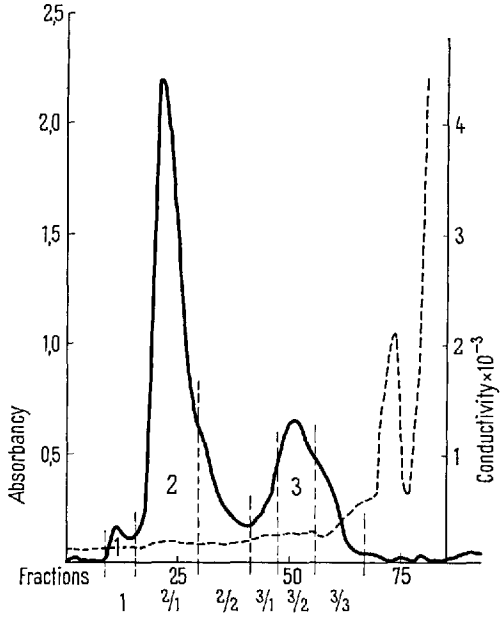

Fig. 1. Gel filtration of 1.5 g/30 ml of inhibitors (IPP) through Sephadex G-25 column (3.2 × 200 cm) equilibrated and eluted with 0.01 *M* acetic acid. Fractions (15 ml) collected at a rate of 60 ml/h. Solid line: absorbancy at 280 mμ, broken line: specific conductivity.

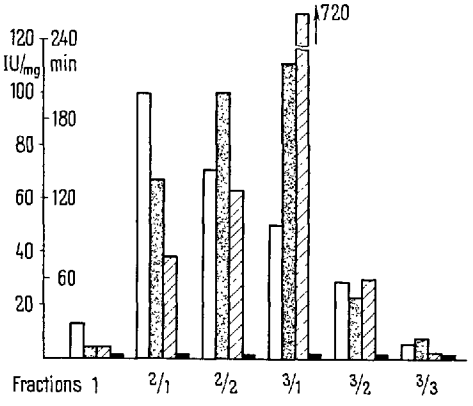

Fig. 2. Inhibitory activity of individual Sephadex-fractions of IPP against some proteolytic enzymes. Equal weights of individual freeze-dried taken into experiment. Trypsin □ and chymotrypsin ⁞⁞⁞: inhibitory activity is relation of the total amount of enzyme to the total amount of inhibitor which decreases its activity to 50%. Plasmin /// and plasmin-activator ●: lysis-time of a standard coagulum in min (control time 5 min).

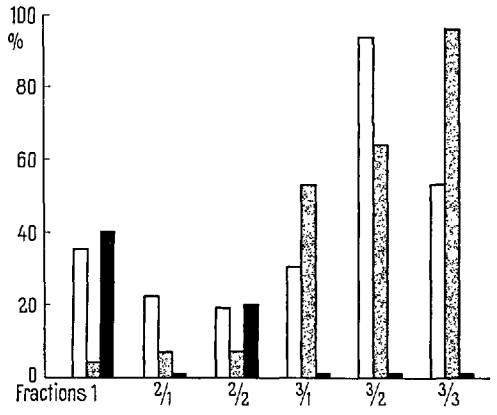

Fig. 3. Some biological properties of individual Sephadex-fractions of IPP. Anti-inflammatory activity □: inhibition of the kaolin inflammation of paw in rats in %. Irritant activity ⁞⁞⁞: mg of material in 0.1 ml of saline causing 3 times area of blueing after saline alone. Haemagglutinating activity ● :amount of the material in mg in the system with 0.05 ml of rabbit blood causing 50% agglutination.

fractions 3/2 and 3/3 of antiphlogistic activity, fraction 3/1 showed an antichymotrypsin and particularly antiplasmin activity. The high-molecular fraction 1 provokes a marked agglutination of erythrocytes. Its antiphlogistic activity is then probably the result of pronounced toxicity. Fractions 2 and 3, on the other hand, are relatively non-toxic. Inhibition of the plasmin-activator did not attain estimable values. More detailed fractionation studies are in progress[4].

Zusammenfassung. Vorläufige Fraktionierung an Dextran-Gel Sephadex G-25 (Pharmacia, Uppsala) ergab eine Trennung des Proteaseinhibitors aus Kartoffeln in die einzelnen Komponenten, die neben der eigentlichen entzündungshemmenden Aktivität noch Antitrypsin- bzw. Antichymotrypsin-, Antiplasmin- und ausgeprägte Hämagglutinierungsaktivität aufwiesen.

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Research Institute for Pharmacy and Biochemistry, Praha (Czechoslovakia), November 2, 1962.

[4] We wish to express our thanks to Mr. D. KAŠÍK and Mrs. L. ŠEBKOVÁ for helpful technical assistance. Biological tests were carried out by Dr. J. HLADOVEC and Dr. Z. HORÁKOVÁ of our Institute; plasmin-activity was determined by Ing. M. RYBÁK from Institute for Haematology and Blood Transfusion.

On the Mechanism of Spore Germination in *B. subtilis*. Permeability and Germination by Pyruvate

L-alanine induces germination in spores of *B. subtilis* incubated at 37° in phosphate buffer at neutral pH[1]. Undoubtedly *L*-alanine acts through a 'substrate' mechanism[2,3], i.e. germination depends upon the breakdown of the aminoacid. The first step which occurs in spores is deamination of alanine by the action of an *L*-alanine oxidase[4] or a DPN-linked *L*-alanine dehydrogenase[5–7]. It has been demonstrated that the production of pyruvate and ammonia does not imply loss of heat resistance and that pyruvate must be further metabolized to have germination[3,4].

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On the other hand, pyruvate, under the same experimental conditions under which alanine is effective, does not induce germination[1,8]. At the present state of our knowledge, two hypotheses can be put forward to explain this discrepancy:

(a) the oxidative deamination of alanine, which has been demonstrated to occur in spores of *B. subtilis*, is an essential step in furthering the metabolism of pyruvate. This reaction, in fact, is strongly exoergonic ($\Delta g = -17{,}9$)[9].

(b) spores are not permeable to pyruvate and, therefore, the ketoacid cannot come in contact with the enzymatic systems inside the cells.

The permeation of spores by solutes depends upon three molecular variables: (1) molecular dissociation and ionic charge; (2) solubility in lipids; (3) molecular weight and shape. The second and third variables are more favourable to the penetration of pyruvate than of alanine into the spores, since pyruvate has a higher solubility in lipids and a lower molecular weight. The first variable, on the contrary, indicates that pyruvate should be taken up much less than alanine. In fact, the spore surface is negatively charged at the usual neutral pH of the germination experiments[10] and, therefore, the strongly acidic molecule of the ketoacid should penetrate only with difficulty because of repulsion between it and the polar groups of the spore surface.

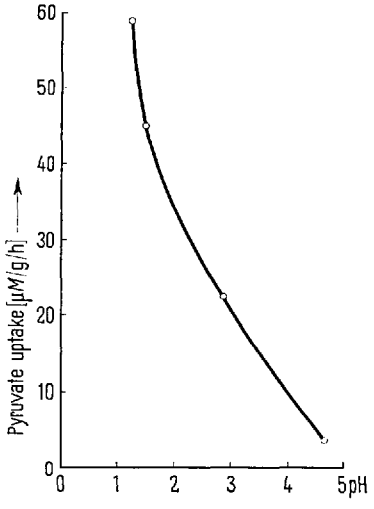

Fig. 1. Pyruvate uptake by spores of *B. subtilis* as a function of pH.

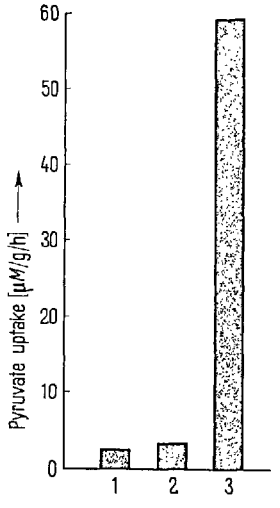

Fig. 2. Pyruvate uptake by spores of *B. subtilis* under different conditions: (1) at pH 7. (2) at pH 7 (after pretreatment of spores at pH 1.2 for 5 min at 37°). (3) at pH 1.2.

Previous experiments, on the other hand, have shown that incubation of spores of *B. megaterium* at pH 4 with pyruvate induces germination[3], and that incubation of spores of *B. subtilis* in HCl-KCl buffer $5 \times 10^{-2} M$ at pH 1.1–1.3 with pyruvate $5 \times 10^{-2} M$ for 5 min at 37°, is effective in inducing germination (about 60%) of the spores, if they are washed after the treatment, resuspended in phosphate buffer $3 \times 10^{-2} M$ at pH 7.3 and incubated at 37°C for 30 min[11].

The presumed difficulty of pyruvate in penetrating the spores under the usual conditions of germination experiments (neutral pH) and the fact that spore germination by pyruvate occurs if a preliminary short contact with this substrate is allowed at a low pH, have led us to investigate: (1) the permeability of spores to pyruvate as a function of the pH; (2) possible irreversible alterations of the spore permeability to pyruvate by treatment at low pH.

Materials and Methods. Clean spores of *B. subtilis* strain 6633 ATCC[4], heat shocked for 60 min at 60°C were suspended at the concentration of 7.5 mg/ml (dry weight) in HCl-KCl buffer $5 \times 10^{-2} M$ (pH 1.1 to 1.5) or in citrate-citric acid buffer $5 \times 10^{-2} M$ (pH 2.8 to 4.6), both containing sodium pyruvate $3.3 \times 10^{-2} M$ and 154×10^3 c.p.m. per ml of pyruvate-2-C^{14}, and incubated at 37°C in a water bath. After 5 min the spore suspensions were rapidly chilled to 0°C and centrifuged at 2° at 16000 *g* for 10 min. The sediments were washed with distilled water in the centrifuge as above. The spores were finally resuspended in distilled water and an aliquot used for radioactivity measurements. A Nuclear-Chicago gas flow counter with automatic sample changer and printing timer was used for the counting. The spores were prepared in a dry ultrathin layer on aluminium sample pans (0.212 mg dry weight/cm²). The μmoles of pyruvate taken up per mg of dry weight spores in 60 min were calculated as follows:

$$\text{Pyruvate uptake} = \frac{\text{radioactivity in spores}}{\text{specific radioactivity of pyruvate in the medium}}$$

Results. Figure 1 shows that spore permeability to pyruvate is strongly influenced by environmental pH. A sharp maximum of uptake of the ketoacid is observed at pH 1.1–1.3; at pH 3 and at pH 4.6 only 29% and 6% respectively of the maximum are taken up by the spores. These results demonstrate that at the usual pH of germination experiments, where pyruvate does not act as a germinating agent, the spores are practically impermeable to the ketoacid, while at pH 1.1–1.3, where a 5 min preincubation with pyruvate allows the spores to germinate after transfer into phosphate buffer at pH 7.3, pyruvate is taken up in a large amount by the spores.

It is to be assumed that the changes in spore permeability to pyruvate shown in Figure 1 are correlated with changes in the electric behaviour of the spore surface and of the permeating molecules. In fact, by lowering the pH,

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the dissociation of the –COOH group of the spore surface decreases. Below pH 2, which corresponds roughly to the isoelectric point of the basic and acidic groups of the spore surface[10], the electric charge is presumably reversed.

The exposure of spores to a low pH, even if for a short time, however, could produce changes of the spore surface other than changes of the electric behaviour. To test this possibility, an experiment was performed, the results of which are shown in Figure 2. Heat-shocked spores were divided into 3 aliquots. The first was incubated at pH 7.3 with pyruvate for 30 min, the second preincubated at pH 1.1 at 37°C for 5 min and than washed and incubated at pH 7 with pyruvate for 30 min; the third was incubated at pH 1.1 at 37°C with pyruvate for 5 min.

The results clearly demonstrate that the exposure for 5 min of spores at pH 1.1 does not produce any change in the permeability behaviour of spores to pyruvate at pH 7.3, and therefore it can be assumed that only reversible changes in the electric behaviour of the spore surface and the pyruvate molecule are responsible for the large keto-acid uptake at pH 1.1.

Discussion. The results reported in this paper strongly support the view that pyruvate does not induce germination under the usual experimental conditions of spore germination (neutral pH) because it cannot penetrate the cell in an amount sufficient to activate the dormant enzymatic systems ('substrate' hypothesis). The difference in germination-inducing capacities of alanine and pyruvate at neutral pH are not to be interpreted, therefore, as a consequence of inability of the spores to metabolize pyruvate, but of the impossibility of an actual contact between the enzymatic systems *inside the cell* and pyruvate *outside the cell.*

When pyruvate is allowed to come inside the cell through an artifice such as a short preincubation with the keto-acid at low pH, or by using alanine which easily penetrates and causes formation of pyruvate inside the cell, germination of the spore occurs.

Riassunto. È stata studiata la permeabilità delle spore batteriche al piruvato. Il piruvato penetra nelle spore soltanto a pH basso, quando la molecola del chetoacido ed i gruppi polari della superficie delle spore sono largamente indissociati. Le modificazioni della superficie, che si verificano nel corso della esposizione a pH acido, non hanno alcuna importanza nel fenomeno. Questi risultati sono messi in relazione con il fatto che il piruvato induce germinazione delle spore soltanto a pH basso e contribuiscono all'ipotesi che un agente germinante, per essere attivo, deve penetrare nella spora ed attivare i sistemi enzimatici quiescenti.

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The Distribution of Nutrients in the Seed Parts of Bengal Gram

In India, Bengal gram (*Cicer arietinum* L.) constitutes a major pulse crop and over 5 million tons are produced annually. Besides other nutrients, it serves as a good source of cheap protein. It is consumed in different forms, generally involving removal of certain component parts, the seed coat and embryo[1].

Investigations on the chemical composition of different anatomical parts of some cereal grains[2–7] have shown that the whole seed is quite an inhomogenous entity and that the various parts differ in respect of their chemical composition. Similar information in respect of Bengal gram, or as a matter of fact of any dicot seed, is not available except for the distribution of nitrogen in the seed parts of ripening pea (*Pisum sativum*)[8].

Seeds of Bengal gram, variety Pb7 were soaked, overnight, in glass distilled water just sufficient to be absorbed completely by the grains. The separation of the seeds into three components, the seed coat, cotyledons, and embryo, was carried out by hand. The components were then dried, weighed and powdered in a coffee grinder to pass a 36 mesh sieve. For chemical analysis, A.O.A.C.[9] methods were employed. The carbohydrate content was, however, obtained by difference.

Analysis for a few constituents of the cotyledons and the seed coat separated mechanically, without soaking the seeds, showed that soaking did not bring about any significant migration of the nutrients. The chemical composition of the different component parts is shown in the Table.

It may be seen from the Table that, although, the embryo is the richest part of the whole seed, it, being smallest

Chemical composition of the component parts of Bengal gram

Constituents	Seed coat	Embryo		Whole seed	
		Cotyledons	Rest of the embryo	Determined	Calculated [a]
Proportion (%)	14.55	84.21	1.22		
Protein (%) (N ×6.25)	2.86	25.00	36.99	21.06	21.92
Ether Extractives (%)	0.22	5.32	13.08	4.73	4.67
Ash (%)	2.82	2.56	5.14	2.74	2.63
Crude Fibre (%)	48.25	1.00	3.10	8.90	7.96
Carbohydrates (%) (by difference)	45.85	66.12	41.69	62.57	62.82
Phosphorus (mg/100 g)	23.0	290.0	744.0	272.0	256.6
Iron (mg/100 g)	7.5	5.1	11.0	5.6	5.5
Calcium (mg/100 g)	1000.0	70.0	110.0	200.0	205.8

[a] Calculated from the content of the different seed parts.

[1] Although botanically the two cotyledons in *Cicer* are part of the whole embryo, in the present paper, where ever embryo has been used it should be taken to mean the rest of the embryo, i.e. the whole embryo minus the cotyledons.

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