Table 1. Acid and neutral amino acids of peptides β Tp XIII of Hb A and Hb $\alpha_2\beta_2^{121\ Lys}$. Because of the specific cleaving action of trypsin on arginine and lysine, and since staining for arginine was negative on these peptides, lysine is assumed to be present also.

Amino acid	Hb A β Tp XIII (molar ratios)	Abnormal Hb β Tp XIII (molar ratios)
Threonine	1.00	0.98
Glutamic acid	2.94	2.11
Proline	1.81	1.93
Alanine	2.01	2.08
Valine	0.93	1.08
Tyrosine	.83	0.71
Phenylalanine	.96	.97

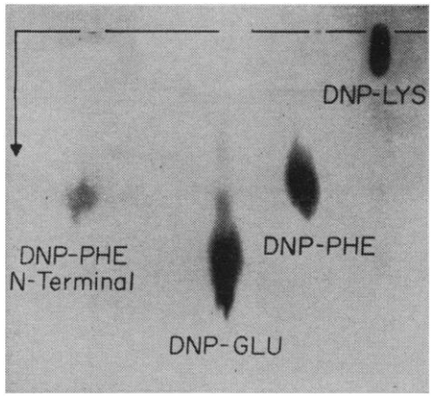

Fig. 2. Chromatogram in 1.5*M* phosphate buffer *p*H 6.0, showing DNP-phenylalanine as the NH_2-terminal amino acid of abnormal peptide β Tp XIII and some other DNP-amino acids as controls.

was in the β-chain. An abnormal α-chain substitution, like that in Hb O Indonesia, which shares similar electrophoretic properties with the above hemoglobin (*3*), was thus excluded.

Tryptic digests of Hb A and Hb E were made as standards to compare with similar preparations of our unknown. High-voltage electrophoresis was done on a sample applied to Schleicher and Schuell filter paper No. 598, cut to a dimension of 58 by 10 cm. The buffer used was *p*H 4.4 (water, pyridine, and acetic acid, 94:2:4) and a potential gradient along the length of the paper was 35 volt/cm held for 75 minutes. After electrophoresis of individual tryptic digests of Hb A, Hb E, and the present unknown, staining with ninhydrin and for arginine revealed that our unknown was not the classic Hb E.

Second-dimension peptide patterns (fingerprints) were formed by using a chromatographic solvent system of butanol, acetic acid, and water (4:1:5), in a recycled ascending procedure. The peptides were more adequately resolved after the second chromatographic run. Figure 1 shows a resulting fingerprint of the abnormal hemoglobin compared to one of Hb A. The peptide of interest, β Tp XIII, was identified by differential staining. It will be noted that this peptide had moved from a relatively uncharged position in Hb A to a less acidic point on the "fingerprint" of the unknown trypsin digest. This shift was suggestive of the abnormality Hb $\alpha_2\beta_2^{121\ Lys}$ (Oβ) sometimes referred to as Hb O Arab, described by Baglioni *et al.* (*3*).

The corresponding peptides were eluted from the chromatograms of digested β-chains (*4*) and further purified by additional electrophoresis in *p*H 3.6 buffer (pyridine, acetic acid, and water; 1:10:90). The isolated peptides were submitted to amino acid analysis (*5*). Comparison of the amino acid compositions revealed a decrease in glutamic acid residues in β Tp XIII of the abnormal hemoglobin (Table 1). The NH_2-terminal amino acid of the abnormal β Tp XIII (*6*) was found to be phenylalanine since its dinitrophenyl (DNP) derivative obtained a similar R_F value as that of standard DNP-L-phenylalanine in 1.5*M* phosphate buffer *p*H 6.0 descending chromatography (Fig. 2). Fingerprints of chymotryptic digests of trypsin-resistant core showed no difference from those of Hb A.

In accordance with Baglioni and Lehmann (*3*), it was now possible to establish that glutamic acid, normally at position 121 in the β-chain, had been replaced by lysine, which was in turn cleaved by trypsin, leaving phenylalanine at the NH_2-terminal end of β Tp XIII. We have demonstrated a tyrosine yield in the mutant peptide quantitatively equal to the normal control (Table 1). This result was different from a corresponding value reported by Baglioni and Lehmann (*3*). Heterozygous carriers of this hemoglobin manifest no clinical symptoms or signs (*1*). However, since no homozygotes have been reported, speculation cannot be made on its pathological effect, if any. We have discussed the anthropological implications of this hemoglobin variant (*7*).

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Seed Dormancy: Breaking by Uncouplers and Inhibitors of Oxidative Phosphorylation

Abstract. *When 2,4-dinitrophenol and carbon dioxide were applied together to dormant seeds of* Trifolium subterraneum *L. (subterranean clover), 2,4-dinitrophenol did not disturb the breaking of dormancy which carbon dioxide usually induces in legume seeds. On the contrary, on its own, it promoted germination in a substantial proportion of seeds; a similar effect was produced by other uncouplers or inhibitors of oxidative phosphorylation.*

The mechanism by which CO_2 acts in breaking the dormancy of small-seeded legumes, especially subterranean clover remains unknown (*1*). While examining what effect metabolic inhibitors might have on this action of CO_2, we obtained the following result using 2,4-dinitrophenol (DNP). The indicated values are the percentages of germination of subterranean clover seed on the 8th day after the named treatments: water, 16; 2.5 percent CO_2, 99; 2.5 percent CO_2 plus 0.1 m*M* DNP, 97; 0.1 m*M* DNP, 54. Not

only did the DNP not interfere with the promotion of germination by CO_2, it also substantially promoted germination on its own. This finding prompted the investigation of the action of other uncoupling agents.

Seeds of a very dormant line of subterranean clover (Commonwealth Plant Introduction No. 19465) were scarified in such a way as not to change markedly their dormancy status (*2*); for 3 hours they were allowed to imbibe either distilled water or the appropriate solution. According to their solubilities, the uncoupling agents were made up in water; dilute alkali adjusted to a final *p*H in the range of 7.0 to 7.5; 0.067*M* phosphate buffer, *p*H 6.5; or 0.05*M* tris (hydroxymethyl) aminomethane HCl buffer, *p*H 7.5. The *p*H of the buffered solutions changed very little during imbibition by the seeds; the *p*H of unbuffered solutions dropped to a *p*H within the range of 6.5 to 7.0 (except for that of DNP, which was approximately 6).

Seeds swollen by the end of 3 hours were transferred to petri dishes (four replicates each with 50 seeds) containing filter paper moistened with water or with the solution used for imbibition. Other seeds soaked in water were treated with 2.5 percent (by volume) CO_2 (four replicates with 25 seeds each). Dishes were maintained at 20°C and moistened with water as required. Seeds whose radicles showed positive geotropism were considered to be germinated and were counted and removed daily.

Of the six compounds investigated four promoted germination substantially, one promoted it less markedly, and one was apparently without effect. However, no compound was as effective as CO_2 (Table 1), and some of them induced production of seedlings with discolored, retarded radicles.

Among them, the substances interfere with at least two of the stages in the reaction forming adenosine triphosphate (ATP) (*3*). The concentrations found effective are in line with the optima established for the uncoupling of phosphorylation in mitochondrial preparations, in bacteria, and in tissues such as cartilage (*4*). It is not obvious why the three substances, DNP, dicoumarol, and carbonyl cyanide *meta*-chlorophenylhydrazone (*m*-Cl-CCP) believed to act at the same site in ATP formation (*3*), should be so differently effective. Differences in their abilities to penetrate to the active site might be responsible, or, as a secondary phenomenon, they might have inhibitory effects on radicle growth. That this might be so is suggested both by the finding that the rate of germination evoked by the uncouplers is slower than that evoked by CO_2 (Table 1) and the fact that delay in the start of germination increases as the concentration is increased (Fig. 1). With some substances, such an inhibition might be extensive enough to prevent emergence of the radicle and thus to mask entirely any breaking of dormancy.

While other substances and conditions break dormancy in these and other seeds, there does not appear to be a common basis for this activity. Interest in our results stems from the fact that the substances shown here to break dormancy, although of diverse chemical structure, are nevertheless linked by having a similar biochemical activity: they are all uncouplers, or inhibitors of oxidative phosphorylation. Our results provide no direct data on mechanisms, but stimulation of respiration by these compounds could have effects similar to those of externally supplied CO_2. The observations that, in lettuce and pea seeds, DNP increased uptake of O_2, output of CO_2, or both, but inhibited germination at the effective concentrations (*5*) need not conflict with this idea, since stimulation of germination by CO_2 in these species has not been recorded. However, this explanation need not be universally valid, since stimulation of respiration is apparently not an obligate feature of uncoupling (*3*).

Table 1. Reduction of dormancy in seed of subterranean clover caused by various uncoupling agents and by CO_2. Seeds were treated with various concentrations (differing by factors of two to ten) of each compound in two to five experiments (one for arsenate). The maximum percentage of seeds that germinated is given along with the concentration of uncoupling agent used in that particular experiment in which this percentage of germination occurred. In each experiment, control groups of seeds were treated either with the solvent system alone or with CO_2. The percentages of germination in response to these treatments are also given.

Day	Germination (%)		
	CO_2 control	Solvent control	Treated
	0.2 mM DNP		
4	77	3	14
13	100	13	60*
	0.2 mM Dicoumarol		
4	98	10	17
9	98	28	38†‡
	0.8 mM Sodium azide		
4	77	3	11
13	100	13	87*
	1.0 mM Sodium arsenate		
4	98	20	51
8	98	23	72*
	4.0 mM Sodium salicylate		
4	94	5	10
9	99	13	66*
	0.01 mM m-Cl-CCP§		
4	100	6	5
8	100	12	16†

* Significance of difference between solvent control and treated, $P < .001$. † Difference between solvent control and treated, not significant. ‡ A similar result was obtained in three other experiments. When all four experiments were combined, the treatment average exceeded the control average ($P < .001$). § Germination in 0.001 m*M* *m*-Cl-CCP was similar, but 0.1 m*M* *m*-Cl-CCP suppressed germination.

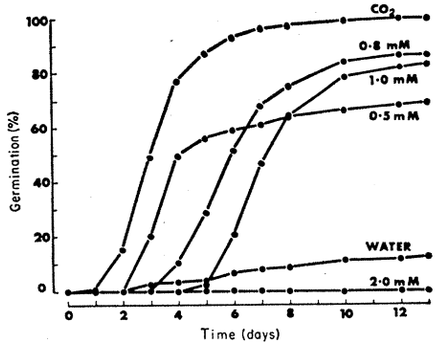

Fig. 1. Reduction of dormancy by sodium azide (various concentrations) and by 2.5 percent CO_2.

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