material collected a considerable time ago and for different purposes.

MATERIAL

The material used in the present investigation was composed of samples of whole blood obtained from different sources:

(A) Samples of citrated horse blood collected by Dr E. G. D. Murray in 1922.

(B) Samples of horse blood received by the late Prof. G. H. F. Nuttall in 1904.

(C) Samples of guinea-pig blood collected by Nuttall in 1902.

(D) Samples of blood of other animals (jackal, ant bear, sea lion and Himalayan bear) received by Nuttall in 1901–3.

These samples of blood were investigated in respect of the properties of (1) haemoglobin, (2) carbonic anhydrase, (3) catalase, (4) glyoxalase and (5) choline esterase. Not every specimen was examined under all these headings, but in every case (except blood samples D) when the properties of haemoglobin or the activities of an enzyme were determined they were invariably compared with those of a corresponding sample of fresh blood.