CXXIV. THE ALKALINE HYDROLYSIS OF LECITHO-VITELLIN WITH PARTICULAR REFERENCE TO THE SEPARATION OF AN ORGANIC FRACTION CONTAINING IRON AND COPPER.

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MIESCHER [1870] observed that after prolonged peptic digestion of egg-yolk an insoluble substance separated out. This substance was found by Bunge [1885] to contain iron in organic combination and was named by him "haematogen" because of its close resemblance to haemoglobin, at least so far as empirical composition was concerned. The chemical nature of this substance was investigated in more detail by Hugounenq and Morel [1905, 1, 2; 1906]. Again, Posternak [1927] has studied a similar iron-containing enzyme-resisting substance. The inconsistent results obtained for the iron content of this material indicate that haematogen is not a pure compound. Haematogen was regarded by these workers as a prosthetic group in the vitellin molecule and as being a precursor of the haemoglobin formed during avian embryonic development. A more detailed discussion of their findings is given by McFarlane, Fulmer and Jukes [1930] and by Jukes and Kay [1932].

Levene and Alsberg [1900] have obtained from vitellin an iron-containing substance, vitellinic acid, by the action of strong ammonium hydroxide. It is probable that vitellinic acid is a purer substance than haematogen since these workers avoided enzymic hydrolysis and employed the protein, vitellin, instead of egg-yolk. All these investigations were, however, carried out without knowledge of the presence of a second protein in egg-yolk. Livetin, this second protein [Plimmer, 1908], was first prepared by Kay and Marshall [1928] in pure form.

The constitution of the egg-yolk-protein has long been considered to resemble that of the milk-proteins. Rimington and Kay [1926] found that very mild hydrolysis of caseinogen with 1 % sodium hydroxide liberates the organic phosphorus from the protein molecule as inorganic phosphorus. We were therefore interested in determining what action dilute sodium hydroxide would have on lecitho-vitellin. In the course of this investigation we obtained results which indicated that the lecitho-vitellin molecule contained copper as well as

iron. These two elements were found in organic combination in the same fraction of a lecitho-vitellin hydrolysate.

Preparation of lecitho-vitellin from egg-yolk.

In endeavouring to improve upon existing methods of preparing the pure protein we have benefited considerably from the observations made by Kay and Marshall in the course of their preparation of livetin.

The intact yolks were freed from egg-white by washing first in running water and then under a slow stream of 0.9 % NaCl on filter-paper. The vitelline membrane was punctured so that the yolk contents were transferred to a beaker without the membrane and chalazae. The yolks, diluted with an equal volume of 10 % NaCl, were repeatedly extracted with ether until the ether extract was colourless. This was carried out, using a mechanical shaker, in a refrigerator room at -9.4° and required five twelve-hour extractions, each with an amount of ether equal to the volume of the egg-yolk solution. The saline solution was dialysed in a large parchment dialyser for two days at about -1.1° against repeated changes of distilled water. A large precipitate of the globulin formed. To complete the precipitation the solution was diluted with four volumes of ice-cold water and after standing overnight was rapidly centrifuged. The protein was dissolved in the minimum amount of 10 % NaCl, transferred to the dialyser, and the precipitation and separation repeated. A third solution and precipitation were carried out with the loss of a small amount of the protein which failed to dissolve in 10 % NaCl. The protein precipitate was transferred to an extraction bottle and extracted with alcohol-ether (seven parts alcohol and three parts ether) at -9.4° . The extraction was repeated until no visible residue was obtained when 50 cc. of the extract were evaporated to dryness. The lecitho-vitellin was next extracted for 24 hours with anhydrous ether in a large glass Soxhlet apparatus with metallic sodium in the extraction flask [Hewitt, 1927]. The lecitho-vitellin was ground to a fine white powder and dried in a vacuum desiccator over sulphuric acid.

A very small portion of the protein thus prepared was insoluble in 0.1 *N* sodium hydroxide. On applying the nitroprusside reaction we found that these suspended particles developed on their surface a strong purple colour indicative of denaturation [Hopkins, 1930]. The soluble protein did not react. The following analyses of the lecitho-vitellin have been made:

	%
Nitrogen	15.71
Phosphorus [Richards and Godden, 1924]	0.59
Lecithin	2.17
Iron [McFarlane, 1932, 2]	0.047

Note. 1 g. of lecitho-vitellin was boiled with absolute alcohol for 24 hours in an atmosphere of an inert gas. The material thus extracted is believed to be lecithin. The dried residue, vitellin, is light reddish-brown in colour. When the extraction is made in the presence of oxygen the vitellin has a pink colour.

The action of 0.25 N sodium hydroxide on lecitho-vitellin.

To 2.0316 g. lecitho-vitellin in a 750 cc. Erlenmeyer flask were added 200 cc. 0.25 *N* sodium hydroxide¹ at 37° . The flask was set in a thermostat at 37° and suction apparatus so arranged that air could be slowly bubbled through concentrated sulphuric acid, then through the lecitho-vitellin solution and finally through 200 cc. *N*/100 HCl. The protein was not completely dissolved until the sixth hour of the experiment. Every 12 hours, from the time the protein solution was placed in the water-bath, the following aliquots were taken for analysis.

¹ Prepared from sodium and found to contain no iron.

1. 15 cc. of the protein digest were slowly added to 50 cc. of 3.5 % trichloroacetic acid¹ in a centrifuge-tube, and the heavy white precipitate was separated.

(a) A 40 cc. aliquot of the clear supernatant liquid was taken for the iron estimation.

(b) A micro-Kjeldahl nitrogen determination was made on a 2 cc. aliquot of the supernatant liquid.

(c) The trichloroacetic acid precipitate was dissolved in the minimum amount of dilute sodium hydroxide and reprecipitated with trichloroacetic acid. The entire precipitate was ashed and the iron content determined.

Fig. 1. Action of 0.25 *N* NaOH on lecitho-vitellin.

2. Amino-nitrogen (micro-Van Slyke) was determined on a 2 cc. aliquot of the protein digest.

3. 10 cc. of the hydrochloric acid solution were titrated with *N*/100 NaOH, using alizarin-red indicator. The ammonia formation during hydrolysis was thus determined.

During the action of 0.25 *N* NaOH on lecitho-vitellin ammonia is slowly evolved and the amount of nitrogen not precipitated with trichloroacetic acid steadily increases (Fig. 1). The two values reach maxima at the same time, *i.e.* after about 60 hours' digestion. The first determination of free amino-nitrogen showed 0.98 mg. in 2.0 cc. of the hydrolysate. This value was not increased up to 48 hours, at which time the determination was discontinued. The results of the micro-iron determinations are shown in Table I.

¹ The trichloroacetic acid was found to contain iron which was removed by distilling the acid.

Table I.

Time hydrolysed with 0.25 <i>N</i> NaOH hours	Amount of iron recovered		Total amount of iron in 15 cc. of the hydrolysate	
	Supernatant liquid mg.	Trichloroacetic acid precipitate mg.	Found mg.	Calculated*
6	0.042	0.032	0.099	0.072
12	0.060	0.007	0.104	0.072
24	0.209	0.002	0.302	0.072
48	0.396	0.002	0.515	0.072

* The lecitho-vitellin contained 0.047 % iron.

Note. The iron content of the supernatant liquid after 12 hours' digestion was greatly increased, from some source other than the lecitho-vitellin. The solution at this time gave an intense red colour with potassium thiocyanate. The source of this inorganic iron was undoubtedly the glass flask.

The values for the iron precipitated by trichloroacetic acid show clearly that after 12 hours' digestion the whole of the lecitho-vitellin iron has been liberated from protein combination. Only a trace of iron (less than 3 % of the original iron content of the protein) was contained in this precipitate after 24 hours' digestion. Repetition of this experiment, using quartz ware, was therefore not considered to be necessary at present. At the completion of the digestion (72 hours) a more detailed study of the products formed from lecitho-vitellin by mild hydrolysis with 0.25 *N* NaOH was undertaken. A preliminary experiment showed that after the precipitation of the protein with trichloroacetic acid the filtrate contained a compound or compounds which could be precipitated from acid solution by a saturated solution of normal lead acetate. This lead salt was decomposed with hydrogen sulphide, and the excess hydrogen sulphide in the filtrate from the lead sulphide was driven off by boiling. This filtrate gave an intense biuret reaction.

In the following experiment the distribution of nitrogen and iron in these fractions of lecitho-vitellin, formed by hydrolysis with dilute alkali, was determined. Aliquots of the filtered hydrolysate were separated into the following fractions and the nitrogen content of each determined by micro-Kjeldahl.

I. *The partition of nitrogen.*

A. 30 cc. of the protein digest (total nitrogen = 55.2 mg.) were added to 50 cc. 3.5 % trichloroacetic acid.

Filtrate (1)	Precipitate (1)
Nitrogen content = 27.2 mg., <i>i.e.</i> 47.6 % of the total nitrogen. To 50 cc. of this solution (10.12 mg. nitrogen) were added 10 cc. saturated normal lead acetate.	Nitrogen content = 28.28 mg., <i>i.e.</i> 51.20 % of the total nitrogen.
Filtrate (2)	Precipitate (2)
Nitrogen content = 6.52 mg., <i>i.e.</i> 11.8 % of the total nitrogen.	Decomposed by H ₂ S, <i>etc.</i> (see above). Nitrogen content = 4.80 mg., <i>i.e.</i> 8.7 % of the total nitrogen.

B. 25 cc. of the protein digest (total nitrogen = 46.0 mg.) were neutralised with 0.25 *N* acetic acid.

Filtrate (100 cc.)	Precipitate
Nitrogen content = 28.4 mg., <i>i.e.</i> 61.7 % of total nitrogen.	Nitrogen content = 16.7 mg., <i>i.e.</i> 36.3 % of total nitrogen.

On adding saturated normal lead acetate to this filtrate a heavy white precipitate was formed. We were unable to separate this precipitate quantitatively, the filtrate being always slightly cloudy. 50 cc. of this filtrate were, however, precipitated with saturated normal lead acetate and the precipitate decomposed with H_2S , *etc.* (see above).

II. The iron distribution.

The trichloroacetic acid precipitate (1) contained little or no iron. The lead acetate precipitate (2) contained the iron in organic combination. The filtrate (2) contained a large amount of ferric iron. The source of this inorganic iron has already been discussed. The substance precipitated on neutralising the hydrolysate with acetic acid contained only a trace of iron. The quantitative results were as follows:

	Iron content mg.
A. Lead acetate precipitate (2) from 50 cc. filtrate (1)	0.082
∴ 80 cc. (approx.) trichloroacetic acid filtrate (1) should contain	0.13
∴ 30 cc. of the original protein hydrolysate contain	0.13
30 cc. of the original protein hydrolysate contain (calculated)	0.14
	Iron content mg.
B. Lead acetate precipitate from 50 cc. acetic acid filtrate	0.071
∴ The acetic acid filtrate (100 cc.) should contain approximately	0.14
∴ 25 cc. of the original protein hydrolysate contain	0.14
25 cc. of the original protein hydrolysate contain (calculated)	0.12

Note. 200 cc. of the original lecitho-vitellin solution contain 0.94 mg. Fe (lecitho-vitellin contains 0.047 % Fe). Allowing for the change in volume from evaporation (measured) during the 72 hours' duration of the digestion 30 cc. of the hydrolysate should contain 0.14 mg. Fe.

It may be concluded from these results that the substance or substances precipitated by normal lead acetate from the trichloroacetic acid filtrate, or from the acetic acid filtrate contain the lecitho-vitellin iron in organic combination.

Copper in lecitho-vitellin.

In the course of investigations on nutritional anaemia we planned to use lecitho-vitellin or its iron-containing fraction as dietary sources of "organic" iron, provided these substances contained no copper. This sample of lecitho-vitellin when analysed by the procedure recommended by McFarlane [1932, 1] was found to contain copper. The amount of copper present, compared with the amount in biological materials in general, was surprisingly high. At this time Dr R. C. Hamilton in this laboratory had prepared another sample of

lecitho-vitellin by essentially the same procedure as already described. The two samples were analysed with the following results:

Sample of lecitho-vitellin	Copper %	Iron*	Ratio Cu : Fe
McFarlane	0.0034	0.047	1 : 13.8
Hamilton	0.0032	0.045	1 : 13.4

* After decomposition of the organic substance the iron was determined by potentiometric titration with titanous sulphate [see McFarlane, 1932, 1].

Egg-yolk, according to McFarlane, Fulmer and Jukes [1930], contains approximately 2.5 g. of protein and about 0.08 mg. copper (average value from their Table IX). They found, however, that by changing the protein in the diet the total protein content of the yolk could be varied as much as 30 %. The copper content of the entire egg was also found to be an extremely variable quantity. Livetin constitutes about one-fifth of the total yolk-protein, according to Kay and Marshall [1928], and the total amount appears to be relatively constant from one egg to another. Assuming that these average values represent approximately the total protein, livetin and copper contents of the egg-yolk; that the lecitho-vitellin contains all the copper, and that the yolk contains only these two proteins, we find:

Average copper content of yolk	0.08 mg.
„ total protein (vitellin and livetin)	2.5 g.
„ amount of livetin	0.5 g.
Vitellin content of yolk	2.0 g.

The copper content of lecitho-vitellin should be approximately

$$\frac{0.08}{2.0} \times 100 = 4.0 \text{ mg./100 g.}$$

Loeschke [1931] has obtained more constant values for the copper content of the hen's egg (0.055–0.095 mg.) than those recorded by McFarlane, Fulmer and Jukes. The copper content of lecitho-vitellin, using the average of Loeschke's values, should be about

$$\frac{0.066}{2.0} \times 100 = 3.3 \text{ mg./100 g.}$$

These calculated values are probably only rough approximations. Since they are, however, of the same order of magnitude as the actual copper content of lecitho-vitellin, it is not improbable that all the copper of the yolk is contained in this protein.

Some preliminary observations on the behaviour of the copper when lecitho-vitellin is hydrolysed by 0.25 N NaOH at 37° have also been made. In the following separations precautions were taken to avoid copper contamination from reagents, glassware, *etc.* All filtrations were made with Jena glass filters.

5 g. lecitho-vitellin were boiled with absolute alcohol for 24 hours. The ash of the alcohol-soluble material contained copper. The amount, too small to determine, must have been less than 0.02 mg. and its presence in this fraction was therefore not considered significant. The vitellin, which was now

pink in colour¹, was hydrolysed with 500 cc. 0.25 *N* sodium hydroxide at 37° for 72 hours. A very small amount of insoluble material was filtered from the digest. The theoretical amount of glacial acetic acid required to neutralise the hydrolysate was added from a burette, drop by drop with constant stirring. A heavy white precipitate formed, which was allowed to settle before filtering. (The acetic acid precipitation is incomplete.) The filtrate was evaporated to a volume of about 100 cc. and the precipitation of the protein completed with trichloroacetic acid.

The two protein fractions were combined, dissolved in the minimum amount of dilute sodium hydroxide and reprecipitated with trichloroacetic acid. The solution and precipitation were repeated once more and the protein fraction finally dried with absolute alcohol. This fraction was also found to contain copper. The amount of copper in 1 g. of the protein was again too small to be accurately determined.

To the protein-free filtrate was added a saturated solution of normal lead acetate and a copious white precipitate formed. The precipitate was decomposed with hydrogen sulphide and the filtrate from the lead sulphide precipitate boiled to remove the hydrogen sulphide. The solution was transferred to a 50 cc. volumetric flask and diluted to volume. This solution was colourless and gave strong biuret, Millon's and glyoxylic acid reactions. It contained a considerable amount of phosphorus, a trace of mercaptan sulphur, and gave a white precipitate with alcohol. This solution was analysed with the following results:

Wt. of substance (or substances) isolated, 0.5440 g.

	% of dry substance
Nitrogen (micro-Kjeldahl)	9.06
Total ash	4.24
Iron [McFarlane, 1932, 2]	1.23
Copper [McFarlane, 1932, 1]	0.0082

Assuming complete precipitation by normal lead acetate, this fraction represents approximately 10 % of the lecitho-vitellin. Only about 25 % of the total copper in lecitho-vitellin was recovered in this fraction. The copper content of the substance or substances isolated is tentatively fixed at 0.008 %. The filtrate from the lead acetate precipitate gave a strong biuret reaction and contained about 10 % of the original lecitho-vitellin nitrogen. Whether the presence of copper in this filtrate is due to incomplete precipitation with lead acetate or to the presence of a second copper-containing fraction in lecitho-vitellin has still to be determined. The presence of lead in this filtrate interferes with the reagent we have used (sodium diethyldithiocarbamate) to detect or determine copper.

The above observations are obviously of a preliminary nature. The composition of this lead acetate precipitate is being investigated.

¹ It was of interest to note that the 72-hour hydrolysate of vitellin (alcohol-extracted lecitho-vitellin) had a fairly deep reddish-brown colour while the same digest of lecitho-vitellin had only a faint yellowish tinge.

DISCUSSION.

During the action of 0.25 *N* sodium hydroxide on lecitho-vitellin an organic iron-containing substance is rapidly liberated from protein combination. The decomposition is accompanied by a more gradual evolution of ammonia and a steady increase in the amount of non-protein nitrogen formed. From the difference in the rates of these reactions it appears unlikely that the latter process is related in any way to that involving the liberation of the iron-containing fraction. No corresponding increase in free NH_2 groups was found. The amount of amino-nitrogen (Van Slyke) was not increased during the hydrolysis.

At the completion of hydrolysis a protein substance can be precipitated by trichloroacetic acid or by neutralising the hydrolysate with acetic acid. This precipitate contains a trace of iron and of phosphorus, the amount of which decreases with repeated precipitation. There is a striking similarity in the action of dilute sodium hydroxide on lecitho-vitellin and on caseinogen.

The filtrate from the trichloroacetic acid precipitate contains about 50 % of the lecitho-vitellin nitrogen. Part of this non-protein nitrogen (about 20 %) is contained in a compound, or compounds, which can be precipitated from acid solution by normal lead acetate. This precipitate contains the lecitho-vitellin iron and part of the copper (approximately 25 %) so combined that no colour is given directly with the sodium diethyldithiocarbamate reagent of Callan and Henderson [1929].

Whilst we have not yet investigated the composition of this lead salt (or salts) in any detail, we have found that some of its properties, particularly so far as its solubility is concerned, differ from those of haematogen. In this respect it resembles more closely the vitellinic acid of Levene and Alsberg [1900] but contains more iron (1.23 %) than that recorded for vitellinic acid (0.57 %).

Having found that the total copper content and the ratio of copper to iron were practically the same in two samples of lecitho-vitellin prepared by different workers, we felt justified in concluding that copper was a constituent of the vitellin molecule. It still remained to be proved, however, that the copper is in chemical union with the vitellin. These results might possibly be explained from the standpoint of adsorption since organic substances and particularly proteins have a great affinity for copper.

We have found that:

1. At least part of the copper of vitellin (25 %) is contained in a fraction which has been separated by precipitation with normal lead acetate.
2. The copper is contained in the filtrate after decomposing the precipitate with hydrogen sulphide in acid solution.
3. The copper is so combined that it does not react with sodium diethyldithiocarbamate, a reagent which will detect less than 0.1 part per million of copper [McFarlane, 1932, 1].

It now appears justifiable to conclude that the copper is present in chemical combination with an organic substance. This copper is present in a substance,

rich in iron, which is generally conceded to be the mother substance of the haemoglobin formed by the avian embryo. It is, therefore, suggested that in the lecitho-vitellin molecule there is a component containing iron and copper in organic combination, which possibly represents, at least in the chick, an intermediate stage in the formation of haemoglobin. In the present state of our knowledge, any further discussion of the possible significance which this fact may have in regard to the action of copper in stimulating haemoglobin synthesis can more appropriately be reserved for a future communication.

Hill [1931] has recently submitted evidence which indicates that the iron of egg-yolk is present as free ferric iron rather than in any stable organic combination, since egg-yolk suspended in acetate buffer does not give the reaction of ferrous iron with $\alpha\alpha'$ -dipyridyl but on adding sodium hydrosulphite a deep red colour is immediately developed. Quantitative determinations of the total iron in egg-yolk by the direct pyridyl reaction, using sodium hydrosulphite and sulphur dioxide or 30 % alcohol, gave results almost identical with those obtained after incineration of the egg-yolk. Needham [1931] accepts these experiments of Hill as disproof of the earlier conclusions of Bunge and Hugounenq and Morel that the iron of haematogen was not merely adsorbed inorganic iron.

All the iron of egg-yolk is not indeed contained in haematogen, and it is not improbable that colloidal ferric hydroxide may also be present. McFarlane *et al.* [1930] have found the iron contents of 38 samples of the total egg-yolk-protein to vary from 0.031 to 0.066 % (average 0.045 % Fe). Taking the average weight of egg-yolk-protein recorded by Kay and Marshall, egg-yolk should contain:

Total protein	4.5 g.
% iron in protein	0.045
Total iron in the yolk-protein	0.002 g.
Total iron content of yolk [Sherman, 1927]	0.0086 g.
" " " [Hill, 1931]	0.0085 g.

Fresh egg-yolk, according to Aggazzotti [1913], has a p_H of about 4.5, while Healy and Peter [1925] record p_H 6.2 to 6.6. The presence of iron as ferric hydroxide is quite possible at the latter p_H but improbable at p_H 4.5. The qualitative reaction with $\alpha\alpha'$ -dipyridyl and egg-yolk in the presence of a reducing agent indicates that at least part of the non-vitellin-iron is present as colloidal ferric hydroxide.

Needham [1931] and Elvehjem [1932] have concluded from Hill's results that egg-yolk contains only inorganic iron. This interpretation of Hill's results involves several very debatable assumptions which the author himself did not choose to make. In his summary Hill says "egg-yolk contains no detectable organic iron analogous to a haematin compound." This, we believe, is a logical interpretation of his quantitative results and of the fact that haematin in the presence of the reducing agent does not react with $\alpha\alpha'$ -dipyridyl.

To consider that these findings constitute proof that the iron in egg-yolk is all in inorganic form is to assume that if egg-yolk contains iron in organic

combination the iron linkage must be the same as that in the haematin molecule. What little evidence we have suggests that in vitellin the iron is less firmly combined than the iron in the haematin molecule. Lintzel [1928] has shown that haemoglobin is extremely resistant to digestion and is not utilised by the organism. According to Elvehjem [1932], the iron of haematin, in contrast with ferric chloride, gave a poor response in haemoglobin production when fed to anaemic rats.

We could find no evidence that $\alpha\alpha'$ -dipyridyl reacts only with inorganic ferrous compounds and does not combine with ferrous organic compounds other than reduced haematin. The ferrous salt of $\alpha\alpha'$ -dipyridyl, according to Blau [1898], is $[\text{Fe}(\text{C}_{10}\text{H}_8\text{N}_2)_3]\text{X}_2$. X may represent a univalent inorganic or organic anion. It is thus possible to have compounds of the type $[\text{Fe}(\text{C}_{10}\text{H}_8\text{N}_2)_3]\text{R}_2$, where R is a negative organic radical.

From our own observations on the cleavage of the vitellin molecule by relatively mild chemical treatment it is reasonable to presume that a strong reducing agent such as sodium hydrosulphite might bring about the same decomposition much more rapidly, *e.g.*,

- (1) Vitellin $\xrightarrow{0.25\text{ }N\text{ NaOH}}$ Protein + FeR_3 (R = organic anion);
- (2) Vitellin $\xrightarrow{\text{Na}_2\text{S}_2\text{O}_4}$ Protein + FeR_2 , and with dipyridyl an addition compound may be formed;
- (3) $\text{FeR}_2 + 3(\text{C}_{10}\text{H}_8\text{N}_2) \rightarrow [\text{Fe}(\text{C}_{10}\text{H}_8\text{N}_2)_3]\text{R}_2$.

The compound finally formed might have a red colour and be soluble in alcohol or extracted from a protein adsorption compound by sulphur dioxide. Since the red colour is probably due to the complex ferrous cation, it is not likely that substitution of an organic anion for an inorganic anion would affect the colour of the compound.

SUMMARY.

1. Some preliminary experiments have shown that during the action of 0.25 *N* NaOH on lecitho-vitellin at 37° there is a gradual evolution of ammonia and a steady increase in the amount of non-protein-nitrogen but no increase in free amino-groups. At the completion of hydrolysis (60–70 hours) the hydrolysate had the following properties.

(a) Trichloroacetic acid, or neutralisation with acetic acid, precipitates a protein substance which contains about 50 % of the lecitho-vitellin-nitrogen but does not contain iron or phosphorus.

(b) Part of the non-protein-nitrogen (about 20 %) is contained in a compound (or compounds) which is precipitated from acid solution by normal lead acetate.

2. Two samples of lecitho-vitellin prepared by different workers contained practically the same amount of copper (0.0033 %) and iron (0.046 %). The iron

and copper are believed to be contained in the vitellin molecule in firm organic union for the following reasons.

(a) All the iron and at least 25 % of the copper are contained in the normal lead acetate precipitate.

(b) The iron and copper are contained in the filtrate after decomposition of the lead salt, or salts, by hydrogen sulphide. This solution gives no direct reaction with potassium thiocyanate or sodium diethyldithiocarbamate. The latter reagent will detect less than 0.1 part per million of copper.

(c) The iron and copper contents of this fraction are tentatively fixed at 1.23 and 0.0082 % respectively of the dry weight of substance.

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