XIV. OBSERVATIONS UPON THE REDUCING SUBSTANCES OF PIGEONS' BLOOD.

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It has been observed from time to time by various investigators, *e.g.* Funk and v. Schönborn [1914], Funk [1920], Honeywell [1922], Riddle and Honeywell [1923], Collazo [1923], Randoin and Lelesz [1925], Marrian, Baker, Drummond and Woollard [1927], Schwarz and Heinrich [1928], and Kinnersley and Peters [1929], that the total reducing value of normal avian blood is considerably higher than that of normal mammalian blood; in fact this value, when expressed in terms of glucose, seems to show that the sugar level in normal birds is roughly the same as that which is characteristic of human diabetics. Schwarz and Heinrich, for example, found that the average total reducing value of hens' blood was as high as 253 mg. of glucose per 100 cc. when estimated by the Hagedorn and Jensen method. Marrian *et al.* obtained values of 180 mg. per 100 cc. for pigeons deprived of food for 24 hours. Collazo found 210 mg. of glucose per 100 cc. for pigeons; and Riddle and Honeywell observed variations in the sugar level according to the breed of pigeons used, their values lying between 149 and 258 mg. of glucose per 100 cc. The question therefore arises whether these high values are caused by an additional amount of glucose as compared with that of mammalian blood, or whether the non-glucose reducing substances in avian blood are present in amounts sufficient to lower the true glucose value to one approaching mammalian limits. Intimately connected with this problem are the unpublished observations (*a*) by Kinnersley and Peters, that the reducing value of the blood of pigeons in insulin convulsions is about 70 mg. of glucose per 100 cc. when estimated by the Hagedorn and Jensen method; and (*b*) by J. T. Irving, that the apparent glucose content of pigeons' blood is about 60 mg. when glycolysis has proceeded until the reducing value has become constant. We take this opportunity to express our indebtedness to Dr. Irving for allowing us to mention his observations. These results seem to show that the amount of non-glucose reducing substances in pigeons' blood is considerable, and it is therefore important to ascertain their nature, relative proportions and, if possible, their function. This information is needed in order to decide what is the best method of estimating the blood-"glucose," but in addition unusual interest centres round the question of function, since

it has been shown by Rüter [1923] that glycolysis of goose and hen blood is slight or absent *in vitro*, and by Bornstein and Ascher [1926] that this is due to the presence of oxygen, observations which we have confirmed in the case of pigeons. It was considered possible that the inhibition of glycolysis and the apparent high content of non-glucose substances might be interdependent.

Experimental.

The reducing substances of normal pigeons' blood.

It is evident that the solutions of the two problems outlined above may be approached by the same route, namely by determining the total "sugar," eliminating the true glucose by means of insulin or glycolysis, and then estimating the substances responsible for the residual reduction. This choice of method of eliminating glucose is justified by the work of Hiller, Linder and Van Slyke [1925], who concluded that glycolysis and the administration of insulin resulted in the destruction of the same amount of glucose. From the figures thus obtained the true glucose content may be calculated. In order to avoid any criticism which might be levelled at this statement, we wish to make it clear that we do not contemplate entering the present controversy on true blood-glucose. For the purpose of the present communication, we shall consider as the blood-glucose the reducing substance (or substances) which disappear during glycolysis or after insulin treatment.

Throughout this work the reducing values have been determined by the Hagedorn and Jensen method. A survey of the literature on the subject of blood-sugar has convinced us that it would be unwise to base conclusions on results derived by different methods. The work of Herbert and Groen [1929] is of special interest in this connection.

The samples have been collected by stunning and guillotining the pigeons (in some cases stunning was omitted), and shedding the blood directly into heparin (6 mg. per bird). This anti-coagulant has no significant reducing value in the Hagedorn and Jensen estimation. Assuming that it survives completely the initial stage of protein precipitation, its presence in a concentration of 1 mg. in 1 cc. of blood would raise the blood-"sugar" by 1–2 mg. per 100 cc. since 0·5 mg. of heparin is equivalent to 0·04 cc. of 0·005 *N* alkaline ferricyanide.

Glycolysis, though absent in aerated avian blood, proceeds normally in presence of potassium cyanide or in absence of oxygen [Bornstein and Ascher, 1926]. In the glycolytic experiments recorded in this paper, it has been found advisable to add 1 mg. of potassium cyanide per cc. of blood. In this concentration potassium cyanide has no significant effect in the Hagedorn and Jensen method, supposing that it survives the initial protein precipitation, since determinations with aqueous solutions have shown that 0·1 mg. of potassium cyanide is equivalent to 0·01 cc. of 0·005 *N* alkaline potassium ferricyanide.

Characteristic curves are given in Fig. 1 for the glycolysis in presence of cyanide of normal pigeons' (A) and of cockerels' blood (C) when agitated gently at 37°. Curve A shows that, in confirmation of Irving's [1926] observations for mammalian blood and unpublished observations for pigeons' blood, glycolysis proceeds in an approximately linear manner. After some 6 hours in pigeons' blood, it stops rather abruptly at 60–70 mg. "apparent" glucose per 100 cc. This value has been regarded throughout this research as the "residual" reduction after glycolysis. Curve C shows that in a cockerel glycolysis stops abruptly at approximately the same level and indicates that the facts here described are also true for hen blood. This matter has not been further investigated.

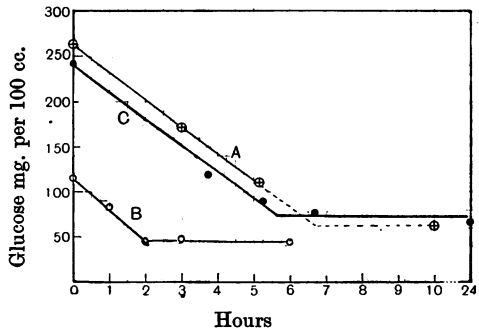

Fig. 1. Glycolysis in presence of cyanide.
A. Normal pigeon. B. Abnormal avitaminous pigeon. C. Normal cockerel.

The curve B represents the glycolysis of the blood of a polyneuritic pigeon under the same conditions, and is included in order to show that the form of the curve is unaltered (as compared with the normal bird). The values in curve B are however exceptional, being the lowest ever recorded for B-avitaminous pigeons in this laboratory. We have been unable to obtain curves for more typical avitaminous pigeons owing to an apparent inefficiency of the glycolytic system in such cases (see p. 101).

The total value of the "residual" reduction in terms of glucose.

In Table I may be seen the effect of eliminating the glucose from the blood of a number of typical normal pigeons. Attention is directed to the high total blood-"sugar" and the comparative constancy of the residual reduction figure after glycolysis.

Table I.

Results in mg. glucose per 100 cc. blood.

Bird	Initial "sugar"	Duration of glycolysis in hours	Residual reduction	
			KCN	Anaerobic
1 Insulinised	(88)*	4	69	71
2 Normal	212	—	—	—
3 „	237	10	74	78
4 „	239	24	68	—
5 „	223	12	82	—

* In convulsions.

The average value for the blood-"sugar" in normal pigeons as taken out of the cage (and therefore feeding) has been found to be 230 mg. ± 20 mg. per 100 cc., and that for birds kept without food for 24 hours 205 mg. ± 15 mg.

Since the residual reduction value represents non-glucose substances, the approximate glucose content of pigeons' blood may be derived from the preceding table. The average value for the total "sugar" is 228 and that of the residual reduction 73, whence the glucose content is 155 mg. per 100 cc. for the feeding bird, a value greater it is true than that of most mammalian bloods, but lower than the uppermost limit for the ox [Schwarz, 1928]. Hence the glucose value for the blood of pigeons deprived of food for 24 hours must be 135 ± 15 mg. per 100 cc. It is clear therefore that the residual reducing value of pigeons' blood is considerably higher than that of mammalian blood, and consequently the true glucose value of birds' blood is not so widely divergent from that of mammals as the Hagedorn and Jensen estimations appear to show [compare Lund and Wolf, 1926].

The nature of the residual reducing substances.

Attention was then directed to the nature of the substances responsible for the residual reduction, and uric acid, glutathione and ergothioneine came into consideration as possibilities. Uric acid has long been known to occur in avian blood, and Randoin and Fabre [1927] have detected aliphatic sulphydryl compounds in pigeons' blood and estimated the average content as 61 mg. per 100 cc. of blood, although individual variations were large. Hunter [1928] found ergothioneine in fowls' blood, and we have isolated some 5 mg. of the hydrochloride from the combined bloods of ten pigeons (see p. 101).

Reducing value of uric acid. Holden [1926] has observed that the reducing power of uric acid in the Hagedorn and Jensen method is 53 % of that of glucose. Flatow [1926] showed that uric acid reduces cold potassium ferricyanide solution. Similar experiments have now been performed from which it has been deduced that uric acid when oxidised by cold alkaline potassium ferricyanide solution has a reducing value of 53 % of that of glucose when oxidised by hot ferricyanide, and that this value remains unchanged when the mixture of uric acid and ferricyanide is heated as in the Hagedorn and Jensen method.

Oxidation by cold alkaline ferricyanide. Since the Hagedorn and Jensen method involves the liberation of free iodine, it is clear that compounds such as ergothioneine and glutathione, which contain sulphydryl groups, will have an "apparent" reducing value even if stable to ferricyanide, since the amount of ferricyanide reduced is measured by estimating by means of thiosulphate the amount of iodine liberated by the excess of ferricyanide. The utilisation of iodine in such oxidations would therefore appear as oxidation by ferricyanide. In the hope of estimating the non-glucose reducing substances by such means, a "cold Hagedorn and Jensen method" has been employed. The sole modification is that the alkaline ferricyanide solution and blood

filtrate are not heated but are at once mixed with potassium iodide-zinc sulphate solution, then with acetic acid and titrated with thiosulphate. The blank estimation is of course a simple titration of unheated ferricyanide with thiosulphate. In Table II are recorded the "cold" reducing values, calculated as mg. glucose in 100 cc. of the bloods of the same pigeons used for the experiments of Table I.

Table II.

1 and 8 insulinised, 2–7 normal. Results in mg. glucose per 100 cc.

Bird	1	2	3	4	5	6	7	8
Initial cold value	32	34	32	—	32	11	30	44
After glycolysis	—	—	—	39	35	14	32	—

It is clear that with one exception, which will be discussed below, there is little significant variation in normal pigeons and that the "cold" value is not affected either by glycolysis in presence of cyanide or by the administration of insulin. It should also be noted that the figures represent only 40–60 % of the total non-glucose reduction. This fact will be referred to later.

Table III has been constructed to show that the substances responsible for the "cold" reducing value and also for the residual reduction by the customary method are present almost entirely in the corpuscles, and that the serum is almost free from these substances.

Table III.

Results in mg. glucose per 100 cc. Bloods centrifuged, relative volumes of corpuscles and serum noted, and plasma pipetted off. Corpuscles usually diluted to original volume of blood with saline. Figures in brackets are samples taken after complete glycolysis.

	Corpuscles		Serum		Whole blood	
Bird	Cold	Hot	Cold	Hot	Cold	Hot
1	34	73	4	—	34	213
2	—	50	—	(10)	—	368
3	—	71 (53)	—	(21)	—	370
4	—	(53)	—	(11)	—	(63)

Table IV.

Amounts in mg. Vol. of solutions, 15 cc.

Method	Ergothioneine added	Glucose added	Apparent glucose	Glucose calc.	Reducing power glucose = 100
Cold*	0·172	None	0·097	—	56
Cold†	0·178	,,	0·099	—	56
Cold‡	0·200	,,	0·108	—	54
Hot*	0·172	,,	0·104	—	61
Hot†	0·178	,,	0·117	—	66
Hot‡	0·200	,,	0·133	—	66
Cold	None	0·299	None	—	—
Hot	,,	0·312	0·229	0·312	—
Hot	0·086	0·149	0·200	0·180	59

* Ergothioneine from ergot.
† Ergothioneine from ergot (different sample).
‡ Ergothioneine from pigs' blood.

Reducing values of ergothioneine. Table IV shows the reducing power of ergothioneine by the "hot" and "cold" Hagedorn and Jensen methods and makes it clear that coupled oxidations with glucose do not occur (cf. Somogyi,

1927]. Holden [1926] has shown that uric acid, cystine, creatinine and amino-acids do not give rise to coupled oxidations. Sjollema [1927] states that ergothioneine in 0·1 % solution has a reducing value of 84 as compared with glucose 100.

Reducing values of glutathione. Table V shows the "hot" and "cold" values for the tripeptide reduced glutathione [Hopkins, 1929].

Table V.

Amounts in mg. Vol. of solutions 15 cc.

Exp.	Method	Glutathione added	Apparent glucose	Reducing power glucose = 100	Remarks
1	Cold	0·500	0·088	17·6	Titrated immediately (30 secs.) after adding alkaline ferricyanide.
2	Cold	0·500	0·064	12·8	Titrated 5–15 mins. after adding alkaline ferricyanide
3	Hot	0·500	0·227	45·4	—

Exps. 1 and 2 show that the speed of estimation makes a considerable difference to the result obtained by the cold method, and Exp. 3 that hot ferricyanide gives a much higher value than cold.

Estimations of non-glucose reducing substances in different filtrates.

In attempting to draw up a balance sheet of non-glucose reducing substances, we were led to examine qualitatively, and later quantitatively, blood-filtrates obtained by different methods. The following table gives a list of the compounds present.

Table VI. *Reducing compounds other than glucose present in blood-filtrates.*

Total: 70 mg. ± 10 mg. per 100 cc. (calculated as glucose).

Substance	Tungstic acid	Trichloroacetic acid	Zinc	Approx. amount in zinc filtrate in mg. per 100 cc.
(1) Uric acid	+(*a*)	?	?	?
(2) —SH compounds	+(*b*)	+(*e*)	—	—
(3) —S.S— compounds	+(*c*)	+	—	—
(4) Ergothioneine	+(*d*)	?	+(*f*)	30
(5) Unknown, reducing cold ferricyanide	?	?	+	10
(6) Unknown, reducing hot ferricyanide	?	?	+	40

(*a*) Benedict [1922]. (*b*) Nitroprusside reaction. (*c*) Walker's reaction [1925]. (*d*), (*f*) Compounds giving G. Hunter's ergothioneine reaction [1928]. (*e*) Less —SH compounds present than in (*b*).

It will be seen that the zinc filtrates (Hagedorn and Jensen) are freer than others from all compounds except ergothioneine, and that for any given blood the variations between the reducing values obtained upon a zinc filtrate and a trichloroacetic acid filtrate will depend upon fluctuations in ergothioneine content and the extent to which other sulphur compounds are present in the

trichloroacetic acid filtrate. This accounts for results of Kinnersley and Peters, in which with pigeons' blood they have found irregular variations between the "sugar" values of these two filtrates respectively, the trichloroacetic acid filtrates being 20–30 mg. higher. Ergothioneine cannot be estimated in the trichloroacetic acid filtrates because this reagent inhibits the diazo-reaction even in neutralised filtrates.

After these experiments were completed, we became aware that Somogyi [1929, 1] had stated that ergothioneine, glutathione and uric acid are absent from zinc filtrates. The discrepancies between the two series of results are doubtless due to the fact that Somogyi precipitated the proteins by shaking at room temperature and not by heating, as in the Hagedorn and Jensen method. The present observations explain the statement by Somogyi in another paper [1929, 2] that the Hagedorn and Jensen technique leaves only about half as much of non-fermentable reducing substances in the filtrate as the tungstic acid procedure.

Bearing in mind that the cold reducing value of uric acid is 53 %, of ergothioneine 56 %, and of glutathione 17 % of that of glucose, we may now compare the amounts of ergothioneine, uric acid and aliphatic —SH compounds as estimated by accepted methods with the values obtained for the cold reduction of various types of filtrate.

Table VII.

The pigeons are the same as those in Tables I and II. Results in mg. per 100 cc. blood. 5–7 normal, 8 insulinised.

	Zinc.	Trichloro-acetic acid.	Tungstic acid			
Bird	Ergo-thioneine	Total —SH as cysteine	Total —SH as cysteine	Indirect uric acid	Rockwood thioneine as uric acid	Ergo-thioneine
6	0	42	61	3·8	1·0	0
7	33	61	85	3·7	1·3	13
5	—	85	—	4·5	1·7	—
8	—	—	—	—	—	10
17	30	—	—	—	—	6
18	27	—	—	—	—	5

Ergothioneine and —SH compounds. Table VII records estimations of these in the bloods of normal pigeons. Ergothioneine was estimated by Hunter's method, using aqueous solutions of ergothioneine hydrochloride as standards, and the total sulphydryl compounds were determined by adding to the solutions an excess of 0·005N iodine in potassium iodide solution and titrating the unused portion with 0·005N thiosulphate solution using starch as indicator. Tungstic acid filtrates were rendered faintly acid before the estimation of sulphydryl compounds. The term "indirect uric acid" has the meaning given by Bulmer, Eagles and Hunter [1925] and represents that portion of the silver precipitate which is decomposed by sodium chloride-hydrochloric acid solution and considered to be true "uric" acid. "Rockwood thioneine" represents the insoluble solid from this treatment, which was regarded as ergothioneine by Rockwood, Turner and Pfiffner [1929]. This

fraction contains a considerable amount of aliphatic sulphydryl compounds or disulphides as may be shown by means of Walker's test, and the figures have no real significance. The "indirect uric acid" fraction also contains traces of these compounds, but when estimated colorimetrically their concentration was about 1 in 8000. Their presence may be neglected, since we have found that one part of uric acid gives the same colour as 125 parts of cystine in the Benedict method [1922] of estimation.

It will be noticed that whereas zinc filtrates tend to give 30 mg. ergothioneine (with exception of bird 6 to which reference is made later), the values for ergothioneine in the tungstic acid filtrate are lower and variable. This is in part due to the difficulty of comparing the colours in the latter filtrates. In the case of 17 and 18, the tungstic acid filtrates gave a colour with Hunter's reagent which matched phenol red of p_H 7·3 and 7·1 respectively, and the values were obtained by matching against a phenol red standard at p_H 7·1. For this purpose we made the assumption that there was present in the p_H 7·1 solution 1/5 phenol red dissociated as the alkaline indicator. The results for the tungstic acid filtrates can be regarded as only approximate, but it is clear that zinc filtrates appear to contain considerably more substances which give Hunter's ergothioneine reaction than do tungstic acid filtrates. These may be ergothioneine itself, or perhaps other thiol-glyoxalines, since it is stated [Benedict and Newton, 1929] that tungstic acid coagulation precipitates a large proportion of the ergothioneine present in blood. In this connection it may be noted that Sjollema [1927] concluded from sulphur analysis that sulphur-containing compounds other than ergothioneine and glutathione are present in blood.

Hot and cold Hagedorn and Jensen estimation of different types of filtrate.

In Table VIII are shown the results of estimations made on the zinc, trichloroacetic acid and tungstic acid blood-filtrates of the same pigeons by the cold and hot Hagedorn and Jensen methods. Though not numerous these results are sufficient to show the type of variation to be expected in general; the "sugar" values of zinc and trichloroacetic acid filtrates differ somewhat by the hot method, the latter being slightly higher, and the results for all three types of filtrate show variations by the cold method. These discrepancies are no doubt due in part to the fluctuating content of aliphatic sulphydryl compounds. Sjollema [1927] has observed a similar increase in non-glucose reducing substances in tungstic as compared with trichloroacetic acid filtrates. In view of the results with pigeon No. 6 it is interesting that Herbert and Groen [1929] have observed that the hot Hagedorn and Jensen method gives figures which are on the average 27 mg. per 100 cc. higher in tungstic acid than in zinc filtrates obtained from human blood.

It will be noted that "cold" values upon the tungstic filtrate give about 30 mg., or approximately the same as the zinc filtrate, though it must be emphasised that the substances responsible are different in the two cases.

Table VIII.

Results as mg. glucose per 100 cc. blood.

Bird	Zinc		Trichloroacetic acid		Tungstic acid.
	Cold	Hot	Cold	Hot	Cold
7	30	227	14	—	—
6	11	253	19	—	34
4	—	239	28	246	—
5	32	223	28	225	—
17	—	—	—	—	31
18	—	—	—	—	30

All trichloroacetic acid filtrates were neutralised before estimation.

Unknown substances reducing "cold" ferricyanide.

Special interest attaches to the estimation of zinc filtrates by the cold method because the amounts of non-glucose substances there present are unable to account for the whole of the reduction. This is best illustrated by reference to pigeon No. 6, from whose blood ergothioneine was absent. The cold value is still 11 mg. of glucose per 100 cc., and the uric acid content (estimated from the tungstic acid filtrate) is of the normal order. Assuming that the same amount is present in the zinc filtrate, it is approximately equivalent to 2 mg. of glucose, so that the equivalent of 9 mg. of glucose per 100 cc. remains unaccounted for. The assumption made here is probably unjustified if interpreted too exactly, but the uric acid content of zinc and tungstic acid filtrates would presumably be of the same order. Now uric acid has half the reducing power of glucose, and creatinine and creatine, which may also be present, are stable to cold alkaline ferricyanide in amounts representing 200 mg. per 100 cc. of blood, whereas Scheunert and Pelzchrzim [1923] have estimated the combined creatine and creatinine as only 4·7 mg. of creatinine per 100 cc., a figure in harmony with the results obtained for other avian bloods by different workers [Hunter, A., 1928]. It follows therefore that zinc filtrates must contain at least one other non-glucose reducing substance which represents about 10 mg. of glucose and is oxidised by cold alkaline potassium ferricyanide or by iodine. This conclusion is supported by the case of bird No. 7 where an approximate balance-sheet for the cold reduction value shows that the equivalent of some 10–12 mg. of glucose is again unaccounted for. Evidence leading to the same conclusion may be derived from a consideration of the cold reducing value for the avitaminous pigeon No. 14, namely 18 mg. of glucose per 100 cc., since ergothioneine was present only in small amount in the zinc filtrate (approximately 8 mg. per 100 cc.).

Unknown substance reducing hot ferricyanide.

Turning now to the residual non-glucose reduction of zinc filtrates by the hot Hagedorn and Jensen method as determined by insulin and glycolysis, the average increase of this over the cold values is 40 mg. of glucose per 100 cc. Part of this increase is no doubt due to creatinine, stated by Holden

[1926] to have 67 % of the reducing power of glucose and by Holmes and Holmes [1926] to have a maximum effect of about the same value. Part corresponds to creatine, which Holmes and Holmes have shown to have a maximal efficiency of about 25 % of that of glucose; and part must represent the increase in the "hot" value over the "cold" for ergothioneine. These corrections cannot amount to more than 3 mg. per 100 cc. in all, and it is therefore evident that a considerable balance remains unassigned, although its nature must at present remain indefinite. Three hypotheses, however, may be cited. The presence of a uric acid-ribose compound such as that isolated from various bloods by Davis, Newton and Benedict [1922], or of other purine- or pyrimidine-carbohydrate compounds, would offer an explanation of the unassigned values in both the cold and hot methods, and is attractive in view of the peculiar position of uric acid in avian metabolism. Alternatively, the view may be taken that the unassigned cold and hot values have no relationship, and that the additional hot value represents either non-glucose carbohydrate or glucosephosphoric esters which have been formed during glycolysis or after the administration of insulin.

The non-glucose reducing substances of B-avitaminous pigeons' blood.

Analyses of the reducing substances in the blood of B-avitaminous pigeons have been made chiefly to determine whether the hot and cold reducing values were altered in these abnormal cases. The results are recorded in Table IX.

Table IX.

Results in mg. per 100 cc. blood.

	Zinc								
	Initial (calc. as glucose)		Glycolysed; time in brackets in hrs.		Trichloroacetic acid. Initial (calc. as glucose, neutralised)		Tungstic acid		
Bird	Cold	Hot	Cold	Hot	Cold	Hot	Total —SH (calc. as cysteine)	Indirect uric acid	Remarks
9	21	336	25 (11)	270	—	—	61	4·6	H.R.
10	34	425	29 (22)	288	—	—	—	—	H.R. *a*
11	—	216	—	—	32	255	—	—	—
12	21	195	—	—	21	207	—	—	H.R. *b*
13	—	115	— (6)	44	—	—	—	—	H.R.
14	18	368	—	48	—	—	—	—	H.R.
15	18	370	—	74	—	—	—	—	H.R.
16	26	287	—	—	26	285	—	—	H.R.

H.R. indicates head retracted. *a*, very abnormal bird. *b*, no symptoms after being on diet 72 days.

Examination of Table IX reveals that there is no very significant variation between undieted and avitaminous pigeons as regards the cold values for zinc or trichloroacetic acid filtrates, and that the hot values for these filtrates in the case of polyneuritic birds have the same inter-relationship as in normal cases. The content of total sulphydryl compounds and of uric acid would seem to be unaffected by the change in diet. The cases have been

selected so that they include not only birds showing polyneuritic hyperglycaemia (Nos. 9, 10, 14, 15), but also those exhibiting normal values (Nos. 11, 12), and one having a very abnormally low blood-sugar (No. 13).

In those pigeons in which the glucose content is very high the glycolytic system may be either impaired or unable through inactivation to deal with the large amounts of glucose, since in several instances (see Nos. 9 and 10) attempts to determine the residual reduction by removing glucose by glycolysis were unavailing. Recourse was therefore made to the following procedure (see Nos. 14 and 15). The blood was centrifuged, the respective volumes of corpuscles and serum noted, and the corpuscles suspended in sufficient isotonic saline to make up the volume of the blood. The glucose was then eliminated by glycolysis from the saline suspension and from a known mixture of a small part of the serum and a larger fraction of the corpuscular suspension. Glycolysis was almost complete in the corpuscle-serum mixture after $2\frac{1}{2}$ hours, as was confirmed by determinations after a further period of $1\frac{1}{2}$ hours. From these results the residual reduction for the whole blood by the hot Hagedorn and Jensen method was calculated. It is clear that the residual reduction, due to non-glucose substances, is of the same order as in normal pigeons, but that it fluctuates more widely.

Isolation of ergothioneine hydrochloride from pigeons' blood.

Ten normal pigeons were stunned and guillotined, and in each case the blood (120–150 cc. in all) was shed into 60 cc. of boiling 0·01*N* acetic acid. After being boiled for one minute to complete coagulation, the combined extracts were filtered and treated exactly as described by Benedict, Newton and Behre [1926] in the isolation of ergothioneine from pigs' blood. The residue (12 mg.) of crude ergothioneine hydrochloride obtained by evaporating the final acid solution to dryness in a vacuum desiccator was almost free from aliphatic disulphide compounds (Walker's test). It crystallised when rubbed with a drop of 0·5*N* hydrochloric acid, and the crystals were pressed on filter paper and dried at 105°. The yield of pure material was 5 mg. This material melted at 207–208° alone or when mixed with a pure specimen of ergothioneine hydrochloride obtained from ergot, M.P. 207–208° (Tanret [1909], compare Newton, Benedict and Dakin [1927]), gave a very positive reaction by Hunter's test in which the odour of trimethylamine was much in evidence, and was free from aliphatic sulphydryl and disulphide compounds. In an attempt to obtain a further supply for analysis, the blood from ten pigeons was worked up in the same way, but the product in this case (10 mg. in all) contained a relatively greater proportion of aliphatic disulphides, detected by Walker's test, and the amount was inadequate for recrystallisation. Since there can be no question that the substance isolated was ergothioneine hydrochloride, we did not think it justifiable to sacrifice the large number of pigeons which would be necessary to enable an analysis to be made.

Discussion.

As a practical issue of the above work, we think that zinc filtrates made by the Hagedorn and Jensen method are the most reliable material for the estimation of glucose in avian blood, because they contain apparently a smaller number of non-glucose reducing substances than other types of filtrate. Somogyi's method of precipitation at room temperature is stated to be reliable for human blood, but on the one occasion on which we tried it we found that the filtrate gave a "cold" value of 16 mg. per 100 cc. (calculated as glucose) and a "hot" value of 299 mg. The figures obtained for the normal Hagedorn and Jensen filtrate of the same blood were 26 and 287 mg. respectively. Since Somogyi's filtrate presumably did not contain uric acid, glutathione, or ergothioneine [Somogyi, 1929, 1], it follows that it must have contained considerable amounts of other non-glucose substances. We regard the value of 16 mg. per 100 cc. obtained by the "cold" estimation of the Somogyi filtrate as additional evidence for the presence of the suggested unknown "cold" reducing substance.

Throughout this paper it has been assumed that absence of Hunter's ergothioneine reaction presupposes absence of thiolglyoxaline compounds, except in the case of trichloroacetic acid filtrates. It is possible, however, that ergothioneine may exist in the corpuscles in equilibrium with its —S.S— form, and that this oxidised form does not give the Hunter reaction. This compound has been obtained by Tanret [1909] and by Barger and Ewins [1911] as a periodide, but has not yet been isolated from blood. No information as to its behaviour towards the diazo-reagent is available, but experiments bearing on this point are in progress.

It is of considerable importance from the standpoint of comparative observations upon birds and mammals to know that the working concentrations of glucose in the tissues of the two classes are not widely different.

The results for the avitaminous birds settle a possible objection to the conclusion of Kinnersley and Peters [1929] that the increased lactic acid in such brains does not depend upon the sugar content of the blood. If the concentration of non-glucose reducing substances in an avitaminous bird had been much less than normal, then an apparently normal "sugar" value would actually mean a higher glucose content. Since this is not true, the conclusions are valid.

Summary.

1. The residual reducing value after glycolysis in pigeons' blood is approximately 70 mg. "glucose" per 100 cc. by the Hagedorn and Jensen method, representing substances mainly in the corpuscles. This agrees with the value reached in insulin convulsions.

2. It has been deduced that the glucose content in the blood of pigeons kept without food for 24 hours is 135 mg. $\pm$ 15 mg. per 100 cc., being not so

widely different from that of mammals as suggested by the total reducing value of approximately 200 mg.

3. Filtrates prepared by different methods contain different proportions of ergothioneine, uric acid and glutathione (aliphatic —SH compounds). These substances can be estimated in terms of glucose (100) by a "cold" Hagedorn and Jensen method, their values being: uric acid 53, glutathione 17 (hot 45), and ergothioneine 56.

4. Zinc filtrates made by the Hagedorn and Jensen method are the most reliable for estimating reducing substances in avian blood, but these contain in addition to glucose, ergothioneine and some other unknown substances reducing the ferricyanide reagent. Approximately 60 % of the residual value is not accounted for by ergothioneine.

5. Zinc filtrates do not contain aliphatic —S.S— and —SH compounds.

6. There are no significant variations from the normal in B-avitaminous birds.

7. Ergothioneine hydrochloride has been isolated from pigeons' blood.

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