Physiology. — Prof. PEKELHARING makes a communication concerning „*pepsin*".

Some years ago I communicated to this meeting a new method for the preparation of pepsin. In artificial gastric juice, obtained by digesting the mucous membrane of the stomach with 0.5% H Cl for some days, I had found a substance, which is little soluble in water, containing about 0.02% HCl, but which becomes more easily soluble as well by a higher as by a lower amount of hydrochloric acid in the fluid. This substance appeared to be an extremely complicated proteid, which possessed to a very high degree the power of digesting proteid matter in acid solution. I supposed this substance might be the enzyme itself and not a proteid matter mixed with the enzyme and my grounds for this supposition were in the first place the extraordinary amount to which this substance was able to digest proteid matter and secondly the observation that the coagulation-temperature of this proteid matter, dissolved in hydrochloric acid, is just the same as the temperature, which makes the enzyme inactive. I had however to confine myself to a supposition, as my material was not of a sufficient degree of purity. I found that it always contained phosphorus, but the amount of phosphorus, though generally about 1%, was variable.

On account of the great importance of the question concerning the nature of the enzymes, I have continued my efforts to obtain the substance in a purer state. At first I used for that purpose the mucous membrane of the stomach of the pig. The method of preparation was altered in some respects in order to improve the purifying of the substance and to increase the amount of pepsin.

I can add to what was stated before, about the nature of the substance, that from its solutions it can also be precipitated by ammoniumsulfate.

After treating the extract of the mucous membrane in the above mentioned way, with basic plumbic-acetate and ammonia and decomposing the precipitate with oxalic acid and dialysing and filtering the thus obtained concentrated solution, there could be obtained out of the filtrate, by saturating with ammonium sulfate, still a considerable quantity of pepsin, which when purified by dissolving in H Cl 0.2% and dialysis, showed exactly the same qualities as the pepsin, prepared directly from the artificial gastric juice and from the lead-precipitate, it especially possessed as great a

[1]) Account of the Meeting on May 30, 1896.

digesting power. Whereas from the solution that was greatly contaminated by products of digestion, a precipitate fit for being filtered was only produced by complete saturation with ammonium sulfate, the pepsin will completely separate from solutions containing but few products of digestion, by half saturating the fluid with this salt.

If the substance is slowly precipitated either by dialysis into water or under the influence of ammoniumsulfate, the precipitate is not amorph, but has the shape of small globules, resembling the globulites of albumen, which after HOFMEISTER'S method can be obtained from egg-albumen. The little globules of pepsin are however smaller, the largest have a diameter of about 15 à 20 μ.

When kept under ammoniumsulfate, pepsin can remain unaltered for a very long time. A preparation, entirely consisting of small globules, repeatedly washed out by decantation with half saturated ammoniumsulfate solution and finally kept in this fluid in an apartment, in which the temperature undergoes great variations, has already remained unaltered for four years. It shows not only no alteration when investigated with the microscope, but when freed from ammoniumsulfate and dissolved in hydrochloric acid, it also digests proteid matter very powerfully.

Though I have treated some hundreds of mucous membranes of pigs' stomachs I have not succeeded in preparing from them a pepsin of constant composition. By analysing some five preparations, purified as much as possible, the values found for the various elements varied between the following numbers:

	C	H	N	P	S
maximum	50.77	7.27	15.06	0.75	1.6
minimum	48.18	6.72	14.02	0.425	1.45

I thus became convinced, that I could not hope for a better result if I continued the purification of the substance by dissolving it again and again in HCl 0.2% and then precipitating it by dialysis into water, on account of the great variability of the substance, the perseverance with which it retains contaminations and the great loss of substance, which is inevitable when purifying it and which would make the preparation of quantities such as are required for analysis, practically impossible.

I therefore resolved to try whether the gastric juice of the dog, which now, owing to PAWLOW'S brilliant researches, can be obtained

in unlimited quantity and without admixtures, should perhaps give more satisfactory results.

My request to Prof. PAWLOW for some further information about the method of operating, was answered with the greatest kindness by a very detailed and accurate account by Dr. WALTHER about everything that must be taken into consideration when applying the gastral fistula and oesophageal fistula, according to PAWLOW's method. Prof. NARATH then kindly applied for me in a dog of 25 KG., first a gastral fistula and when the wound was completely healed and the silver cannula was fixed in it, also an oesophageal fistula. Besides to the gentlemen already mentioned, I am also greatly obliged to Messrs. DE BRUIN, SCHIMMEL and THOMASSEN, lecturers at the State Veterinary School at Utrecht, who when the good healing of the wound in the stomach was in some danger, repeatedly gave me excellent assistance.

Throughout the whole of the year 1901 the dog, who was always in an excellent state of health, was regularly used, generally three times a week, for the producing of gastric juice. By a pseudo-feeding with meat there was obtained in the first hour an average of 200 cc., in the second hour still an average of 100 cc. of gastric juice. Each time the fluid obtained in a quarter of an hour, was filtered in order to free it from little flakes of mucus. The filtrate was then perfectly colourless and clear. Only occasionally when the amount of pepsin was great and the temperature of the room was low, it showed some opalescence, which however, when heated to the temperature of the body, disappeared at once without leaving a trace, to reappear again when cooled. The amount of acid was on an average 0.16 norm. H Cl.

As NENCKI and SIEBER already stated a short time ago, the pepsin is precipitated for the greater part from the pure gastric juice of the dog, in the same way as from the extract of the mucous membrane of the stomach, prepared with hydrochloric acid, as soon as the amount of acid is decreased to about 0.02 %: the best by dialysis. In the dialysator the substance is then deposited just like the pepsin out of the mucous membrane of the pig, in the shape of transparent small globules. While however the pepsin of the pig, at least when dried, was always somewhat coloured, even when it was separated out of a solution, in which no decided colour could be seen, the pepsin of the dog was always,

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[1]) Zeitschr. f. Physiol. Chemie, Bd. XXXII, S. 291.

even after drying, of a pure white colour. This was only then not the case, when, what occurred a few times, the gastric juice was mixed with bile. Gastric juice which, by admixture with bile, was distinctly coloured yellow, was never used for the preparation of pepsin. But it could be shown that even the smallest traces of bile-pigment, were retained by the pepsin. When the gastric juice contained but so little of it, that the yellow colour could not clearly be distinguished, the pepsin, precipitated from it by dialysis, yet showed a yellow tint which became greenish when dried. For analysis I have exclusively used entirely colourless pepsin.

The fluid, separated from pepsin which had been precipitated by dialysis, was half saturated with ammoniumsulfate. Thus a not unimportant precipitate was again obtained. This was dissolved, when liberated from ammoniumsulfate by dialysis into 0.2 % H Cl, at 37° C. in as little hydrochloric acid as possible of the same strength, filtered and subjected to dialysis into distilled water. The substance thus precipitated was dried and separately collected for analysis.

When the pure gastric juice was half saturated with ammoniumsulfate, in the filtrate of this no perceptible precipitate could any more be produced by complete saturation with this salt. The fluid, dialysed for 24 hours, filtered and then half saturated with ammonium sulfate, still after total saturation showed a certain amount of turbidness. When dialysed at a low temperature, a very small portion of the substance is always decomposed, whereby albumose is liberated.

In the first place I have investigated the amount of phosphorus. The continued investigation of the pepsin, obtained out of pigs' stomachs, had already made me doubtful, whether the phosphorus I found in it, really owed its origin to the highly complicated proteid, of which this pepsin principally consists. As I have formerly stated the amount of P of my first preparations was about 1%, but already then I observed that a more or less considerable contamination with substances containing phosphorus, was likely. The more the pepsin was purified, the smaller the amount of phosphorus was generally found to be, though I found the pepsin out of the pig's stomach never free from it. Moreover I had become convinced that I had erroneously placed the coagulation-product, which is obtained by heating of the acid solution of pepsin, on one line with the well-known nucleo-proteids. Later on I shall again refer to that.

I now found the pepsin of the dog free from phosphorus. The substance separated by dialysis from the gastric juice, still contained a trace of it, but such a small quantity (about 0.01 %) as can only be attributed to impurity.

This impurity was apparently quite removed together with the precipitate, which was formed when dialysing. For when afterwards the pepsin which was still in solution, was precipitated by half saturating with ammoniumsulfate and purified by dissolving it in hydrochloric acid and dialysis, there was not found in it the slightest trace of phosphorus. Nevertheless this pepsin was equally able to digest proteid matter as was that which was separated directly by dialysis out of the gastric juice.

NENCKI and SIEBER did find phosphorus in the pepsin, prepared by them out of the gastric juice of the dog, even when the pepsin was washed out with alcohol and had thus been altered. However they found the amount of P not only small, but also in various preparations very different. In the pepsin, precipitated by dialysis and not washed out, it varied between 0.073% and 0.148%, in the preparations washed out with alcohol between 0.045% and 0.091%.

They take for granted that pepsin contains lecithine; partly at least, not as an impurity, but in a combination to be compared with the compounds of lecithine with glycose, morphin, etc., especially studied by BING.

I am not prepared to deny the possibility of the presence of a compound of pepsin with lecithine in the gastric juice, nevertheless I wish to lay stress on this : that the existence of the enzyme should not be considered as being connected with the presence of lecithine or any other P-containing group in the molecule of the pepsin, now that I have succeeded in preparing very powerful pepsin, in which either no phosphorus at all, or only a very insignificant trace of it could be shown.

Contamination of the pepsin with phosphorus can, besides by lecithine, also be caused by other substances. In the gastric juice, obtained by pseudo-feeding, there always occurs, at least in the dog used by me, some mucus, which can easily be removed by filtration. This consists of a P-containing proteid matter. When taken from the filter and washed out with water and alcohol, it scarcely dissolves at all in diluted hydrochloric acid, but by digesting with pepsin and hydrochloric acid, it gradually loses the gelatinous character and dissolves for the greater part, while a sediment, easily soluble in alkali, is formed.

This mucine is probably a nucleo-proteid and it is certainly possible, that when kept in contact with the gastric juice for some time and partly digested, it may yield *P*-containing decomposition-products to the solution. I have therefore immediately filtered the gastric juice, obtained in each quarter of an hour.

As to the amount of chlorine my statements agree with those of NENCKI and SIEBER. While the latter found in five determinations, either 0.47 % or 0.48 %, I found 0.49 %. To the reasons, given by these investigators, for the opinion, that chlorine forms an element of the molecule of pepsin I may add another. The pepsin that was separated either directly by dialysis, or first by ammonium sulfate, was then dissolved, in as great a concentration as possible, at 37° C. in 1 % oxalic acid and precipitated from this solution by dialysis into distilled water. This precipitate rapidly digested fibrin, when dissolved in oxalic acid. If now the chlorine found in the pepsin, which was separated out of the solution in hydrochloric acid, did owe its origin to hydrochloric acid that was not sufficiently washed out, then we might expect, that in the substance, separated out of oxalic acid, the clorine could no more be found. Nevertheless chlorine could repeatedly be shown very distinctly in this.

The results of the analysis of six preparations follow here.

	C	H	N	S	
I.	52.13	7.06	14.13	1.66	Pepsin precipitated by dialysis of the gastric suice.
	52.06	7.19	14.33		
	51.81	7.09			
II.	51.92	7.14	14.50		
III.	52.13	7 01	14.58	1.63	
IV.	51.61	6.93	14.57	1.61	
V.	52.32	7.16	14.75	1.83	Pepsin precipitated by half saturating the dialysed gastric suice with ammoniumsulfate.
VI.	52.01	7.02	14.65		

That the amount of sulfur in V was found a little higher than in the other preparations, may perhaps be attributed to contamination with ammonium sulfate. This supposition is perhaps supported by the amount of nitrogen. With 0.2 % S derived from $(NH_4)_2 SO_4$ corresponds 0.175 % N. So the amount of N whould become 14.575 % and would better agree with the amount of nitrogen of the other preparations.

The quantity of preparation VI was not sufficient also to determine the amount of sulfur. The amount of ash is not, as with all the other preparations, taken into account here. Herein lies however no objection of any significance, because the amount of ash was

always found to be very slight. In the pepsin precipitated by dialysis it was ± 0.1%, in that which was first precipitated by ammonium sulfate ± 0.2%.

Mme. SCHOUMOW—SIMANOWSKI [1]) found for the pepsin precipitated by cooling out of the gastric juice of the dog and washed with alcohol:

C	H
50.71	7.17

and for pepsin precipitated from the gastric juice, by saturating with ammoniumsulfate:

C	H	N	S
50.37	6.88	14.55	1.35
		15.0	1.24

where the amount of ash was not taken into account, whereas NENCKI and SIEBER found, when analysing the substance precipitated by dialysis:

	C	H	N	S
	51.26	6.74	14.33	1.5
and				
	51.99	7.07	14.44	1.63

is the average result of the four preparations analysed by me, of pepsin precipitated by dialysis.

The difference concerns especially the amount of carbon, which was found by me in all my preparations higher than by the Russian investigators, though my figures are already very near those of NENCKI and SIEBER. On account of the absence of phosphorus in my preparations and also of the smaller amount of ash (N and S found 0.57%) I think I may consider the substance prepared by me, as being better purified. Also in the pepsin out of the mucous membrane of the pig's stomach, which I did not succeed in liberating from phosphorus, I found a lower amount of carbon, varying between 48.18 and 50.77%.

In the ash I could detect iron in accordance with NENCKI and SIEBER. I have not made determinations of the amount of iron.

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[1]) Arch. des Sc. biol. T. II, p. 463.

From the cleavage-products, into which the substance is split by heating, I have up till now only studied the coagulation-product more accurately, which is precipitated when the heating is done rapidly. Formerly I already stated that by boiling with mineral acids, purine-bases can be obtained out of this. To this I can only add that I have been able to prepare out of the pepsin of the pig a basis which was found to be xanthine. At first I found no reducing substance after boiling the coagulation-product with mineral acid. Further investigation however taught me, as is already stated by FRIEDENTHAL [1]) and also by NENCKI and SIEBER, that when so treated a reducing substance is indeed liberated, which shows the qualities of a pentose. Nevertheless, although this proteid matter contains purinebases and a carbohydrate group, it will not do to continue to group it any longer among the nucleo-proteids, as it contains no phosphorus. That no phosphorus was found in it, when it was prepared out of the pure pepsin of the dog, is a matter of course. But also when it was precipitated from the fresh gastric juice by boiling and then carefully washed, successively with water, alcohol and ether, I could not find a trace of phosphorus in it. When investigating the coagulation-product out of the pepsin of the mucous membrane of the pig I had already been doubtful, whether this substance might indeed be considered to be a nucleo-proteid, on account of the failing of all my efforts, to prepare a nucleic acid out of it.

It did produce an acid, when treated with alkali, but this acid was a proteid matter, which proved to be little soluble in water, insoluble in diluted acid and easily soluble in warm alcohol.

This acid is best prepared in the following way:

The coagulation product is dissolved in 1% hydrate of potassium and is boiled with this in the water-bath for five minutes. The fluid which was first entirely colourless, then acquires a light yellow tint. It is now made acid with hydrochloric acid. Hereby a considerable precipitate is formed under development of sulfureted hydrogen, which, when cooled, is filtered off. It is of a pure white colour. The filtrate is also colourless, but this becomes yellow again with alcalic reaction. The filtrate gives biuret reaction and produces, after boiling with hydrochloric acid, pentose.

The precipitate is washed out with 0.5% HCl, dissolved in boiling alcohol of 85% and filtered, when hot. On cooling it, the

[1]) ENGELMANN's Archiv. f. Physiol. 1900, S. 189.

substance is precipitated as a gelatinous mass, at the surface of which a clear layer of alcohol gradually separates, which retains but little of the substance in solution. The precipitate, was first washed by decantation with 96 % alcohol and then mixed with equal volumes of alcohol and ether, when it becomes flaky and deposits well. It is at last brought on the filter, washed with pure ether and dried. When drying the precipitate which was first of a pure white, sometimes acquires a light yellow tint.

This substance has the qualities of an acid. Brought into water, it is dissolved, with an acid reaction if an alkali be carefully added. When this acid reacting solution is subjected to electrolysis, under a tension of ± 50 Volt, in the way lately described by HUISKAMP [1]) after being freed as much as possible from other salts, by dialysing into repeatedly renewed distilled water for a few days, then the proteid is transported to the anode and is there deposited as a gelatinous lump, while at the negative pole alkali is accumulated.

I propose to give to this substance the name of *pepsinic acid.*

Out of the alcoholic solution the substance separates by evaporation of the alcohol, as a varnish-like substance. It gives biuret and xantho-protein-reaction and the reactions of ADAMKIEWICZ and of MILLON. As might be expected it does not yield sulfur, when boiled with hydrate of potassium. By boiling for a long time with alkali it is changed further. Addition of hydrochloric acid then no more causes any precipitate.

Out of pepsin solutions, which are changed by heating slowly without becoming turbid by this, this substance cannot be obtained. I first became acquainted with the pepsinic acid as a decomposition product of the pepsin, prepared out of the mucous membrane of the pig's stomach and I afterwards obtained it in exactly the same way out of the pepsin of the dog. However I never succeeded in preparing the pepsinic acid of the pig entirely colourless. Therefore I did not use this for elementary analysis.

The analysis of the pepsinic acid of the dog gave the following results:

C	H	N	S
50.79	7.02	14.44	1.08

whereas for the coagulation-product, out of which this acid is prepared and which may be considered as an acid proteid matter itself, was found:

C	H	N	S
50.35	6.98	14.90	1.64

[1]) Zeitschr. f. Physiol. Chemie, Bd. XXXIV, S. 32.

The highly complicated proteid matter, which besides mucine, is the only albuminous element of the gastric juice of the dog, could only in so far be said to differ from the substance, prepared out of the mucous membrane of the pig's stomach, by the fact that the latter was not sufficiently pure. As to the qualities and the decomposition-products, as far as the investigation of these was possible, they were exactly the same. As I mentioned before, such a substance may also be prepared out of the mucous membrane of the stomach of the dog and of the calf.

This substance is, like other proteid substances, levogyr. I have not been able to detect any influence of the reaction of the solution upon the degree of rotation.

When it is now taken into consideration that the substance obtained out of the gastric juice of the dog can be made to acquire a degree of purity that is satisfactory for proteid matters, the hypothesis that this substance is the enzyme itself and does not own its activity to admixtures, does not appear to be a very bold one.

The substance loses the action of pepsin by heating, at the exact temperature which decomposes it.

When gastric juice, by half saturating it with ammoniumsulfate, is liberated from this substance, it loses its capacity to digest proteid. With respect to this it shonld be taken into consideration that the presence of ammoniumsulfate is highly detrimental to the action of pepsin. But I have repeatedly convinced myself, that the fluid, also when the salt was removed to a trace by dialysis, was quite unable to digest fibrin. If we now consider that $^1/_{1000}$ mgr. of the substance prepared by me still shows a distinct though weak action on fibrin in 6 ccm. H Cl 0.2 %, then we may rightly conclude, that the filtrate freed from ammoniumsulfate by dialysis, does not contain any pepsin, all the enzyme thus being precipitated by the salt out of the gastric juice. And in this precipitate we find nothing but the proteid matter, which shows the action of the enzyme as strongly as possible.

I find another argument for my view in the observation, that the digesting power of the gastric juice keeps pace with the quantity of the coagulation-product precipitated from it by heating. This is especially clear, when in the dog the secretion of gastric juice is increased by injection of diluted alcohol into the rectum during the pseudo-feeding.

The gastric juice secreted under the influence of alcohol, is more considerable in quantity and surely as rich, but generally a little richer in acid than that secreted before; but poorer in pepsin. The coagulation-

product, which doubtless owes its origin to the substance which I consider to be the enzyme, is then always precipitated in a perceptibly smaller quantity. The determination of the relative quantity of pepsine in the gastric juice took place after the method of METT, whereas the quantity of the coagulation-product was determined by boiling 50 cc. of the gastric juice over the flame and bringing the precipitate, after cooling, on a weighed filter, washing it out successively with water, alcohol and ether, drying it at 110° C. and weighing.

That solutions of pepsine can be prepared, which act powerfully and yet show no proteid-reactions is, as I pointed out before and as has also been stated lately by NENCKI and SIEBER, no reason to deny to pepsin the nature of a proteid matter.

By BLISS and NOVY an observation is mentioned, which might raise a doubt, as to whether pepsin can indeed be considered to be a proteid matter [1]). They found namely, that pepsin was not changed at all by formaldehyde, although this substance affects various proteid matters and makes them insoluble. I convinced myself of the justness of the observation. Solutions of pepsin in hydrochloric acid, to which formol is added to an amount of 2 to 3 %, may be kept for days, without perceptibly losing any digesting power. Of course the amount of formaldehyde must be considerably decreased, either by diluting or by dialysis, before the solution is brought in contact with fibrin, because otherwise the fibrin itself would be affected by formol and made unsusceptible for digestion.

It can not be maintained, however, that all proteidmatters are changed by formaldehyde. It is especially the proteidmatter here treated, which is not affected by this. I have dissolved the purified matter in 0.2 % H Cl, while adding formol, and been able to precipitate it again from this solution, as well by dialysis, as by ammoniumsulfate, without it having lost any of the qualities of pepsin.

The substance possesses not only the power of digesting albumen in an acid solution, but it also causes milk to coagulate, as I stated before. In accordance with NENCKI and SIEBER I also found that it forms "plastein" out of albumose.

NENCKI and SIEBER have argued, that there is no serious objection to the supposition, that one and the same molecule may have various enzym-actions. With that argument I quite agree. To stick

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[1]) Journal of exp. med. Vol. IV. p. 47.

to the well-known analogy, given by E. FISCHER, there are keys, which consist of a ring with different pieces attached to it, which each fit into a different lock. Even when one or more of those pieces are bent or made useless in some other way, those that are left can still be used.

While the gastric juice of the dog used by me, showed very clearly the fat-decomposing action, described by VOLLHARD[1]), the pure pepsin, prepared out of it, had not the slightest action on fat, neither by neutral nor by acid reaction.

Mathematics. — "*The differential equation of* MONGE." By Prof. W. KAPTEYN.

In our communication of June 6th 1901 we gave the results of our investigation of the differential equation

$$r - \lambda^2 t + \mu = 0,$$

in which λ and μ were supposed to be dependent only on p and q or on x, y and z.

If we now assume no limiting conditions with respect to λ and μ, we shall find that the above mentioned equation can possess two intermediate integrals in the following case only.

Suppose c, h, v and ϱ to be any functions respectively of x, of y, of x, y, z and of v; then putting

$$G = \frac{\partial}{dx} + p\frac{\partial}{\partial z} \quad , \qquad H = \frac{\partial}{\partial y} + q\frac{\partial}{\partial z},$$

λ must be equal to $\frac{P}{Q}$ and μ equal to $\lambda^2 Q_1 + P_1$,

where

$$P = \frac{c^2 G^2(v) - 1}{c\, v_3^2} \qquad Q = \frac{h^2 H^2(v) - 1}{h\, v_3^2}$$

$$P_1 = \frac{\varrho'}{\varrho}\frac{v_3}{c} P + \frac{c_1}{c\, v_3} G(v) + \frac{1}{v_3} G\, G(v)$$

$$-Q_1 = \frac{\varrho'}{\varrho}\frac{v_3}{h} Q + \frac{h_2}{h\, v_3} H(v) + \frac{1}{v_3} H\, H(v),$$

[2]) Munch. med. Wochenschr., 1901, S. 141 and Zeitschr. f. klin. Med. Bd. XLII, S. 414.

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