nant, untreated women and seven bloods from normal males. This seemed a sufficient number of control subjects since the normal range in cholesterol levels is firmly established and we sought only to check our own methods.

The method used in testing for serum cholesterol blood levels was that described by SCHOENHEIMER and SPERRY[15]. The proteins are precipitated from the serum by an acetone-alcohol mixture and simultaneously the cholesterol and cholesterol esters are extracted. Digitonin is used to precipitate the cholesterol after saponification and this is tested as to color development with acetic anhydride-sulfuric acid reagent, in comparison with color produced in a standard cholesterol solution. Color readings were made with a Bausch and Lomb spectrophotometer. HAWK, OSER, and SUMMERSON[16] give a range of 150 to 300 mg cholesterol in 100 ml blood as normal.

As will be seen from Table I, only five tests made on bloods from treated, pregnant women exceeded the normal range. This is of interest since DEUEL[14] states that cholesterol levels are normally higher in pregnancy. Four of these tests are on bloods from the same woman, drawn at different times. In Table II, which deals with treated non-pregnant women, there is one specimen of blood in which the normal cholesterol range is exceeded. Among the pregnant, non-treated group (Table III) there are two bloods which exceed normal levels. One of these findings, at a level of 873 mg cholesterol, was confirmed in another laboratory. None of the normal bloods exceed the range for cholesterol.

Apparently the oral administration of Rh hapten does not affect serum cholesterol levels. Instead, there was a certain amount of fluctuation from one test to another on the same individual, nearly all of these within the normal range.

In summary, 61 bloods from Rh sensitized women treated with Rh hapten given orally were tested for serum cholesterol levels, using 17 control bloods from pregnant, non-treated women, non-pregnant women and normal males. Differences in cholesterol levels between the treated and control groups were not significant.

A. F. LEWIS and BETTINA B. CARTER

Charles C. Adams Ecological Center, Western Michigan University, Kalamazoo (Michigan), February 4, 1958.

Zusammenfassung

Cholesterin lässt sich als Bestandteil des ungereinigten Rh-Haptens nachweisen. Deshalb wurde geprüft, ob die perorale Aufnahme dieser Fraktion den Blutcholesterinspiegel beeinflusst. Bei 17 Menschen, die kein Hapten erhielten, und bei 61 Hapten-Empfangenden wurde keine Erhöhung des Cholesterinspiegels festgestellt.

[15] R. SCHOENHEIMER and W. M. SPERRY, J. biol. Chem. *106*, 745 (1934).

[16] P. B. HAWK, B. L. OSER, and W. H. SUMMERSON, *Practical Physiological Chemistry* (Blakiston, New York 1954).

Influence of Blood Sugar Level on the Glycolytic Activity of Human Red Cells

Considerable evidence has been collected in the last few years on the importance of glucose as the main source of energy for the red cells[1]. Mature erythrocytes utilize glucose mainly by means of glycolysis, i.e. by anaerobic fermentation to pyruvic and lactic acids, only a very small fraction being oxydized aerobically. The hexose-monophosphate shunt has been recently demonstrated to occur also in the red cells and appears to be an important metabolic pathway[2]. Abnormalities of the glycolysis and related enzymes have been described in the *in vitro* storage as well as in a number of hemolytic conditions and are likely to occur also during the *in vivo* ageing.

But the ultimate significance of glycolysis deserves further study. Among the many problems which have still to be answered, an important point is the following: do red cells utilize a fixed amount of glucose for their metabolic requirements regardless of external factors? Or do they have to be considered as simple enzyme parcels passively reacting in a peripheral medium and metabolizing any suitable substrate? Or might their metabolic activity be influenced by the environmental conditions and adjust itself to plasma composition?

The statement generally reported, that the glycolysis rate does not depend upon the initial glucose level within normal ranges, would support the first possibility aforementioned. In the present paper some contradictory results are reported.

Materials and methods.—The investigations have been carried out in normal individuals ranging from 22 to 35 years of age. Glycolysis has been determined according to HOLLINGSWORTH's technique[3]. Blood sugar was measured in duplicate before incubation, and after 1 and 2 h, by means of the SOMOGY-NELSON's method. The standard error for the determination of glucose in duplicate was $\pm$ 2·7 mg%.

Results.—The samples were withdrawn 1–4 h after lunch. In the reconstituted samples, the red cell count ranged from 3 360 000 to 5 200 000 and the hemoglobin from 9·9 to 14·9 g%. The white cells were found between 0 and 800 per mm^3. The RBC: WC ratio ranged from 4000 to 50 000. No changes of the hematocrit were observed at the end of the experiments.

The mean initial glucose level was 76·2 mg% with a standard deviation of $\pm$ 17·9. It dropped to 40·2 mg% $\pm$ 11·4 after 2 h. Glycolysis, expressed as mg of glucose utilized by 100 ml of packed red cells per hour, has been found to be 43·9 mg with a standard deviation of $\pm$ 13·6. The percentage in respect to the initial glucose level was 23·7 $\pm$ 4·6.

By plotting, on a normal graphic, the glycolysis values against the initial glucose level, a fairly good correlation was observed and confirmed statistically. In our 40 normal controls, the correlation coefficient (r) was found to be + 0·742 and the calculated 't' value (6·822) was highly significative ($P < 0{\cdot}001$; Figure). The regression line is given by the formula: $y = 7{\cdot}4 + 0{\cdot}48\,x$, where y is the expected glycolysis and x the initial glucose level. The confidence limits ($2\,\sigma$) are $\pm$ 15.

According to these data, glycolysis could be tentatively interpreted as a first order enzymatic reaction. Since, in this case, the amount of substrate (S) present at the time t is given by the formula: $S_t = S_0\, e^{-kt}$, k being the velocity constant, the reaction itself could be better expressed

[1] O. F. DENSTEDT in J. L. TULLIS, *Blood cells and plasma proteins* (Academic Press Inc., New York 1953), p. 223. – S. GRANICK, Blood *4*, 404 (1949). – T. A. J. PRANKERD, Brit. J. Haematol. *1*, 131 (1955).

[2] O. F. DENSTEDT, in J. L. TULLIS, *Blood cells and plasma proteins* (Academic Press Inc., New York 1953), p. 223.

[3] J. W. HOLLINGSWORTH, J. Lab. clin. Med. *45*, 920 (1955).

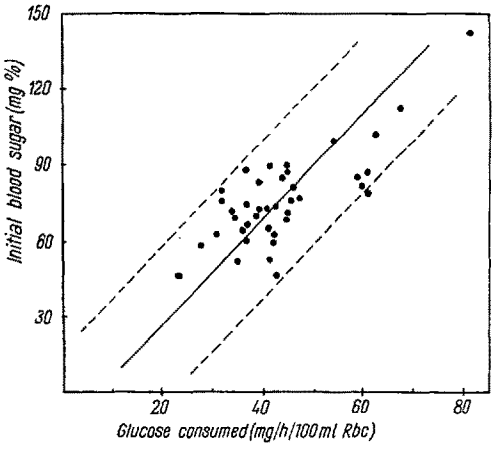
Modifications of glycolysis during glucose tolerance test

Case	Time 0		After 30 min		After 60 min		After 120 min	
	Initial glucose	Glycolysis	Initial glucose	Glycolysis	Initial glucose	Glycolysis	Initial glucose	Glycolysis
1	75	55	—	—	47	44	51	46
2	137	76	142	41	95	45	84	32
3	108	61	116	66	—	—	75	39
4	78	42	115	51	98	32	70	84
5	79	39	120	38	137	36	93	28
6	90	61	84	63	118	45	59	42
7	74	43	176	47	132	30	—	—

Glycolysis is expressed as mg of glucose/100 ml RBC/h.

by the formula: $k = \frac{\log N_0 - \log N_t}{t} \times 2{\cdot}303$.

Since a perfect linear correlation has been found between the k and the hematocrit of samples with different RBC: plasma ratio, in order to have comparable results, the values of k have been corrected for an hematocrit of 50. In the 40 normal subjects of our group, the velocity constant/h was $0{\cdot}0066 \pm 0{\cdot}0018$. A relatively slower speed of the reaction was found in the first hour presumably due to some delay in the equilibration of the temperature between blood and waterbath.



Correlation between initial glucose level and glycolysis. Solid line is the calculated regression line for the points shown and the dashed lines the 2 σ confidence limits.

In order to confirm the existence of a correlation between the initial glucose level and the rate of glycolysis, experiments were carried out both *in vivo* and *in vitro*. By adding *in vitro* measured amounts of glucose to samples of blood, no changes of the glycolysis were observed as compared with that of the blood alone. Glycolysis was also determined in normal individuals during a glucose tolerance test. 40–60 g of glucose were given by mouth and glycolysis was measured in the fasting state and after 30, 60, and 120 min. No consistent, pattern was obtained. In three subjects (cases 1, 2, 3 of the Table) a fairly good correlation was found between the initial glucose level and the glycolysis: in the single cases the points approximated a straight line. In other three instances (cases 4, 5, 6 of the Table), the glycolysis rate changed quite unpredictably with no constant relationship to the variations of the blood sugar. No constant changes were observed in one case given 25 g of glucose intravenously (case 7 of the Table). It may be of interest to note that the subjects in whom a correlation could be elicited were allowed to perform normal muscular activity during the test while the other four cases were kept resting in bed.

Discussion.—Our results expressed as mg/100 ml RBC/h are in perfect agreement with the ones reported by others with the same technique[4].

As to the concentration of glucose, it is generally reported even in recent papers[5] that the initial glucose level, if below 500 mg%, has no effect on the glycolysis whereas higher values inhibit it. But from earlier authors, it was reported that glycolysis could be considered as a first order reaction, where the amount of glucose utilized is proportional within the limits of the experimental error to the amount of the available substrate[6]. While the data concerning experiments *in vitro* are rather conflicting, the results *in vivo* appear more uniform, even if not all authors called attention to it[7]. The possible correlation between glycolysis and initial glucose level should be considered when glycolysis is followed under the influence of certain drugs or in different experimental conditions; in many instances the observed values would become not significant if expressed as velocity constant.

One wonders if the intimate mechanism of the changes of blood sugar concentration may account for the different behaviour of glycolysis. As a matter of fact, when glucose level was artificially increased by administration of sugar in resting patients, no constant modifications could be detected. In the cases in which the changes occurred during muscular activity, as also reported in animals in which the blood sugar was altered by endogenous mobilization, a fairly good correlation could be elicited.

On the basis of the available data, the concentration of glucose appears to be an important factor but not the only one. Further studies are in progress in order to ascertain the possible influence both *in vivo* and *in vitro* of some hormonal substances.

[4] J. W. HOLLINGSWORTH, J. Lab. clin. Med. *45*, 920 (1955). – J. G. SELWYN and J. V. DACIE, Blood *9*, 414 (1954). – J. E. ULTMAN, G. A. HYMAN, J. L. HARVEY, and A. R. DENTE, Blood *12*, 1114 (1957).

[5] R. M. BIRD, J. biol. Chem. *169*, 492 (1947). – J. W. HOLLINGWORTH, J. Lab. clin. Med. *45*, 920 (1955). – E. PONDER, *Hemolysis and related phenomena* (Grune & Stratton, New York 1948). – J. G. SELWYN and J. V. DACIE, Blood *9*, 414 (1954). – J. E. ULTMAN, G. A. HYMAN, J. L. HARVEY, and A. R. DENTE, Blood *12*, 1114 (1957).

[6] A. KANITZ, Biochem. Z. *57*, 437 (1913). – P. RONA and A. DÖBLIN, Biochem. Z. *32*, 489 (1911). – P. RONA and G. G. WILENKO, Biochem. Z. *62*, 1 (1914).

[7] C. COLIZZI and G. TUSINI, Atti Soc. lombarda Sci. med. Biol. *8*, 238 (1953). – F. VACCARI and M. ROSSANDA, Arch. int. Pharmacodyn. *90*, 421 (1952). – F. VACCARI, M. ROSSANDA, and G. MALAGUTI, Arch. int. Pharmacodyn. *99*, 234 (1954).

By expressing the glycolysis through the velocity constant corrected for the hematocrit, the variations due to the concentration of glucose are automatically eliminated. However, it has to be kept in mind that glycolysis is not a simple process but consists of a chain of interrelated reactions and therefore the use of a single velocity constant may be an oversimplification which is not free from reasonable criticism.

Whereas the relationship between initial glucose level and glycolysis is strongly suggestive of a first order reaction, the disappearance of the glucose followed at shorter intervals during the first 2 h approximates to a straight line more consistent with a reaction of order zero. We have no satisfactory explanation for this discrepancy.

Since under normal circumstances the range of the sugar levels in the blood is relatively small, no significant error can be made if one expresses the glycolysis in the conventional way. However, this method becomes unreliable when the glycolysis is followed during spontaneous or induced sugar variations.

F. Buffa and G. Maraini

Istituto di Patologia speciale medica e metodologia clinica, Università di Torino (Italy), May 27, 1958.

Riassunto

Studiando la glicolisi di globuli rossi umani è stata notata una correlazione statisticamente significativa tra livello iniziale di glucosio e glicolisi, espressa in mg di glucosio utilizzati da 100 ml di globuli rossi per ora. In condizioni di carico sperimentale con glucosio la correlazione ha avuto un comportamento variabile.

Activation of Cathepsin in Fatty Liver

It has been shown in recent years that cathepsin activity of liver cells is mainly located within mitochondria[1]. De Duve *et al.*[2] have found that the cathepsin which is active on haemoglobin is bound to a special type of particles, which have the same sedimentation characteristics such as light mitochondria. These particles have been called *lysosomes*. They contain practically all acid phosphatase, β-glucuronidase, ribonuclease, desoxyribonuclease and cathepsin activities of the liver cells. The activity of these enzymes is very low when the lysosomes are intact, but increase many times as a result of damaging treatments. These also produce the displacement of the bound enzymes from the particles to the suspension fluid. Many different treatments produce similar effects: homogenization in Waring blendor, suspension in hypotonic solutions, incubation at 37°C for a short time, addition of salts, addition of substances which decrease the surface tension.

The presence of mitochondrial damage in fatty livers has been described[3]. Morphological modifications of mitochondria consist mainly in the swelling of the particles. Swollen mitochondria show higher permeabilities to many substances originally contained inside their body, as ribonucleic acid[4], cytochrome *c*[5], pyridine nucleotides[6], adenosine phosphates[7], thiamine pyrophosphate[8], citrate[9]. These substances are displaced from the mitochondrial body to the suspension fluid as a consequence of treatments which produce mitochondrial swelling. These treatments resemble strongly those which produce lysosome damage. It seems then not improbable that lysosome damage exists in fatty livers. Strong increase of acid phosphatase activity of fatty livers has been reported[10]. In these conditions, a redistribution of the enzyme takes place, as a large part of it is displaced from mitochondria into the fluid part of the homogenate. An increase of β-glucuronidase in fatty livers has been reported by Mills *et al.*[11].

Thus it was interesting to study the behaviour of cathepsin, another enzyme present in lysosomes. Albino rats weighing 150–180 g were used. They were fed on a standard diet. Fatty infiltration of the liver was obtained either by 2 subcutaneous injections of carbon tetrachloride (0·2 ml of a 20% solution in olive oil, each day), or also by one subcutaneous injection of white phosphorus (0·1 ml of the 0·5% solution in olive oil). The rats were killed by decapitation 24 h after the last injection. The liver was immediately dissected and transferred to a cold room at 2°C.

10% homogenates were prepared in a Potter-Elvehjem homogenizer, two types of tissue suspensions being prepared in each case: the first one was prepared with 0·25 *M* sucrose, the second one with 0·25 *M* sucrose containing 0·1% *Triton X–100* (obtained through the courtesy of Prof. H. G. K. Westenbrink). This was used to produce maximum activation of cathepsin, as a result of the disintegration of the particles (Wattiaux and De Duve[12]). In order to avoid mechanical damage to the particles in the case of the homogenates prepared with 0·25 *M* sucrose, mitochondria were not isolated and the enzyme determinations were performed on the whole homogenates. These were used within 10 min after the death of the animal.

Cathepsin activity was determined at 37°C by a modification of the method of Anson[13] described by Gianetto and De Duve[14]. The reaction mixture had the following composition: 0·17 *M* acetate buffer, pH 5·0, 0·00026 *M* haemoglobin, the enzyme and 0·25 *M* sucrose to 3 ml. The reaction was stopped by adding 4 ml of ice-cold 0·3 *M* trichloroacetic acid. The mixture was cooled immediately and filtered in the ice box, in order to decrease the hydrolysis of sucrose, the products of which interfer with the

[1] M. E. Maver and A. E. Greco, J. nat. Cancer Inst. *12*, 37 (1951). – J. T. Finkenstaedt, Proc. Soc. exp. Biol. Med. N. Y. *95*, 302 (1957).

[2] C. De Duve, R. Gianetto, F. Appelmans, and R. Wattiaux, Nature *172*, 1143 (1953). – R. Gianetto and C. De Duve, Biochem. J. *59*, 433 (1955).

[3] H. C. Zollinger, Revue Hématol. *5*, 696 (1950). – M. U. Dianzani, G. Biochim. *2*, 180 (1953). – M. U. Dianzani and G. F. Bahr, Acta path. microbiol. scand. *35*, 25 (1954). – E. Levin, R. M. Johnson, and S. Albert, J. biol. Chem. *228*, 15 (1957).

[4] M. U. Dianzani, Biochim. biophys. Acta *11*, 353 (1953).

[5] M. U. Dianzani and I. Viti, Biochem. J. *59*, 141 (1955).

[6] G. S. Christie and J. D. Judah, Proc. Roy. Soc. (London) [B] *907*, 241 (1954). – M. U. Dianzani, Biochim. biophys. Acta *17*, 391 (1955). – F. E. Hunter and L. Ford, J. biol. Chem. *216*, 357 (1955). – P. Siekevitz and V. R. Potter, J. biol. Chem. *215*, 221 (1955). – G. F. Maley and H. A. Lardy, J. biol. Chem. *215*, 377 (1955).

[7] H. Blaschko, G. V. R. Born, A. D'Iorio, and N. R. Eade, Biochem. J. *62*, 18P (1956). – M. U. Dianzani, Biochem. J. *65*, 116 (1957).

[8] M. U. Dianzani and M. A. Dianzani Mor, Biochim. biophys. Acta *24*, 564 (1957).

[9] W. C. Schneider, M. Striebich, and G. H. Hogeboom, J. biol. Chem. *222*, 969 (1956).

[10] M. U. Dianzani, Biochim. biophys. Acta *14*, 514 (1954).

[11] G. T. Mills, J. Paul, and E. E. B. Smith, Biochem. J. *53*, 245 (1953).

[12] R. Wattiaux and C. De Duve, Biochem. J. *63*, 606 (1956).

[13] M. L. Anson, J. gen. Physiol. *20*, 565 (1937).

[14] R. Gianetto and C. De Duve, Biochem. J. *59*, 433 (1955).